Marsh, W. H., & Fitzgerald, P. J. (1973) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 32*, 2119-2125.
Mathis, D. J., & Chambon, P. (1981) *Nature (London) 290*, 310-315.
Mathis, D. J., Oudet, P., Wasylyk, B., & Chambon, P. (1978) *Nucleic Acids Res. 5*, 3523-3547.
McGhee, J. D., & Felsenfeld, G. (1980) *Annu. Rev. Biochem. 49*, 1115-1156.
McGhee, J. D., Rau, D. C., Charney, E., & Felsenfeld, G. (1980) *Cell (Cambridge, Mass.) 22*, 87-96.
Nelson, D. A., Perry, M., Sealy, L., & Chalkley, R. (1978) *Biochem. Biophys. Res. Commun. 82*, 1346-1353.
Noll, M., Thomas, J. O., & Kornberg, R. D. (1975) *Science (Washington, D.C.) 187*, 1203-1206.
Pehrson, J., & Cole, R. D. (1980) *Nature (London) 285*, 43-44.
Perry, M., & Chalkley, R. (1981) *J. Biol. Chem. 256*, 3313-3318.
Perry, M., Nelson, D., Moore, M., & Chalkley, R. (1979) *Biochim. Biophys. Acta 561*, 517-525.
Poupko, J. M., Kostellon, A. B., & Morrill, G. A. (1977) *Differentiation (Berlin) 8*, 167-174.
Prasad, K. N., & Sinha, P. K. (1976) *In Vitro 12*, 125-132.
Reeves, R., & Candido, E. P. M. (1979) *Biochem. Biophys. Res. Commun. 89*, 571-579.
Reeves, R., & Cserjesi, P. (1979) *J. Biol. Chem. 254*, 4283-4290.
Riggs, M. G., Whittaker, R. G., Neumann, J. R., & Ingram, V. M. (1977) *Nature (London) 268*, 462-464.
Ruiz-Carrillo, A., Wangh, L. J., & Allfrey, V. G. (1975) *Science (Washington, D.C.) 190*, 117-128.
Schaffhausen, B., & Benjamin, T. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1092-1096.
Sealy, L., & Chalkley, R. (1978a) *Cell (Cambridge, Mass.) 14*, 115-121.
Sealy, L., & Chalkley, R. (1978b) *Nucleic Acids Res. 5*, 1863-1876.
Shewmaker, C. K., & Wagner, T. E. (1980) *Eur. J. Biochem. 107*, 505-510.
Simpson, R. T. (1978) *Cell (Cambridge, Mass.) 13*, 691-699.
Tack, L. O., & Simpson, R. T. (1979) *Biochemistry 18*, 3110-3118.
Thoma, F., Koller, Th., & Klug, A. (1979) *J. Cell Biol. 83*, 403-427.
Traub, P., & Boeckmann, G. (1978) *Hoppe-Seyler's Z. Physiol. Chem. 359*, 571-579.
Vidali, G., Boffa, L. C., Bradbury, E. M., & Allfrey, V. G. (1978a) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2239-2243.
Vidali, G., Boffa, L. C., Mann, R. S., & Allfrey, V. G. (1978b) *Biochem. Biophys. Res. Commun. 82*, 223-227.
Weintraub, H. (1980) *Nucleic Acids Res. 8*, 4745-4756.
Weisbrod, S., Groudine, M., & Weintraub, H. (1980) *Cell (Cambridge, Mass.) 19*, 289-301.
Whitlock, J. P., Augustine, R., & Schulman, H. (1980) *Nature (London) 287*, 74-76.
Wintrobe, M. M., Ed. (1974) *Clinical Hematology*, 7th ed., p 9, Lea & Febiger, Philadelphia, PA.
Xue, S., & Rao, P. N. (1981) *J. Cell Sci. 51*, 163-171.

