Fidelity of Fractionated Deoxyribonucleic Acid Polymerases from Human Placenta†

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ABSTRACT: Deoxyribonucleic acid (DNA) polymerase activities from human placenta have been fractionated and classified among the α, β, and γ types by using the criteria of size, ability to utilize various synthetic template–primers, and sensitivity to phosphate, *N*-ethylmaleimide, aphidicolin, and 2′,3′-dideoxythymidine 5′-triphosphate. Reverse transcriptase and δ-polymerase activities were not detected. α activity resolved into two fractions on diethylaminoethylcellulose which could be distinguished catalytically by their template–primer preferences. Similarly, multiple species of β-polymerase were obtained upon purification by phosphocellulose or glycerol gradient sedimentation. Each DNA polymerase fraction was tested for misincorporation frequencies with synthetic template–primers and either Mn^{2+} or Mg^{2+}. All α-polymerases copied the templates with relatively high fidelity, though absolute values depended upon the length of primer and the primer/template ratio. With Mn^{2+} present, an 8.6S species of β-polymerase copied the synthetic templates very accurately, but 6.3S and 4.6S forms copied the same polymers relatively unfaithfully. The 8.6S form could be converted to a 4.8S species with a concomitant loss of fidelity. γ-Polymerase also copied deoxyribopolymers relatively inaccurately in the presence of Mn^{2+}. The relative concentrations of complementary to noncomplementary triphosphates affected the frequency of misincorporation for β and γ activities. Conversely, α-polymerase did not show similar behavior at saturating triphosphate concentrations. None of the placental polymerase fractions contained exonuclease activity that could discriminate between complementary and noncomplementary 3′ ends.

The DNA polymerases of procaryotes have an intrinsic 3′ → 5′ exonuclease activity which removes nucleotides that are incorrectly inserted during polymerization (Brutlag & Kornberg, 1972; Gefter, 1975). Error-free DNA synthesis also has been suggested to result from "kinetic proofreading" in the case of T4 phage polymerase (Hopfield, 1974; Gillin & Nossal, 1976a,b). However, the mechanism by which eucaryotes ensure a high degree of accuracy during DNA replication has not been identified yet. Neither partially purified nor homogeneous preparations of α-, β-, or γ-polymerases appear to have intrinsic 3′ → 5′ exodeoxyribonuclease activity. In the case where a 3′ → 5′ exonuclease activity has been reported to be associated with a polymerase ("δ"; Byrnes et al., 1976), it has not been shown to be correction-specific. Moreover, Chang (1973) ruled out forms of editing which involve DNA hydrolysis by determining that nucleoside monophosphates are not generated during polymerization reactions with either the 3.4S or the 6.8S calf thymus polymerases. Therefore, it would appear that for eucaryotic DNA polymerases, either extrinsic factors act to assure fidelity or fidelity is controlled prior to phosphodiester bond formation.

Recently, it was reported that DNA polymerase activity present in extracts from late-passage cultured MCR-5 human fibroblasts had levels of incorporation of incorrect nucleotides in the presence of a defined synthetic DNA template that were up to 10-fold higher than those observed with extracts from an early passage of these cells (Linn et al., 1976). These results are consistent with suggestions that alterations in the fidelity of DNA polymerase(s) could be important in aging or age-related diseases (Szilard, 1959; Burnet, 1974). However, the preliminary observations must be further refined in order to determine whether the changes noted were due to primary or secondary structural changes in the polymerases, to post-translational modifications of normal polymerases, to the appearance of a new, error-prone polymerase species, or to an alteration of other polypeptide components that are involved in assuring fidelity during DNA replication. In this context, we have isolated DNA polymerases from human placenta and examined the accuracy with which they copy synthetic templates. As reported here, under comparable assay conditions, α-polymerases copied a variety of synthetic templates with relatively high fidelity, whereas β- and γ-polymerases copied the same templates less faithfully. However, with β-polymerase, the particular form of the enzyme has a profound effect on its fidelity. In addition, the fidelities of the polymerases have varying sensitivities to the relative concentrations of complementary and noncomplementary triphosphates, the primer lengths, and the primer/template ratios. With this information and the observation that the polymerase fractionation applies also to cultured fibroblasts, we should be able to study more completely the observations made with the aging cells.

Experimental Procedures

Materials. DEAE-cellulose, type 40, was obtained from Brown Co., Berlin, NH; phosphocellulose (P11) was from Whatman. All synthetic polynucleotides and oligonucleotides were from P-L Biochemicals. [^{3}H]Deoxyribonucleoside triphosphates were from Amersham, and [α-^{32}P]dATP was from New England Nuclear. Unlabeled deoxyribonucleoside triphosphates were from P-L Biochemicals. Salmon sperm DNA was activated according to the procedure of Schlabach et al. (1971). Markers for glycerol gradient sedimentation were bacterial alkaline phosphatase (Worthington) and ovalbumin (Sigma). Homogeneous *Escherichia coli* polymerase I was the generous gift of Dr. Arthur Kornberg, Stanford University. Terminal deoxyribonucleotidyltransferase was kindly provided by Dr. Robert Ratliff, Los Alamos, NM.

Poly(dA-[^{3}H]dT) was synthesized according to Modrich & Lehman (1970). The final product had a specific activity of 23 000 cpm/nmol. [α-^{32}P]dGTP was added to 3′ termini of

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poly(dA-[^{3}H]dT) with terminal transferase in a 50-μL reaction which contained 0.2 M potassium cacodylate, pH 7.1, 1 mM $CoCl_2$, 1 mM 2-mercaptoethanol, 30 nmol of poly(dA-[^{3}H]-dT), and 13 μM [α-^{32}P]dGTP. The number of residues added per molecule was calculated after determining the number of 5′ termini of the poly(dA-[^{3}H]dT) with the polynucleotide kinase exchange reaction (Berkner & Folk, 1977). The polymer was extracted with phenol and dialyzed. Poly(dA-dT)–[^{3}H]dT and poly(dA-dT)–[^{3}H]dG were synthesized by the addition of the nucleoside triphosphate to commercial poly(dA-dT). [^{3}H]dGTP was added by using terminal transferase in a 0.2-mL reaction mixture containing 0.2 M potassium cacodylate, pH 7.1, 1 mM $CoCl_2$, 1 mM 2-mercaptoethanol, 55 nmol of poly(dA-dT), and 10 μM [^{3}H]dGTP (12.6 Ci/mmol). [^{3}H]dTTP was added by using *E. coli* DNA polymerase I in a 0.2-mL reaction containing 80 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 50 nmol of poly(dA-dT), and 20 μM [^{3}H]dTTP (30 Ci/mmol). All polymers were extracted with phenol and dialyzed extensively before use.

Buffer I contained 0.4 M potassium phosphate, pH 7.5, 0.5 mM dithiothreitol, and 10 mM 2-mercaptoethanol. Buffer II contained 0.02 M potassium phosphate, pH 7.5, 0.5 mM dithiothreitol, and 1 mM 2-mercaptoethanol. Buffer III contained 0.05 M potassium phosphate, pH 7.5, 0.5 mM dithiothreitol, and 1 mM 2-mercaptoethanol. Buffer IV contained 0.05 M potassium phosphate, pH 7.5, 0.5 mM dithiothreitol, 1 mM 2-mercaptoethanol, and 20% glycerol.