Changes in DNA Polymerases α, β, and γ during the Replicative Life Span of Cultured Human Fibroblasts†

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ABSTRACT: DNA polymerases from IMR-90 human diploid fibroblasts at various passage levels and from HeLa cells were purified and fractionated into α_1, α_2, α_3, β, and γ species and subspecies, and then the accuracy with which each one copied synthetic template-primers was measured in the presence of Mn^{2+} or Mg^{2+}. All activities from fibroblasts of later population doubling levels incorporated noncomplementary triphosphates more frequently than did the same polymerase type from earlier population doubling levels. HeLa polymerase activities copied several different templates in the presence of Mn^{2+} with greater fidelity than enzymes from fibroblasts of population doubling level 27 or greater. The total DNA polymerase activity extracted from IMR-90 cells decreased with increasing population doubling levels. The α-polymerase activity generally declined with increasing population doubling levels, while β-polymerase activity remained relatively constant, except at the very end of the cellular replicative life span. In addition, the amounts of α_2 and α_3 became progressively lower relative to α_1, and a new α-type polymerase activity, α_0, appeared upon diethylaminoethylcellulose chromatography. HeLa cells also contained three α species, though two of them eluted from diethylaminoethylcellulose at higher phosphate concentrations than α species from fibroblasts. Postconfluent IMR-90 cells of population doubling level 21 had a decreased level of α-polymerase relative to that recovered from rapidly growing cells. This polymerase activity had some chromatographic properties similar to enzyme from late-passage cells. In addition, the α-, β-, and γ-polymerases from these cells had decreased fidelities relative to those isolated from subconfluent cells.

Alterations in the fidelity of DNA polymerases are expected to engender profound biological consequences. For example, several T4 mutants exhibit a relationship between changes in DNA polymerase fidelity and an increased or decreased mutation frequency (Speyer et al., 1966; Hall & Lehman, 1968). These altered mutation frequencies are often due to abnormal ratios of DNA polymerase activity to editing exonuclease activity (Muzyczka et al., 1972), though at least one of the T4 mutant enzymes selects noncomplementary triphosphates more frequently than does wild-type enzyme (Gillin & Nossal,

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0006-2960/82/0421-1002$01.25/0

1976). In mammalian systems, DNA polymerases with decreased accuracy have been isolated from MRC-5 human diploid fibroblasts at late passage levels (Linn et al., 1976; Murray & Holliday, 1981), from livers of aged rats (Barton et al., 1974), from rat liver after exposure to the carcinogen *N*-(2-fluoroethyl)acetamide (Chan & Becker, 1979), and from rat liver fibroblasts after treatment with mitomycin C (Bozhkov et al., 1978). These findings suggest that an accumulation of mutations might have a role in degenerative aging processes, certain genetic diseases, or in the genesis of cancer (Szilard, 1959; Burnet, 1974; Medvedev, 1972a,b). However, the biochemical basis for the decreased polymerase fidelity in the mammalian situations has not been elucidated, though one might hypothesize that it is due to primary or secondary structural alterations in the polymerase, to posttranslational modifications of normal polymerase, to the appearance of new, error-prone polymerase species, or to an alteration of polypeptide components involved in assuring fidelity during DNA replication.

In order to distinguish among these possibilities directly, individual α-, β-, and γ-polymerase activities with altered fidelities must be examined and their properties compared with those of highly accurate enzymes. Therefore, the initial observation of Linn et al. (1976), that DNA polymerase activity from late-passage human fibroblasts incorporates incorrect nucleotides as much as 10 times more frequently than enzyme from a low population doubling level, has been extended. First, procedures were developed with human placental DNA polymerases to fractionate the activities, rigorously identify their type, and measure their relative fidelities under standardized assay conditions with defined template-primers (Krauss & Linn, 1980). Now, the adaptation of these procedures to α, β, and γ types of DNA polymerase obtained from IMR-90 fibroblasts at several population doubling levels and from HeLa cells is reported. The former are normal diploid human fibroblasts of limited replication potential (a model system for in vitro aging studies), whereas the latter cells originate from a human tumor and can replicate indefinitely. Additional studies also compare the properties of DNA polymerases from postconfluent to those of proliferating IMR-90 cells at a low population doubling level.

Experimental Procedures

Materials. DEAE-cellulose, type 40, was from Brown Co., Berlin, NH; phosphocellulose (P11) was from Whatman. All synthetic polynucleotides, oligonucleotides, and unlabeled triphosphates were from P-L Biochemicals. ^{3}H-Labeled deoxyribonucleoside triphosphates were from Amersham or New England Nuclear. Salmon sperm DNA was activated according to the procedure of Schlabach et al. (1971). Homogeneous *Escherichia coli* polymerase I was the generous gift of Dr. Arthur Kornberg, Stanford University.

Buffer I contained 0.02 M potassium phosphate, pH 7.6, 0.5 mM dithiothreitol, and 10 mM 2-mercaptoethanol. Buffer II contained 0.4 M potassium phosphate, pH 7.6, 0.5 mM dithiothreitol, and 10 mM 2-mercaptoethanol. Buffer III contained 0.02 M potassium phosphate, pH 7.6, 0.5 mM dithiothreitol, and 1 mM 2-mercaptoethanol. Buffer IV contained 0.05 M potassium phosphate, pH 7.6, 0.5 mM dithiothreitol, and 1 mM 2-mercaptoethanol. Buffer V contained 0.05 M potassium phosphate, pH 7.6, 0.5 mM dithiothreitol, 1 mM 2-mercaptoethanol, and 20% glycerol.

Polymerase Assay. Enzyme activity was assayed by using activated salmon sperm DNA as described by Linn et al. (1976). KCl was omitted when measuring α-polymerase activity. One unit of enzyme incorporates 1 nmol of total nucleotide in 30 min at 37 °C. Activities were calculated from assays which were within the linear ranges of time and enzyme concentration.

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. Reactions with homopolymer pairs contained a template:primer ratio of 1:1 (nucleotide residues). Reactions with poly(dT)·oligo(dA)$_{19-24}$ contained a template:primer ratio of 10:1. All preparations of polymers and triphosphates were tested in misincorporation assays with *E. coli* polymerase I with which there was no significant misincorporation. 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-polymerases contained 0.5 mM $MnCl_2$ and 100 mM KCl. Assays with Mg^{2+} instead of Mn^{2+} contained 1 mM $MgCl_2$.

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-labeled deoxyribonucleoside triphosphate (80–150 cpm/pmol) to measure total polymer synthesis and the other with noncomplementary ^{3}H-labeled deoxyribonucleoside triphosphate (12–30 cpm/fmol) to measure nonfaithful synthesis. The use of biased nucleotide pools in determining error frequencies has been discussed previously (Krauss & Linn, 1980). 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 by using controls from which template-primer was omitted and for terminal transferase by using controls with 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). Reactions were generally carried out with several levels of enzyme to assure a constant misincorporation frequency vs. the level of synthesis, but for brevity, only the highest levels are given in Tables I and II.

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

Cell Culture. IMR-90 human fetal lung fibroblasts, obtained from the Institute for Medical Research, Camden, NJ, were grown in Delbecco's modified Eagle's medium containing 10% (v/v) fetal calf serum (Irvine Scientific). When the cells reached confluence, they were subcultured by using trypsin at a 1:4 split ratio or, at passages above population doubling level (PDL)[1] = 38, a 1:2 split ratio. Under these conditions, the replicative life span was PDL = 46 ± 2. Cell cultures were monitored for mycoplasma contamination by the Institute for Medical Research, Camden, NJ.

HeLa cells in suspension culture were grown to densities of (2.5–7) × 10^5/mL in Joklik's modified Eagle's medium containing 5% fetal calf serum.

Fractionation of Polymerases from Cultured Cells. For harvesting, fibroblasts were rinsed with chilled 25 mM potassium phosphate, pH 7.0, 0.15 M NaCl, and 0.15 M citrate, detached by gently scraping with a rubber blade, and then washed twice with the above buffer after collection in an International clinical centrifuge (Model CL) at half-maximal

[1] Abbreviation: PDL, population doubling level.

speed for 3 min. HeLa cells were collected by centrifugation at 3300g for 10 min in a Sorvall centrifuge. The cells were washed twice with the above buffer after centrifugation at 700g for 10 min.

(*A*) *Step I.* The cell pellet was suspended in buffer I (7–10 mL/g wet weight cells), and the suspension was sonicated for six 10-s intervals by using the microtip of a Biosonic sonifier (Bronwill Scientific, Rochester, NY) at a probe intensity of "35". The suspension was then brought to 0.4 M phosphate by the addition of 1 M potassium phosphate, pH 7.6, left on ice for 30 min, and then centrifuged at 12000g for 10 min.

(*B*) *Step II.* The crude extract was passed over a column of DEAE-cellulose (10 mg of protein/mL of packed resin) previously equilibrated with buffer II. Flow-through fractions containing the majority of enzyme and protein were pooled and dialyzed for 3 h against 4 L of buffer III.