Polymerase Assay. Enzyme activity was assayed by using activated salmon sperm DNA under the conditions described by Linn et al. (1976). When measuring α-polymerase activity, KCl was omitted. One unit of enzyme incorporates 1 nmol of total nucleotide in 30 min at 37 °C.

Fidelity Assay. Reaction mixtures (0.1 mL) contained 50 mM Tris-HCl, pH 7.5, 0.5 mM dithiothreitol, 0.5 mg/mL bovine serum albumin, 10 nmol of synthetic template–primer, 50 μM complementary deoxyribonucleoside triphosphate, 6 μM noncomplementary deoxyribonucleoside triphosphate, and enzyme as indicated. Initally, noncomplementary triphosphates were purified as described by Lehman et al. (1958) and tested in misincorporation assays with homogeneous *E. coli* DNA polymerase I and placental polymerases. Subsequently, unpurified triphosphates were used in misincorporation assays only if there were no detectable differences in error frequencies relative to purified triphosphates. (The controls with the *E. coli* enzyme also served to assure that the polymers were free of contaminating nucleotides.) Unless otherwise noted, assays of α-polymerases also contained 0.25 mM $MnCl_2$ and no KCl, assays of β-polymerases contained 0.5 mM $MnCl_2$ and 50 mM KCl, and assays of γ-polymerase contained 0.5 mM $MnCl_2$ and 100 mM KCl. In assays with Mg^{2+} instead of Mn^{2+}, $MgCl_2$ was 1 mM with poly(dA)·poly(dT), poly-(dA-dT), or poly(dT)·oligo$(dA)_{19-24}$, 2 mM with poly(dI)·poly(dC) or poly(dI-dC), and 7.5 mM with poly(rA)·oligo-$(dT)_{10}$. Before the assay, template–primers were heated for 10 min at 70 °C and slowly cooled to assure reproducible secondary structures. Each misincorporation ratio was obtained from parallel reactions, one with complementary [^{3}H]deoxyribonucleoside triphosphate (80–150 cpm/pmol) to measure total polymer synthesis and the other with noncomplementary [^{3}H]deoxyribonucleoside triphosphate (12–30 cpm/fmol) to measure nonfaithful synthesis. Blank values for misincorporated nucleotide were reproducible to within 20% in an individual experiment, and values 50% above the blank were considered significant. Each enzyme sample was tested for contaminating endogenous template with controls in which template–primer was omitted and for terminal transferase with controls using single-stranded homopolymer. After incubation for 4 h at 37 °C, reaction mixtures were precipitated and collected onto glass fiber filters and radioactivity was measured as described by Linn et al. (1976). Synthetic template–primer concentrations are expressed as nucleotide residues. Reactions were generally done with several levels of enzyme to assure a constant misincorporation frequency vs. the level of synthesis. For brevity, however, only the highest levels of reaction are noted in the tables.

3′ → 5′ Exonuclease Assay. Reaction mixtures (0.1 mL) contained 80 mM Hepes,[1] pH 7.0, 1 mM $MnCl_2$, 120 mM KCl, 0.5 mM dithiothreitol, enzyme as indicated, and either 10 nmol of poly(dA-[^{3}H]dT)–[^{32}P]dG, 0.5 nmol of poly(dA-dT)–[^{3}H]$d(T)_{0.2}$, or 0.7 nmol of poly(dA-dT)–[^{3}H]$d(G)_{19}$. At various times after incubation at 37 °C, 20-μL aliquots were precipitated onto glass fiber filters and radioactivity was determined as described by Linn et al. (1976).

Protein Assay. Protein was determined according to Lowry et al. (1951) with bovine serum albumin as a standard.

Fractionation of Placental Polymerases. All operations were at 0–4 °C. In a typical preparation (Table I) a human placenta was immersed in ice-cold 25 mM potassium phosphate, pH 7.0, 0.15 M NaCl, and 0.015 M sodium citrate within 10 min of delivery by Caesarian section. Approximately 400 g of tissue was excised, rinsed, suspended in 250 mL of buffer I, and homogenized in a Waring blender 4 times for 30 s. The homogenate was centrifuged for 30 min at 13000*g*, and the supernatant was saved. The pellet was resuspended in 300 mL of buffer I and blended as previously described, and then this suspension was sonicated for four, 30-s intervals by using the large probe of a Branson sonifier cell disrupter (Model W185D) at 75 W. The sonicate was centrifuged as above, and the supernatant was combined with the first supernatant.

The crude extract was passed at 2 mL/min over a column of DEAE-cellulose (5.5 × 27.5 cm) previously equilibrated with buffer I. Fractions containing significant red hemoglobin color were pooled, concentrated threefold by dialysis against 30% polyethylene glycol (w/v), 0.05 M potassium phosphate, pH 7.5, 0.5 mM dithiothreitol, and 10 mM 2-mercaptoethanol, and then dialyzed extensively against buffer II.

The dialyzed extract was loaded onto a second DEAE-cellulose column (4 × 36 cm) which had been equilibrated with buffer II. The column was washed with 350 mL of this buffer, and fractions containing the red hemoglobin were pooled. A 2-L linear gradient of potassium phosphate, pH 7.5, from 0.02 to 0.40 M containing 0.5 mM dithiothreitol and 1 mM 2-mercaptoethanol was finally passed through the column, and fractions of 12 mL were collected into plastic tubes. Four peaks of polymerase activity were recovered that eluted in the flow through and at 0.10, 0.19, and 0.23 M phosphate (Figure 1). These were subsequently shown to be β-, γ-, and two α-polymerases (called α_1 and α_2), respectively (see below). Each pool of polymerase activity was concentrated two- to threefold by dialysis against 30% polyethylene glycol (w/v in buffer IV), dialyzed extensively against buffer III, and stored at −80 °C.

For further purification, individual polymerase pools were rapidly thawed, diluted with an equal volume of buffer IV, and adsorbed onto phosphocellulose columns (10 mg of protein

[1] Abbreviation used: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Table I: Purification of Polymerase Activities[a]

	fraction	vol (mL)	protein (mg)	act. (units)	sp act. (units/mg)
(I)	crude extract	730	10300	4510	0.26
(II)	first DEAE-cellulose	620		4090	
	dialyzed concentrate	200	6440	1000	0.15
(III)	second DEAE-cellulose				
	β	132	1190	494	0.41
	γ	140	630	178	0.28
	α_1	100	190	97 (400)	0.51 (2.1)
	α_2	60	46	94 (850)	2.0 (18)
(IV)	phosphocellulose				
	β_1	24	33	13.1	0.39
	γ	23	50	130	2.6
	α_{1a}	2.4	3.6	17 (20)	4.7 (13)
	α_{1b}	2.4	2.6	15 (26)	5.6 (23)
	α_{2a}	11	2.3	15 (133)	6.5 (59)
	α_{2b}	5.9	1.2	5.9 (59)	5.0 (50)
(V)	glycerol gradients				
	β_1	0.26	<0.005	0.13	>26
	γ	0.26	<0.005	0.10	>20
	α_{1a}	0.26	<0.005	(0.16)	(>32)
	α_{1b}	0.26	<0.005	(0.13)	(>26)
	α_{2a}	0.26	<0.005	(0.27)	(>54)
	α_{2b}	0.26	<0.005	(0.10)	(>20)