(*C*) *Step III.* The dialyzed extract was loaded onto a second DEAE-cellulose column (10 mg of protein/mL of packed resin) which had been equilibrated with buffer III. The column was washed with 2 column volumes of this buffer, and an 8 column volume linear gradient of potassium phosphate, pH 7.6, from 0.02 to 0.4 M containing 0.5 mM dithiothreitol and 1 mM 2-mercaptoethanol was applied. Fractions were collected into plastic tubes. β activity was eluted in the flow through and γ and α activities, respectively, during the gradient. If polymerase fractions were to be stored frozen, glycerol was added to 10% (v/v).

(*D*) *Step IV.* For further purification, the individual polymerase species were diluted to a conductivity equal to that of buffer IV, and glycerol was added to 20% (v/v). The enzyme was adsorbed onto phosphocellulose (10 mg of protein/mL of packed resin) and the column washed with buffer V and then eluted with a linear gradient (4 column volumes) from 0.02 to 0.4 M potassium phosphate, pH 7.6, containing 0.5 mM dithiothreitol, 1 mM 2-mercaptoethanol, and 20% glycerol. Fractions were collected into plastic tubes.

Results

Fractionation of DNA Polymerase Activities upon DEAE-cellulose. Previously, we fractionated DNA polymerase activities from human placenta and classified them among the α, β, and γ types by the criteria of size, by their ability to utilize various synthetic template-primers, and by sensitivity to phosphate, *N*-ethylmaleimide, aphidicolin, and 2′,3′-dideoxythymidine 5′-triphosphate (Krauss & Linn, 1980). This fractionation scheme has now been applied to human fibroblasts and to HeLa cells (see Experimental Procedures). Polymerase fractions from the cultured cells were verified as to their class by testing their sensitivity to aphidicolin (5 μg/mL) and 2′,3′-dideoxythymidine 5′-triphosphate (200 μM in the presence of 50 μM dTTP).

After chromatography of extracts from cultured cells on DEAE-cellulose (step III), three peaks of α-polymerase (α_1, α_2, and α_3) were usually obtained (Figure 1). For IMR-90 cells at low population doubling levels (PDL = 27, Figure 1; see also PDL = 21, Figure 2 below), these peaks eluted at 0.16, 0.22, and 0.27 M phosphate. Extracts from cells after further passaging (PDL = 37–45, Figure 1) contained decreased amounts of α_2 and α_3 relative to that of the α_1 fraction. In addition, there was an increase in α-polymerase activity eluting before 0.16 M phosphate. Since this activity preceded α_1, it was called α_0. The chromatographic profiles have been reproducible for several years, and peak fractions when reapplied to DEAE-cellulose eluted at the same salt concentration as previously. HeLa extracts contained α-polymerase activities which eluted at 0.23, 0.29, and 0.33 M phosphate (Figure 1).

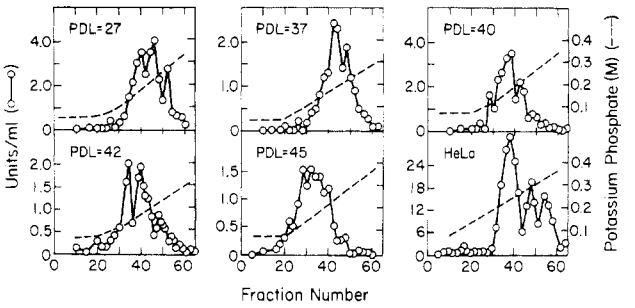

FIGURE 1: DEAE-cellulose chromatography. Dialyzed fractions of step II from IMR-90 fibroblasts and HeLa cells were applied to the columns as follows: PDL = 27, 37 units; PDL = 37, 15 units; PDL = 40, 136 units; PDL = 42, 36 units; PDL = 45, 29 units; HeLa, 690 units. Fractions were pooled as follows: PDL = 27; γ, 27–29; α_1, 36–41; α_2, 44–48; α_3, 41–43; PDL = 37; γ, 27–29; α_0 = 36–39; α_1, 41–45; α_2, 47–50; PDL = 40; γ, 23–25; α_0, 27–29; α_1, 32–39; α_2, 41–45; α_3, 48–50; PDL = 42; α_0, 30–35; α_1, 37–44; α_2, 46–50; α_3, 51–53; PDL = 45; γ, 21–23; α_0, 26–29; α_1, 31–36; α_2, 39–42; α_3, 47–49. Fraction volumes were as follows: PDL = 27, 0.33 mL; PDL = 37, 0.40 mL; PDL = 40, 0.70 mL; PDL = 42, 0.65 mL; PDL = 45, 0.48 mL; HeLa, 0.85 mL.