[a] DNA polymerase activities were isolated from 400 g of human placental tissue as described in the text. Numbers in parentheses indicate polymerase activity in the absence of KCl. DEAE-cellulose fractions (step III) were pooled as indicated in Figure 1, and values for phosphocellulose fractions (step IV) have been normalized to these total pools. Less than 5 μg of protein could be detected by a Lowry assay of 200 μL of glycerol gradient fractions. Only 0.2 mL of each phosphocellulose fraction was used for each glycerol gradient.

per mL of packed resin). The columns were washed with buffer IV and then eluted with a linear gradient (4 column volumes) from 0.02 to 0.4 M potassium phosphate, pH 7.5, containing 0.5 mM dithiothreitol, 1 mM 2-mercaptoethanol, and 20% glycerol. About 50–60 fractions were collected into plastic tubes, and active fractions were pooled. The α- and β-polymerases each separated further into two fractions during phosphocellulose chromatography to yield α_{1a} and α_{1b}, α_{2a} and α_{2b}, and β_1 and β_2, respectively; γ-polymerase eluted as a single peak (Figure 2).

Phosphocellulose pools (0.2 mL) were layered onto 5.2 mL of glycerol gradients (20–40% v/v) containing 0.5 mM dithiothreitol, 1 mM 2-mercaptoethanol, and buffer and KCl as indicated. After sedimentation at 4 °C for 20 h at 50000 rpm in a Spinco SW50.1 rotor, fractions (~0.26 mL) were collected from the tube bottom. Ovalbumin ($s_{20,w}$ = 6.3 S) and bacterial alkaline phosphatase ($s_{20,w}$ = 3.6 S) were sedimented simultaneously in a separate tube to serve as sedimentation references.

Glycerol gradient fractions were stored at −20 °C. Fractions from DEAE-cellulose (step III) and phosphocellulose (step IV) were stored at −80 °C and were stable for at least 6 months.

Results

Identification of the Placental DNA Polymerase Classes. The various DNA polymerase activities separated upon DEAE-cellulose (Figure 1) were classified on the basis of the following characteristics previously reported (Weissbach et al., 1975; Edenberg et al., 1978; Ohashi et al., 1978). The flow-through fraction was designated β because it had a relatively low sedimentation coefficient (Table II), it was not absorbed by DEAE-cellulose, it was not inhibited by 2.5 mM *N*-ethylmaleimide or 5 μg/mL aphidicolin, and it was inhibited 95% by 200 μM 2′,3′-dideoxythymidine 5′-triphosphate in the presence of 50 μM dTTP. The peak eluting from DEAE-cellulose at 0.10 M phosphate was classified as a γ-polymerase because it could polymerize dTTP with poly(rA)·oligo(dT) as a template–primer, it had a relatively high sedimentation coefficient (Table II), it was 100% inhibited by 2.5 mM *N*-ethylmaleimide, and it was 90% inhibited by 2′,3′-dideoxythymidine 5′-triphosphate but was resistant to aphidicolin. The remaining two activities from DEAE-cellulose were classified as α-polymerases because they had relatively high sedimentation coefficients (measured after further resolution on

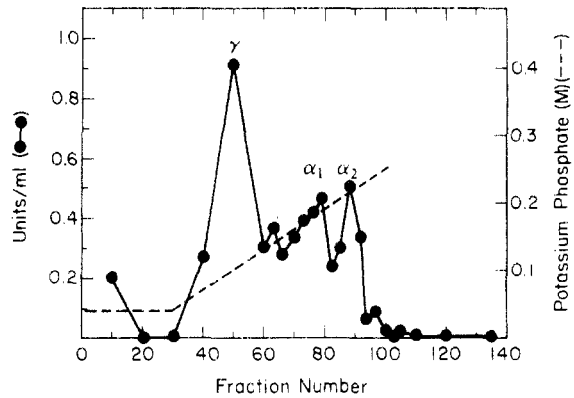

FIGURE 1: DEAE-cellulose chromatography. The dialyzed concentrate of step II containing 1000 units was applied to the column. Fractions were pooled as follows: γ, 40–50; α_1, 70–80; α_2, 82–92.

Table II: Sedimentation Coefficients of Polymerase Activities[a]

polymerase fraction	sedimentation coeff (S) in gradient buffer: 0.2 M potassium phosphate, pH 7.5	0.5 M KCl, 0.02 M Tris-HCl, pH 7.5	0.02 M Tris-HCl, pH 7.5
α_{1a}	6.5		10.7
α_{1b}	6.3		
α_{2a}	6.9		10.4
α_{2b}	6.9		
β_1		4.6	6.3
β_2		4.8	8.6 (4.8, 5.2)
γ	9.8 (3.6)	6.6 (4.0, 9.2)	

[a] Sedimentation was performed as described under Experimental Procedures with phosphocellulose fractions (Figure 2). Numbers in parentheses are minor peaks of activity which were also observed.

Table III: Template-Primer Preferences of α-Polymerases[a]

template-primer	pmol incorpd			
	α_1		α_2	
	Mn^{2+}	Mg^{2+}	Mn^{2+}	Mg^{2+}
activated DNA		370		172
poly(dA)·poly(dT)	176	164	46	36
poly(dA-dT)	124	654	36	243
poly(dT)·oligo(dA)$_{19-24}$	1044	152	967	189
poly(dI)·poly(dC)	315	69	13	8
poly(dI-dC)	846	123	10	9
poly(dC)·oligo(dG)$_{12-18}$	143	54	72	28

[a] Reactions with synthetic templates were performed under the fidelity assay conditions. The α_{1a} and α_{2a} phosphocellulose fractions were used; in other experiments, α_{1b} and α_{2b} showed similar distinctive template-primer preferences. Ratios of polymer/nucleotide for poly(dA)·poly(dT), poly(dI)·poly(dC), and poly(dC)·oligo(dG)$_{12-18}$ were 1:1; that for poly(dT)·oligo(dA)$_{19-24}$ was 10:1. The particular nucleotides incorporated were as follows: for activated DNA, all four deoxyribonucleoside triphosphates; for poly(dA)·poly(dT) and poly(dT)·oligo(dA)$_{19-24}$, dAMP; for poly(dA-dT), dAMP and dTMP; for poly(dI)·poly(dC) and poly(dC)·oligo(dG)$_{12-18}$, dGMP; for poly(dI-dC), dGMP and dCMP.

phosphocellulose, Table II), they were inhibited 100% by 2.5 mM *N*-ethylmaleimide, they were inhibited 90% by aphidicolin but only ~20% by 2′,3′-dideoxythymidine 5′-triphosphate, and they could not utilize polyribonucleotides as templates.

Multiple Species of α-Polymerase. The two α fractions that were isolated after DEAE-cellulose chromatography (Figure 1) each resolved further into two subclasses when chromatographed on phosphocellulose (α_{1a}, α_{1b} and α_{2a}, α_{2b}, respectively) (Figure 2). However, the members of each pair behaved identically when further characterized. On the other hand, the α_1 species sediment through 0.2 M potassium phosphate glycerol gradients somewhat slower than do the α_2 fractions (Table II). In addition, the two polymerase fractions can be distinguished by their pattern of template–primer utilization (Table III). α_1-Polymerase is active with poly(dA)·poly(dT), poly(dA-dT), poly(dI)·poly(dC), poly(dI-dC), poly(dT)·oligo(dA), and poly(dC)·oligo(DG); however, α_2-polymerase has a strong preference for polymeric template–oligomeric primer pairs or activated salmon sperm DNA. This difference is not due to differing DNase content, as neither fraction significantly degrades poly(dA-[^{3}H]dT) (see below) and endonuclease was not found in any polymerase fraction when assayed by using a closed, circular PM2 DNA as substrate (Kuhnlein et al., 1976).

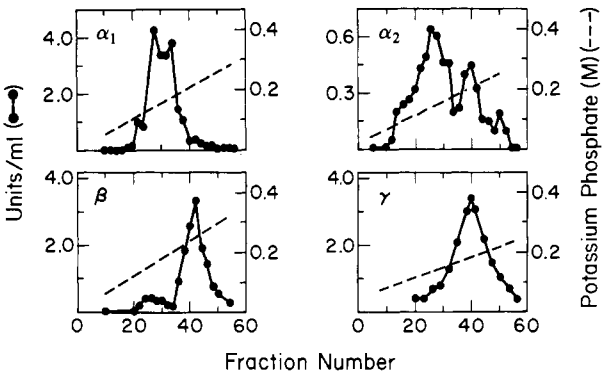

FIGURE 2: Phosphocellulose chromatography. Polymerase pools containing the indicated amounts of activity were applied to phosphocellulose columns: α_1, 48 units; α_2, 42 units; β, 53 units; γ, 69 units. Fractions were pooled as follows: α_{1a}, 27 and 28; α_{1b}, 34 and 35; α_{2a}, 14–30; α_{2b}, 36–44; β_1, 20–28; β_2, 36–47; γ, 32–48.

Multiple Species of β-Polymerase. The two β fractions that are isolated after phosphocellulose chromatography (Figure 2) are both resistant to *N*-ethylmaleimide and stimulated by 50–100 mM KCl. In glycerol gradients containing 0.02 M Tris, β_1 has a major peak at 6.3 S with a minor peak at 4.8 S, whereas β_2 has a major peak at 8.6 S with minor peaks at 4.8 and 5.2 S (Table II). However, in glycerol gradients containing 0.55 M KCl, both species similarly dissociate: β_1 sediments at 4.6 S while β_2 sediments at 4.8 S (Table II).

Fidelity of Polymerase Fractions. Misincorporation frequencies were computed from measurements of the polymerization of nonhomologous nucleotides using synthetic template–primers with Mg^{2+} or Mn^{2+} present (Linn et al., 1976). For assurance that there were no interfering impurities in the template–primers and deoxyribonucleoside triphosphate preparations, error frequencies were determined by using homogeneous *E. coli* polymerase I with which there was no significant misincorporation.

The placental polymerase fractions were tested for fidelity with poly(dA)·poly(dT) and poly(dA-dT) (Table IV) with Mn^{2+} or Mg^{2+} present. All polymerases preferred to incorporate dAMP rather than dTMP with the poly(dA)·poly(dT). Relatively high levels of misincorporation were observed only in the case of the β_1- and γ-polymerases when Mn^{2+} was present. There was no detectable misincorporation by α_1, α_2, or β_2 in any case. [In the case of β-polymerases with Mg^{2+}, activity was too low [see Wang et al. (1977)] to give significant sensitivity, however.]

Table IV: Misincorporation Frequencies with Deoxyadenylate-Deoxythymidylate Polymers[a]

polymerase fraction	divalent cation	poly(dA)·poly(dT) incorpn dAMP (pmol)	dGMP (fmol)	dCMP (fmol)	error frequency	poly(dA-dT) incorpn dAMP and dTMP (pmol)	dGMP (fmol)	error frequency
α_1	Mn^{2+}	110	<8		<1/14000	176	<5	<1/35000
	Mg^{2+}	116	<10		<1/12000	104	<10	<1/10000
α_2	Mn^{2+}	47	<6		<1/7800	36	<6	<1/6000
	Mg^{2+}	54	<11		<1/4900	118	<10	<1/12000
β_1	Mn^{2+}	131	32		1/4100	83	8	1/10000
		53		30	1/1800			
	Mg^{2+}	8	<11		(<1/730)	2	<10	(<1/200)
β_2	Mn^{2+}	86	<4		<1/22000			
		75		<8	<1/9400			
	Mg^{2+}	41	<11		<1/3700	2	<10	(<1/200)
γ	Mn^{2+}	142	71		1/2000	61	17	1/3600
	Mg^{2+}	84	<15		<1/5600	47	<10	<1/4700

[a] Phosphocellulose fractions were used as described under Experimental Procedures.

Table V: Misincorporation Frequencies with Deoxycytidylate–Deoxyinosinate Polymers[a]

		polymer					
		poly(dI)·poly(dC)			poly(dI-dC)		
		incorpn			incorpn		
polymerase fraction	divalent cation	dGMP (pmol)	dTMP (fmol)	error frequency	dGMP and dCMP (pmol)	dTMP (fmol)	error frequency
α_1	Mn^{2+}	226	16	1/13000	826	25	1/33000
	Mg^{2+}	823	<16	<1/51000			
β_1	Mn^{2+}	30	27	1/1100	95	<7	<1/14000
	Mg^{2+}	27	<14	<1/1900	6	<14	(<1/430)
β_2	Mn^{2+}	244	<6	<1/41000			
	Mg^{2+}	113	<14	<1/8000	125	<14	<1/9000
γ	Mn^{2+}	157	21	1/7500	342	<10	<1/34000
					658	<7	<1/94000
	Mg^{2+}	248	20	1/12000	42	<14	<1/3000

[a] Phosphocellulose fractions were used as described under Experimental Procedures.

Table VI: Misincorporation Frequencies with Polymer–Oligomer Template-Primers[a]

			incorpn				
polymer present	template/primer ratio (nucleotide residues)	polymerase	dAMP (pmol)	dTMP (pmol)	dGMP (fmol)	dCMP (fmol)	error frequency
poly(rA)·oligo(dT)$_{10}$		γ		243	9		1/27000[b]
				243		10	1/24000[b]
				209	<16		<1/13000[b,c]
poly(dT)·oligo(dA)$_{19-24}$	10:1	α_1	390		15		1/26000
			152		<15		<1/10000[d]
		α_{2a}	34		<4		<1/8000
		α_{2b}	108		<4		<1/27000
			189		<15		<1/13000[d]
poly(dT)·oligo(dA)$_{19-24}$	1:1	α_1	203		73		1/2800
		α_{2a}	30		10		1/3000
		α_{2b}	131		27		1/4800
poly(dT)·oligo(dA)$_{10}$	1:10	α_{2a}	770		88		1/8800
		α_{2b}	910		96		1/9500
poly(dT)·oligo(dA)$_{10}$	1:1	α_{2a}	380		203		1/1900
		α_{2b}	349		195		1/1800
poly(dC)·oligo(dG)$_{12-18}$	1:1	α_1		<6[e]	64[f]		<1/10000
		α_{2a}		<6[e]	28[f]		<1/5000
				<6[e]	61[f]		<1/10000
		α_{2b}		<6[e]	28[f]		<1/5000
				<6[e]	62[f]		<1/10000

[a] Phosphocellulose fractions were used; except where indicated, assays are described under Experimental Procedures. [b] Reactions under conditions optimal for γ-polymerase (Knopf et al., 1976) for 4 h at 30 °C. [c] Reactions with 7.5 mM $MgCl_2$ in place of $MnCl_2$. [d] Reactions with 1 mM $MgCl_2$ in place of $MnCl_2$. [e] In femtomoles. [f] In picomoles.