In all cases, γ-polymerase was adsorbed by DEAE-cellulose less strongly than α activity, and β-polymerase was not adsorbed.

Yields of Polymerase Activities. The yields and specific activity of total DNA polymerase assayed in crude extracts (step I) of IMR-90 cells decreased 6-fold from 174 units/g of cells (5.6 units/mg of protein) when population doubling levels increased from 21 to 45. Yields of α-polymerase activity isolated in step III decreased 8-fold from 54 units/g of cells, while the amount of β-polymerase activity remained relatively constant (10 units/g of cells) until near the end of the cellular replicative lifespan (PDL = 42 and 45). (γ-Polymerase was present in low amounts in these extracts since the sonication level during Step I was optimized for α and β recovery, and therefore may not have sufficiently disrupted mitochondria to release γ-polymerase). The yield of β activity from HeLa cells was comparable to that of IMR-90 cells, while the yield of α activity was 3-fold higher than PDL = 21, IMR-90 cells.

Fidelity of Polymerase Fractions. Misincorporation frequencies were measured by utilizing the polymerization of nonhomologous nucleotides into synthetic template-primers with either Mg^{2+} of Mn^{2+} present (Linn et al., 1976; Krauss & Linn, 1980). As a control for possibly interfering impurities in the template-primers or deoxyribonucleoside triphosphate preparations, error frequencies were determined with homogeneous *E. coli* polymerase I which synthesizes DNA very accurately.

After separation by DEAE-cellulose chromatography, DNA polymerase activities from IMR-90 and HeLa cells were tested for fidelity, in assays containing poly(dA-dT), poly(dA)·poly(dT), poly(rA)·oligo(dT)$_{10}$, poly(dT)·oligo(dA)$_{19-24}$, and poly(dI)·poly(dC) (Table I). In assays with Mn^{2+} present, increased frequencies of misincorporation were observed with each polymerase fraction isolated from IMR-90 cells at later passages compared to those isolated from cells of lower population doubling levels. Polymerase activity isolated from the α_0 region obtained from the low population doubling cells was relatively faithful; however, an increased error frequency was observed in extracts from cells at later passages (from 1/38000 to 1/3000). α_3, which was more unfaithful than α_1 and α_2 even at low population doubling levels, exhibited a further dramatic loss of fidelity at PDL = 40, 42, and 45 with poly(dT)·oligo(dA)$_{19-24}$. α_1 and α_2 were less faithful from PDL = 45 than from PDL = 27 cells by using poly(dA-dT) with

Table I: Misincorporation Frequencies of DNA Polymerase Fractions after DEAE-cellulose Chromatography[a]