The polymerases were also tested for the misincorporation of dTMP into poly(dI)·poly(dC) and poly(dI-dC) (Table V). With poly(dI)·poly(dC) and Mn^{2+}, again relatively high misincorporation frequencies were observed with the β_1- and γ-polymerases, whereas the α_1- and β_2-polymerases were more faithful. Double-label experiments with [^{3}H]dGMP and [α-^{32}P]dATP or [α-^{32}P]dTTP confirmed that the misincorporation frequency for the α_1- polymerase was not greater than 1/10 000 (data not shown). In all reactions with Mg^{2+} or with Mn^{2+} and poly(dI-dC), incorporation of dTMP was not detectable or was very low.

Misincorporation frequencies for the α- and γ-polymerases were also determined with homopolymeric templates and oligomeric primers (Table VI). With poly(rA)·oligo(dT)$_{10}$, γ-polymerase incorporated dGMP or dCMP relatively infrequently in contrast to reactions with Mn^{2+} and poly(dA)·poly(dT) or poly(dA-dT) (Table IV). The α-polymerases had optimal polymerizing activity with poly(dT)·oligo(dA)$_{19-24}$ in a 10:1 template/primer ratio (nucleotide residues) and had relatively high fidelity under the assay conditions used. Increasing the amount of oligo(dA)$_{19-24}$ primer to a 1:1 ratio decreased enzyme activity but increased significantly the frequency of misincorporation. With poly(dT)·oligo(dA)$_{10}$, a 1:10 template/primer ratio was necessary for optimal activity and the α_2 fractions were somewhat unfaithful. A ratio of 1:1 further increased the misincorporation frequency four- to fivefold. Poly(dC)·oligo(dG)$_{12-18}$ was found to give optimal activity at a 1:1 ratio (within a range of 1:10–10:1). In no case was dTMP incorporation detected by using this template–primer.

Misincorporation assays also were performed with α, β, and γ preparations from glycerol gradients because some dATPase activity was observed in α_1 preparations from phosphocellulose and the γ fraction from phosphocellulose contained a 3′ → 5′ exonuclease activity (see below). (No other phosphocellulose fractions had detectable dNTPase.) After these contaminating activities were separated by sedimentation, polymerase fidelities did not change (data not shown, but see Table VII for β-polymerase).

Dependence of β-Polymerase Fidelity upon Enzyme Species. The various forms of β-polymerase differed in their fidelities in the presence of Mn^{2+}. (Comparisons in Mg^{2+} were precluded by low activity.) With either poly(dA)·poly(dT) or poly(dI)·poly(dC), misincorporation by β_1 was significantly greater than that by β_2 with fractions from phosphocellulose (Tables IV and V) or after sedimentation through glycerol

Table VII: Misincorporation by β-Polymerase Species[a]

polymerase fraction	poly(dA)·poly(dT) incorpn dAMP (pmol)	dGMP (fmol)	dCMP (fmol)	error frequency	poly(dI)·poly(dC) incorpn dGMP (pmol)	dTMP (fmol)	error frequency
0.02 M Tris-glycerol gradient fraction							
β_1 (6.3 S)	18	12		1/1500	22	19	1/1200
β_2 (8.6 S)	85	<10		<1/8500	124	<13	<1/9600
β_2 (4.8 S)	211	38		1/5600			
0.55 M KCl-glycerol gradient fraction							
β_1 (4.6 S)	186	42		1/4400			
β_2 (4.8 S)	91	15		1/6000	86	9	1/9500
"dissociation" in vitro of β_2							
undiluted; no preincubn	86	<4		<1/22000			
undiluted; preincubd	50	<4		<1/13000			
diluted; preincubd at 23 °C	56	11		1/5100			
diluted; preincubd at 30 °C	57	14		1/4100			
diluted; preincubd at 37 °C	57	17		1/3400			
oligo(dT)–cellulose chromatography of β_1 (3.2 S)	132	104		1/1300			
phosphocellulose rechromatography of β_2 (3.6 S)	167	8		1/21000			
	148		8	1/19000			

[a] Glycerol gradient fractions are described under Experimental Procedures. For in vitro dissociation experiments, the enzyme was diluted two- to threefold with buffer IV and preincubated for 30 min at 23, 30, or 37 °C. To obtain the oligo(dT)-cellulose fraction, β_1 was adsorbed onto an oligo(dT)–cellulose column and eluted with a linear gradient from 0.05 to 0.5 M KCl in 0.01 M Tris-HCl, pH 7.4, 0.5 mM dithiothreitol, 1 mM 2-mercaptoethanol, and 10% glycerol (v/v). The resulting activity sedimented at 3.2 S in glycerol gradients containing 0.5 M KCl. For rechromatography of β_2 on phosphocellulose, the enzyme was incubated at 4 °C for 4 h with 0.5 M NaCl, diluted eightfold with buffer IV, and subjected to phosphocellulose chromatography as previously described. The resulting activity sedimented at 3.6 S in 0.5 M KCl-glycerol gradients. Assays are described under Experimental Procedures.

gradients containing low salt (Table VII). However, after dissociation of the 8.6S β_2 species to a 4.8S form by sedimentation through glycerol gradients containing 0.55 M KCl, the fidelity decreased and approached that of β_1 (Table VII). [The small amount of β_2 that sedimented in low salt gradients at 4.8 S also had low fidelity (Table VII)]. Misincorporation by the β_1 species was unchanged after dissociation from the 6.3S to a 4.6S form by sedimentation in high salt (Table VII).

For a test of whether the β_2 species could be "dissociated" without sedimentation, it was incubated with 0.5 M NaCl and then diluted and assayed for fidelity. Misincorporation did increase, although greater than 50% of the enzymatic activity was lost (data not shown). Subsequently, it was found that a simple 1:3 dilution of enzyme followed by 30 min at 23–37 °C before assay induced a decrease in fidelity (Table VII). Sedimentation of enzyme in low salt after this treatment showed a broadening of the enzyme peak toward the 6.3S region (data not shown). The preincubation alone resulted in a 35% decrease in activity but had no effect upon fidelity (Table VII).

Other forms of β-polymerase were also observed to have altered fidelities. A species which sedimented at 3.2 S in glycerol gradients containing high salt concentration was obtained after chromatography of the β_1-polymerase upon oligo(dT)–cellulose. Also a 3.6S form was isolated after rechromatography of the β_2 species upon phosphocellulose after exposure in high salt. The 3.2S form was extremely unfaithful (Table VII), whereas the misincorporation frequency of the 3.6S species, roughly 1/20000 (Table VII), is comparable to that observed in a recent report for placental β-polymerase (Seal et al., 1979). Homogeneous β-polymerase (3.4S) from calf thymus and KB cells incorporated dCMP at a frequency of 1/10000 with poly(dA)·oligo(dT) (Wang et al., 1977; Chang, 1973).

Effect of Nucleoside Triphosphate Concentrations upon Fidelity. The incorporation of complementary and noncomplementary nucleotides was measured with various relative

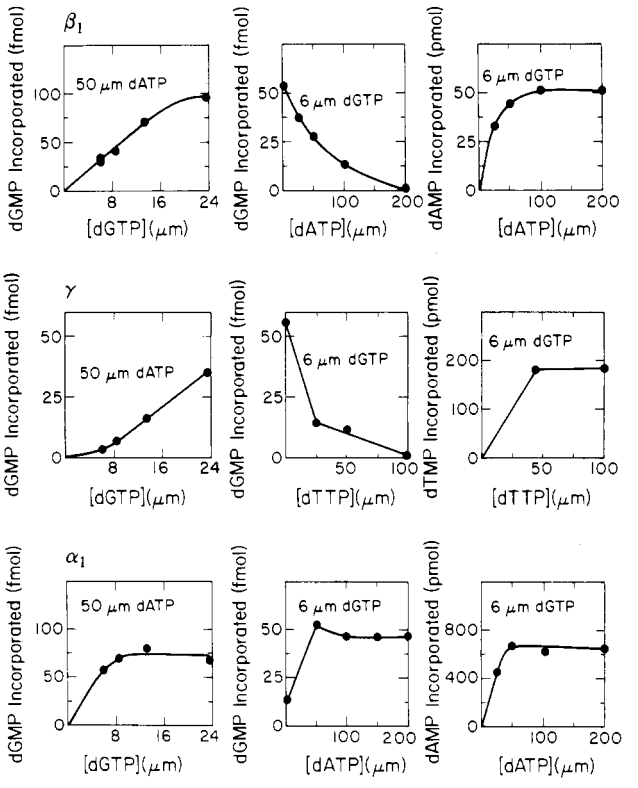

FIGURE 3: Effect of varying triphosphate concentrations upon incorporation. Amounts of enzymes used were as follows: β_1 and γ, 0.04 unit; α_1, 0.7 unit. The β_1 and α_1 fractions were assayed with poly(dA)·poly(dT). In the absence of enzyme, 15 fmol of dGMP was "acid insoluble". γ-Polymerase was assayed with poly(rA)·oligo(dT) under the conditions of Byrnes et al. (1976), and 20 fmol of dGMP was acid insoluble in the absence of enzyme.

concentrations of the triphosphates (Figure 3). For the β_1- and γ-polymerases, increasing the concentration of the noncomplementary triphosphate (dGTP) from 6 to 24 μM resulted

in an increasing level of incorporation of the noncomplementary nucleotide. However, for α_1-polymerase, a limit was reached at 10 μM dGTP. In these reactions, the level of complementary triphosphates was saturating (50 μM).

In another series of experiments, the concentration of noncomplementary triphosphate (dGTP) was held constant at 6 μM and the concentration of complementary triphosphate (dATP or dTTP) was increased from 50 to 200 μM (Figure 3). The effect on misincorporation also differed with the polymerase being tested (Figure 3). In the case of the β- and γ-polymerases, misincorporation steadily decreased to zero with increased concentrations of complementary triphosphates. (The level of incorporation of complementary triphosphate remained constant as seen in the right-hand panels.) By contrast, with the α-polymerase misincorporation increased and reached a maximum where total synthesis was maximal; then it was *not* reduced by further increases in the amount of complementary triphosphate. In addition, with α-polymerase raising the dGTP from 6 to 200 μM did not reduce the incorporation of the complementary dATP (data not shown). It would appear that triphosphate selection is less "competitive" with the α activity than with the β- and γ-polymerase fractions.

Exonuclease Activities of the Fractions. Each placental polymerase fraction was assayed for 3′ → 5′ exonuclease activity to test for the presence of an unassociated 3′ → 5′ exonuclease activity that might function as a proofreader. Phosphocellulose fractions were incubated with poly(dA-[^{3}H]dT) labeled at the 3′ end with [^{32}P]dG, and the release of each label was monitored. α-Polymerase fractions were generally free of nuclease, but some β preparations, particularly β_1, contained small amounts of DNase activity; however, this activity did not appear to be exclusively 3′ → 5′ in polarity as there was no preferential release of the terminal ^{32}P label. In addition, the nuclease activities associated with β fractions did not show a selectivity for the removal of mismatched (dGMP) termini vs. matched termini from poly(dA-dT).

The γ fraction preferentially released ^{32}P from the 3′ termini. Since polymerization fidelity or γ might be affected by this 3′ → 5′ exonuclease activity, we tested whether the exonuclease of the γ fraction could "edit" mismatched 3′-terminal deoxyribonucleotides under conditions of polymerization. When samples of γ phosphocellulose fractions were incubated with either poly(dA-dT)–[^{3}H]d(G)$_{1,9}$ or poly(dA-dT)–[^{3}H]-d(T)$_{0,2}$ in the presence of complementary triphosphates, neither label was released under conditions for polymerization. Furthermore, as noted above, removal of the nuclease from the γ-polymerase by glycerol gradient sedimentation had no effect upon fidelity. Thus, the 3′ → 5′ exonuclease activity in the γ fraction does not appear to edit in the presence of complementary triphosphates.

Discussion

Only placentas obtained within minutes of delivery by Caesarian section were used because extracts from frozen tissue gave variable activity profiles after DEAE-cellulose chromatography. The placental extracts contained more γ activity relative to α and β than is generally reported with other tissue or cell sources; this difference could be due to the sonication procedure which would rupture mitochondrial membranes, releasing the γ-type mitochondrial polymerase (Knopf et al., 1976).

Placental extracts possibly could contain viral reverse transcriptase since viral-type C particles are sometimes detected in normal human placenta. However, only one polymerase fraction (γ) was highly active with poly(rA)·oligo(dT), and this fraction was not inhibited by 5 mM phosphate as are the C-viral reverse transcriptases (Modak & Marcus, 1977). Furthermore, increased concentrations of complementary triphosphates decreased the level of misincorporation in the γ fraction, whereas with avian myeloblastosis virus polymerase the incorporation of noncomplementary triphosphates was not reduced by a 20-fold excess of complementary triphosphates (Battula et al., 1975).

The placental γ-polymerase fraction from phosphocellulose contained 3′ → 5′ exonuclease activity, and Byrnes et al. (1976) have described the isolation from erythroid hyperplastic bone marrow of a large polymerase, δ, that has an associated 3′ → 5′ exonuclease activity. The placental enzyme fraction was not δ-polymerase, however, as it did not prefer poly(dA-dT) over "activated" DNA under δ conditions and it was active with poly(rA)·oligo(dT). Perhaps the placental γ fraction was contaminated with one of the placental exonucleases described by Hamilton & Grossman (1978).