polymerase fraction	cell source	poly(dA·dT) dTMP + dAMP (pmol)	poly(dA·dT) dGMP (fmol)	poly(dA·dT) error frequency	poly(dA)·poly(dT) dAMP (pmol)	poly(dA)·poly(dT) dCMP (fmol)	poly(dA)·poly(dT) error frequency	poly(rA)·oligo(dT)$_{10}$ dTMP (pmol)	poly(rA)·oligo(dT)$_{10}$ dGMP (fmol)	poly(rA)·oligo(dT)$_{10}$ error frequency	poly(dT)·oligo(dA)$_{19-24}$ dAMP (pmol)	poly(dT)·oligo(dA)$_{19-24}$ dGMP (fmol)	poly(dT)·oligo(dA)$_{19-24}$ error frequency	poly(dI)·poly(dC) dGMP (pmol)	poly(dI)·poly(dC) dTMP (fmol)	poly(dI)·poly(dC) error frequency
α_0	I-MR-90, PDL = 21										530	14	1/38000			
	40										168	18	1/9300			
	42										260	32	1/8100			
	45										220	73	1/3000			
	HeLa ∞										56	<11	<1/5100			
α_1	I-MR-90, PDL = 27	90	7	1/13000	157	<5	<1/31000				832	34	1/24000	401	19	1/21000
	27	232	<12	<1/19000[b]												
	37										276	14	1/20000	204	20	1/10000
	40	61	8	1/7600	61	7	1/8700				216	17	1/13000	233	25	1/9300
	42	73	9	1/8100							163	16	1/10000			
	45	61	7	1/8700	62	27	1/2300				181	21	1/8600	276	36	1/7700
	45	132	18	1/7300[b]												
	HeLa ∞	106	<6	<1/18000	73	<5	<1/15000				851	26	1/33000	614	24	1/26000
α_2	I-MR-90, PDL = 27	109	8	1/14000							327	22	1/15000	452	12	1/38000
	27	351	<12	<1/29000[b]												
	37	99	10	1/9900							181	18	1/10000	130	13	1/10000
	40	48	5	1/9600	47	9	1/5200				154	37	1/4200	291	24	1/12000
	42	46	7	1/6600							95	25	1/3800			
	45	24	15	1/1600	26	28	1/930				81	37	1/2200	128	19	1/6700
	45	63	36	1/1800[b]												
	HeLa ∞	91	<6	<1/15000							546	17	1/32000	1000	<13	<1/77000
α_3	I-MR-90, PDL = 27	45	8	1/5600	24	<5	<1/4800				134	20	1/6700			
	37															
	40	28	9	1/3100	28	10	1/2800				15	28	1/540	103	<7	<1/15000
	42	30	11	1/2700							18	26	1/690			
	45										4	23	1/170	29	11	1/2600
	HeLa ∞	65	<6	<1/11000							238	19	1/12000	376	<13	<1/29000
β	I-MR-90, PDL = 27	88	<6	<1/15000	11	<5	<1/2200				69	<12	<1/5800	53	<9	<1/5900
	37				10	8	1/1200							41	12	1/3400
	40	171	14	1/12000												
	42	49	20	1/2400							40	21	1/1900			
	45	14	9	1/1600												
	HeLa ∞	104	<6	<1/17000							37	<12	<1/3100	179	15	1/12000
γ	I-MR-90, PDL = 27	25	8	1/3100				73	<6	<1/12000[c]	74	<12	<1/6200	223	<10	<1/22000
	37							45	11	1/4100[c]				43	12	1/3600
	40	9	13	1/690	10	6	1/1700				57	14	1/4100			
	45	1	13	1/80	1	20	1/50				18	15	1/1200			
	HeLa ∞	20	<6	<1/3300							78	<6	<1/13000			

[a] Assays were as described under Experimental Procedures. [b] Reactions with 1 mM $MgCl_2$ in place of $MnCl_2$. [c] Reactions under conditions optimal for γ-polymerase (Knopf et al., 1976).

Table II: Misincorporation Frequencies of DNA Polymerase Fractions after Phosphocellulose Chromatography[a]

polymerase fraction	PDL	poly(dT)·oligo(dA)$_{19-24}$ dAMP (pmol)	poly(dT)·oligo(dA)$_{19-24}$ dGMP (fmol)	poly(dT)·oligo(dA)$_{19-24}$ error frequency	poly(dI)·poly(dC) dGMP (pmol)	poly(dI)·poly(dC) dTMP (fmol)	poly(dI)·poly(dC) error frequency
α_{0a}	27				6	<12	<1/500
	37				0.5	<12	<1/40
α_{0b}	27				8	16	1/500
	37				1	15	1/70
α_{1a}	27	177	13	1/14000	192	<12	<1/16000
	37	42	12	1/3500	22	<12	<1/1800
α_{1b}	27	66	<9	<1/7300	20	<12	<1/1700
	37	44	18	1/2400	7	<12	<1/580
α_{2a}	27	49	<5	<1/9800	29	<11	<1/2600
	37	16	8	1/2000	8	20	1/400
α_{2b}	27	15	10	1/1500	7	16	1/440
	37	14	8	1/1800	6	14	1/430
α_{3a}	27	27	<9	<1/3000			
β_1	27	9	9	1/1000			
	37	9	59	1/150			
β_2	27	25	<5	<1/5000			
	37	27	13	1/2100			

[a] Assays were as described under Experimental Procedures.

Mg^{2+} present. In fact, with these late-passage fractions, it is noteworthy that Mg^{2+} did not confer higher fidelity than Mn^{2+}, a conclusion which also can be made from data of Linn et al. (1976) and Murray & Holliday (1981). Apparently, the Mg^{2+}-dependent fidelity determinants are more perturbed in these later passage extracts. (Determinations for β-polymerase with Mg^{2+} present could not be made, because its activity is greatly reduced with Mg^{2+} in the presence of synthetic template-primers).