Although it is premature to determine a correspondence between the partially purified placental α species and those obtained in other systems by procedures which are not identical with those described here, our extraction procedures when applied to IMR-90-cultured human fibroblasts yield two fractions which appear to be analogous to α_1 and α_2. Multiple α-polymerases have also been observed in murine, avian, and bovine tissues (Hackmann & Lezius, 1975; Fry & Weisman-Shomer, 1976; Matsukage et al., 1976; Pedrali-Noy & Weissbach, 1977; Wickremasinghe et al., 1977) and recently in a transformed human cell line from which one species has been purified to homogeneity (Fisher & Korn, 1977). The heterogeneity could be physiologically significant because an increase in the activity of α-polymerase correlates with increased cellular and viral DNA synthesis (Pritchard et al., 1978; Edenberg et al., 1978; Chiu & Baril, 1975; Hübscher et al., 1977).

While both α_1 and α_2 use polymeric templates with oligomeric primers, the rates of incorporation and misincorporation are dependent upon the length of the primer, the primer/template molar ratio, and the divalent cation. Lengthening the primer ensures formation of a more stable duplex, and changing the relative concentration of primer to template could cause a shift from triple- to double-stranded complexes (Cassani & Bollum, 1969). Likewise, in reactions with Mg^{2+} and poly(dT)·oligo(dA)$_{19-24}$, the α activity was decreased, perhaps due to the formation of triple-stranded complexes (Cassani & Bollum, 1969).

The relatively high fidelities of the α-polymerases reported here, while in agreement with those for α-polymerase from regenerating rat liver (Salisbury et al., 1978), contrast with determinations for the human placental enzyme by Seal et al. (1979). Since primer length as well as the primer/template molar ratio affects fidelity (Table VI), the nuclease reported in the latter placental preparations might have been responsible for the higher misincorporation in those studies utilizing oligomer–polymer primer–templates. On the other hand, the higher error frequency of those preparations with poly(dA-dT) in the presence of Mn^{2+} (1/3000) raises the interesting possibility that, apart from differences in assay conditions, fidelity of α-polymerase may be dependent upon "factors" which can be removed during isolation. Conversely, the higher misincorporation could have been due to a small amount of γ-polymerase present in the α preparation. (A distinct peak of γ-polymerase was not observed in that purification procedure.)

The multiple forms of β-polymerase are of interest with regard to fidelity variations. Multiple forms of β-polymerase have been observed for calf thymus (Chang, 1973), human

KB cells (Wang et al., 1975), murine tissues (Hecht, 1975), and Novikoff hepatoma cells (Mosbaugh et al., 1977). In these cases, the enzyme was shown to undergo salt-dependent disaggregation, and specific proteins have been reported that promote reaggregation, stimulate enzymatic activity, confer stability, or convert one form of the enzyme to another (Probst et al., 1975; Wang et al., 1975; Mosbaugh et al., 1977). Now it appears that enzyme specificity also correlates with the β-enzyme form. Conversely, while the placental γ-polymerase also had altered sedimentation characteristics after exposure to high salt or oligo(dT)–cellulose, alteration of fidelity was not observed (unpublished observations).

While standardized conditions were utilized to measure the relative fidelity of the polymerases, several potential uncertainties in the interpretation of such data might be pointed out. First, the replacement of Mg^{2+} by Mn^{2+} often leads to enhanced misincorporation by DNA polymerases (Berg et al., 1962; Hall & Lehman, 1968; Dube & Loeb, 1975), and the most clear-cut differences among the placental enzymes were with Mn^{2+} present. However, only quantitative effects have been reported when substituting Mn^{2+} for Mg^{2+}: Hall & Lehman (1968) showed that mutagenic T4 phage DNA polymerases exhibited the expected changes—although magnified—with Mn^{2+} present; Linn et al. (1976) found that fidelity changes with aging were demonstrated with either Mg^{2+} or Mn^{2+} present; Wang et al. (1977) found that the frequency of noncomplementary nucleotides adjacent to complementary nucleotides in β-polymerase reactions is the same with either Mg^{2+} or Mn^{2+}.

A second caution is that studies using synthetic polymers may not accurately reflect the events occurring during synthesis of natural DNA, since the nature of the template has been found to affect the error frequency. Loeb has developed a misincorporation assay in which ϕX174 DNA containing an amber mutation is copied in vitro by DNA polymerase and errors are determined by measuring the reversion of progeny phage to wild type (Weymouth & Loeb, 1978). With both *E. coli* polymerase I and AMV reverse transcriptase, results with ϕX174 correlated well with those obtained with synthetic polymers (Loeb, 1978).

Finally, the relative and absolute concentrations of complementary and noncomplementary triphosphates, the length of primer, and the primer/template ratio affected the error frequency. These effects might account in part for the range of misincorporation frequencies reported in the literature for a particular type of eucaryotic polymerase. In addition, the qualitatively different response to changes in triphosphate concentration by α- vs. β- or γ-polymerases requires that any comparison of fidelities of various enzyme fractions be limited to a defined set of conditions. The effect of nucleotide concentration on polymerization fidelity may be pertinent to the suggestion that in vivo mutagenesis is influenced by the relative levels of the dNTP pools (Peterson et al., 1978).

Besides the effect of relative triphosphate concentrations, several other fundamental differences of mechanism have also been reported that distinguish α- from β- and γ-polymerases. The "6–8" calf thymus polymerase carries out pyrophosphorolysis and pyrophosphate exchange, whereas the "3.4S" enzyme does neither (Chang & Bollum, 1973). The large cytoplasmic "C" polymerase from KB cells also carries out PP_i exchange (Sedwick et al., 1972). Inhibition by 0.2 M PP_i is noncompetitive with respect to triphosphate for the 6–8S polymerase but is competitive for the 3.4S polymerase (Chang & Bollum, 1973). α-Polymerase is uniquely resistant to inhibition by 2′,3′-dideoxythymidine 5′-triphosphate whereas the β and γ enzymes are inhibited (Edenberg et al., 1978; Waquar et al., 1978). Edenberg et al. (1978) suggest that since 2′,3′-dideoxythymidine 5′-triphosphate is a chain terminator once incorporated, the resistance of α-polymerase to this analogue is the result of an enhanced ability to discriminate between the analogue and TTP. Finally, the small polymerases of calf thymus and KB cells, as well as γ-polymerase, can effectively utilize mispaired primer termini, whereas the large cytoplasmic enzymes cannot (Sedwick et al., 1972; Chang, 1973). How these differences might relate to the fidelity differences observed between the classes of enzymes remains to be determined.

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References

Battula, N., Dube, D., & Loeb, L. A. (1975) *J. Biol. Chem. 250*, 8404–8408.

Berg, P., Fancher, H., & Chamberlin, M. (1962) *Informational Macromolecules* (Vogel, H. J., Bryson, V., & Lampen, J., Eds.) p 1467, Academic Press, New York.

Berkner, K. L., & Folk, W. R. (1977) *J. Biol. Chem. 252*, 3176–3184.

Brutlag, D., & Kornberg, A. (1972) *J. Biol. Chem. 247*, 241–248.

Burnet, M. F. (1974) *Intrinsic Mutagenesis*, Medical and Technical Publishing Co., Ltd., Lancaster, Great Britain.

Byrnes, J. J., Downey, K. M., Black, V. L., & So, A. G. (1976) *Biochemistry 15*, 2817–2823.

Cassani, G. R., & Bollum, F. J. (1969) *Biochemistry 8*, 3928–3936.

Chang, L. M. S. (1973) *J. Biol. Chem. 248*, 6983–6992.

Chang, L. M. S., & Bollum, F. J. (1973) *J. Biol. Chem. 248*, 3398–3404.

Chiu, R. W., & Baril, E. F. (1975) *J. Biol. Chem. 250*, 7951–7957.

Dube, D. K., & Loeb, L. A. (1975) *Biochem. Biophys. Res. Commun. 67*, 1041–1046.

Edenberg, H. J., Anderson, S., & DePamphilis, M. L. (1978) *J. Biol. Chem. 253*, 3273–3280.

Fisher, P. A., & Korn, D. (1977) *J. Biol. Chem. 252*, 6528–6535.

Fry, M., & Weisman-Shomer, P. (1976) *Biochemistry 15*, 4319–4328.

Gefter, M. L. (1975) *Annu. Rev. Biochem. 44*, 45–77.

Gillin, F. D., & Nossal, N. G. (1976a) *J. Biol. Chem. 251*, 5219–5224.

Gillin, F. D., & Nossal, N. G. (1976b) *J. Biol. Chem. 251*, 5225–5232.

Hackmann, H., & Lezius, A. (1975) *Eur. J. Biochem. 50*, 357–366.

Hall, Z. W., & Lehman, I. R. (1968) *J. Mol. Biol. 36*, 321–333.

Hamilton, L., & Grossman, L. (1978) *Mutat. Res. 53*, 195.

Hecht, N. B. (1975) *Biochim. Biophys. Acta 383*, 388–398.

Hopfield, J. J. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4135–4139.

Hübscher, U., Kuenzle, C. C., & Spadari, S. (1977) *Nucleic Acids Res. 4*, 2917–2929.

Knopf, K-W., Yamada, M., & Weissbach, A. (1976) *Biochemistry 15*, 4540–4548.

Kuhnlein, U., Penhoet, E. E., & Linn, S. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1169–1173.

Lehman, I. R., Bessman, M. J., Simms, E. S., & Kornberg, A. (1958) *J. Biol. Chem. 233*, 163-170.
Linn, S., Kairis, M., & Holliday, R. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2818-2622.
Loeb, L. (1978) *J. Supramol. Struct., Suppl. 2*, 284 (abstract).
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265-275.
Matsukage, A., Sivarajan, M., & Wilson, S. H. (1976) *Biochemistry 15*, 5305-5314.
Modak, M. J., & Marcus, S. L. (1977) *J. Biol. Chem. 252*, 11-19.
Modrich, P., & Lehman, I. R. (1970) *J. Biol. Chem. 245*, 3626-3631.
Mosbaugh, D. W., Stalker, D. M., Probst, G. S., & Meyer, R. R. (1977) *Biochemistry 16*, 1512-1518.
Ohashi, M., Takahiko, T., & Ikegami, S. (1978) *Biochim. Biophys. Acta 82*, 1084-1090.
Pedrali-Noy, G., & Weissbach, A. (1977) *Biochim. Biophys. Acta 477*, 70-83.
Peterson, A. R., Landolph, J. R., Peterson, H., & Heidelberger, C. (1978) *Nature (London) 276*, 508-510.
Pritchard, C., Bates, R. C., & Stout, E. R. (1978) *Virology 27*, 258-261.
Probst, G. S., Stalker, D. M., Mosbaugh, D. W., & Meyer, R. R. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 1171-1174.
Salisbury, J. C., O'Connor, P. J., & Saffhill, R. (1978) *Biochim. Biophys. Acta 517*, 181-185.
Schlabach, A., Fridlender, B., Bolden, A., & Weissbach, A. (1971) *Biochem. Biophys. Res. Commun. 44*, 879-885.
Seal, G., Shearman, C. W., & Loeb, L. A. (1979) *J. Biol. Chem. 254*, 5229-5237.
Sedwick, W. D., Wang, T. S.-F., & Korn, D. (1972) *J. Biol. Chem. 247*, 5026-5033.
Szilard, L. (1959) *Proc. Natl. Acad. Sci. U.S.A. 45*, 30-45.
Wang, T. S.-F., Sedwick, W. D., & Korn, D. (1975) *J. Biol. Chem. 250*, 7040-7044.
Wang, T. S.-F., Eichler, D. C., & Korn, D. (1977) *Biochemistry 16*, 4927-4933.
Waquar, M. A., Evans, M. J., & Huberman, J. A. (1978) *Nucleic Acids Res. 5*, 1933-1946.
Weissbach, A., Baltimore, D., Bollum, F. J., Gallo, R., & Korn, D. (1975) *Eur. J. Biochem. 59*, 1-2.
Weymouth, L. A., & Loeb, L. A. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 1924-1928.
Wickremasinghe, R., Hesselwood, I., Holmes, A., & Johnston, I. (1977) *FEBS Lett. 78*, 139-142.

Modification of Cytidines in a Qβ Replicase Template: Analysis of Conformation and Localization of Lethal Nucleotide Substitutions†

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ABSTRACT: The solution conformation of MDV-1(+) RNA, a small RNA template replicated autocatalytically in vitro by Qβ replicase, was investigated with sodium bisulfite, a reagent that selectively converts single-stranded cytidines to uridines. The reactivity of 45 of the 76 cytidines in MDV-1(+) RNA was determined by nucleotide sequence analysis. Only 14 of these 45 cytidines were converted to uridine. Treatment of the RNA with methoxyamine, another single-strand-specific cytidine modification reagent, gave results in good agreement with the bisulfite data. The limited reactivity of MDV-1(+) RNA with these reagents indicates that it is a highly structured molecule. A secondary structure consistent with the chemical modification data is proposed. Modification of MDV-1(+) RNA by bisulfite renders it inactive as a template for RNA replication. This inactivation and the modification of the cytidines at the 3′ end of the molecule occur at very similar rates. By using a short complementary RNA "mask" to protect just these cytidines, we demonstrated that the loss of activity resulted from their modification. This implies that one or more of the cytidines in the 3′-terminal sequence is required for template activity and that changes within this sequence can have lethal consequences. The effects of modification elsewhere in the sequence are discussed.

Qβ replicase is the RNA-directed RNA polymerase that replicates the single-stranded RNA genome of coliphage Qβ [Haruna & Spiegelman, 1965; see Blumenthal & Carmichael (1979) for a review]. This enzyme displays a high degree of template specificity for Qβ RNA and its complement over cellular or other phage RNAs (Haruna & Spiegelman, 1965; Feix et al., 1968; Okada et al., 1971). Only two other types of RNAs are templates for autocatalytic replication by Qβ replicase in vitro: the "Qβ RNA variants" and the so-called "6S RNAs".[1] The Qβ variants were generated in vitro by extensive replication of Qβ RNA (Mills et al., 1967). These RNAs, which hybridize to Qβ RNA (Mills et al., 1968), are approximately 550 nucleotides long. The 6S RNAs actually range in size from 91 to 221 nucleotides. They were originally isolated from in vitro Qβ replicase reactions that did not

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[1] Qβ replicase carries out a limited polymerization on certain other templates, but they are not replicated autocatalytically [see Schaffner et al. (1977) for a summary].

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