The α_1, α_2, α_3 and γ species isolated from HeLa cells were generally more faithful than those determined for comparable species from IMR-90, PDL = 27; HeLa β-polymerase also had a greater fidelity than the fibroblast enzyme when assayed with poly(dA-dT) or poly(dI)·poly(dC).

Further Fractionation of Polymerase Species. The individual α-, β-, and γ-polymerase fractions from DEAE-cellulose chromatography of extracts from IMR-90 PDL = 27 and PDL = 37 cells were adsorbed to phosphocellulose and then eluted with a gradient of increasing phosphate molarity. As observed previously with human placental polymerases (Krauss & Linn, 1980), α_1 and α_2 each resolved further into two subspecies, α_{1a}, α_{1b} and α_{2a}, α_{2b}, respectively. The α_1 fractions from PDL = 27 eluted at similar positions and proportions when compared to those from PDL = 37. The α_2 subspecies emerged slightly earlier than α_{1a} and α_{1b}, and the activity of the α_{2b} fraction became reduced in extracts from PDL = 37. α_3 activity from PDL = 27 extracts likewise exhibited subspecies. No α_3 activity was found in the PDL = 37 extracts. α_0 activity from PDL = 27 and 37 emerged from phosphocellulose at a lower concentration than α_1 or α_2 and also resolved into two subspecies in both cases. Finally, the proportion of the β_1 and β_2 activities from PDL = 27 was similar to those resolved from PDL = 37 preparations.

Fidelity of Polymerase Fractions after Phosphocellulose Chromatography. The polymerase activities obtained from phosphocellulose fractionation were assayed for misincorporation with poly(dT)·oligo(dA)$_{19-24}$ and poly(dI)·poly(dC) in the presence of Mn^{2+} (Table II). Decreases in fidelity of α_{0b}, α_{1a}, α_{1b}, α_{2a}, β_1, and β_2 fractions from PDL = 37 were observed when compared to the error frequencies of the analogous PDL = 27 species, though for α_{2b} there was no detectable change in fidelity. Comparisons for the α_3 species were obviated by low enzymatic activity. There were also differences in fidelity among subclasses at the same passage level. For example, the misincorporation frequency of α_{2b} from PDL = 27 with either poly(dT)·oligo(dA)$_{19-24}$ or poly(dI)·poly(dC) was higher than those of α_{1a}, α_{1b}, α_{2a}, or α_{3a}. In addition, for both α_1 and α_2 from PDL = 37, both subclasses obtained after phosphocellulose chromatography displayed lower fidelity than the activity of the DEAE-cellulose fractions from which they originated. This latter observation might imply that the catalytic specificity of the enzyme species from later passage cells is more labile to stresses of purification or that a "fidelity factor" is removed or damaged for each of these subspecies.

β_1 activity from human placenta was previously observed to be less faithful than that of β_2 (Krauss & Linn, 1980). Similarly, β_1 isolated from a given population doubling level of cultured fibroblasts misincorporates more frequently than β_2 activity from the same preparation (Table II). However, both β_1 and β_2 activities exhibited losses of fidelity when isolated from PDL = 37 vs. PDL = 27 cells.

Polymerases from Subconfluent and Postconfluent Cultured Fibroblasts. IMR-90 fibroblasts at PDL = 19 were split 1:4, and then half were harvested after 3 days when the cells were judged to be 80–90% confluent by microscopic observation ("subconfluent"). The remaining cells were allowed to reach confluency, incubated for 5 days further, and then harvested ("postconfluent"). When extracts (step I) were prepared, the postconfluent cells yielded approximately one-tenth the DNA polymerase activity of the subconfluent cells, although similar amounts of protein were found from the two types.

Upon DEAE-cellulose chromatography, the amount of α-polymerase activity in the postconfluent extract was found to be approximately 5-fold less relative to that from the subconfluent cells, whereas the amounts of β-polymerase were similar. In addition, with the postconfluent extracts, there was a broadening of the α_1 region toward the α_0 region and a relative diminution of the α_3 peak (Figure 2). Furthermore, all of the α fractions as well as the β- and γ-polymerases from postconfluent cells misincorporated more frequently than the comparable enzyme fractions isolated from the subconfluent cells when poly(dT)·oligo(dA)$_{19-24}$ was utilized (Table III) even in the presence of Mg^{2+}. Thus, in terms of total amounts of activity, chromatographic properties upon DEAE-cellulose, and fidelities during synthesis, the polymerases from PDL = 21 postconfluent cells behaved qualitatively like enzyme from subconfluent cells of higher doubling number.

Table III: Misincorporation Frequencies of DNA Polymerase Fractions from Subconfluent and Postconfluent Fibroblast Cultures at PDL = 21 with Poly(dT)·Oligo(dA)$_{19-24}$[a]

polymerase species	DEAE-cellulose fraction	divalent cation	subconfluent dAMP (pmol)	subconfluent dGMP (fmol)	subconfluent error frequency	postconfluent dAMP (pmol)	postconfluent dGMP (fmol)	postconfluent error frequency
α_0	28	Mn^{2+}	271	<9	<1/30000			
	30	Mn^{2+}	263	<9	<1/29000	503	47	1/11000
	32	Mn^{2+}	198	<9	<1/22000	355	49	1/7200
	32	Mn^{2+}	530	14	1/38000	537	72	1/7500
	32	Mg^{2+}	82	<8	<1/10000	87	10	1/8700
α_1	35	Mn^{2+}				500	48	1/10000
	36	Mn^{2+}	351	<9	<1/39000	252	27	1/9300
	36	Mn^{2+}	848	21	1/40000	481	60	1/8000
	36	Mg^{2+}	98	<8	<1/12000	89	12	1/7400
α_2	40	Mn^{2+}	227	<9	<1/25000	123	16	1/7700
	40	Mn^{2+}	528	<9	<1/59000	297	40	1/7400
	40	Mg^{2+}	73	<8	<1/9100	83	11	1/7500
	41	Mn^{2+}	487	<9	<1/54000	222	28	1/7900
α_3	50	Mn^{2+}	33	<9	<1/3700	69	22	1/3100
	50	Mn^{2+}	74	<9	<1/8200	140	39	1/3600
	50	Mg^{2+}	19	<8	<1/2400	28	17	1/1600
β	flow through	Mn^{2+}	298	<12	<1/25000	116	46	1/2500
γ	16	Mn^{2+}	15	<12	<1/1200	10	18	1/560
		Mg^{2+}	5	<8	<1/620	3	11	1/270

[a] DEAE-cellulose fractions from chromatograms such as those shown in Figure 2 were assayed as described under Experimental Procedures.

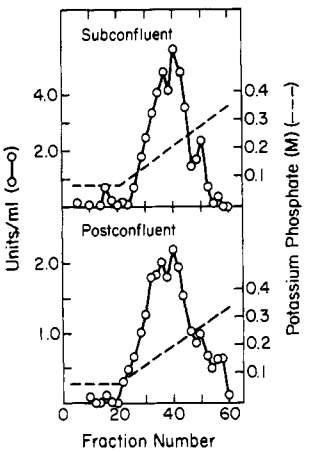

FIGURE 2: DEAE-cellulose chromatography of polymerase activity from subconfluent and postconfluent fibroblasts. Dialyzed extracts from subconfluent (53 units) and postconfluent (29 units) IMR-90, PDL = 21 cells were used.

Discussion

Cultured human fibroblasts (IMR-90, as well as MRC-5 and F65 cell strains not used in this report) and HeLa cells contain α-, β-, and γ-polymerase activities which distributed similarly to placental enzymes upon DEAE-cellulose (Krauss & Linn, 1980), though in some cases the α-polymerase species eluted from DEAE-cellulose at somewhat different phosphate concentrations. Three α-polymerase forms were consistently observed in early-passage fibroblasts and in HeLa cells, and, as with other cell types, the significance of this multiplicity is unclear. Inclusion during initial extraction of the protease inhibitors phenylmethanesulfonyl fluoride, leupeptin, pepstatin, and ethylenediaminetetraacetic acid did not result in changes in the chromatographic profiles or yields. In experiments in which fibroblast nuclei and cytoplasm were separately extracted and then processed identically, no discrete partitioning of α_1, α_2, or α_3 was observed (unpublished experiments).

A major conclusion to be drawn from the fidelity measurements is that *each* DNA polymerase species displayed increased error frequencies as the replicative potential of the cell population decreased. The fidelities were determined in vitro by using defined synthetic template-primers and Mn^{2+} or Mg^{2+}. The system has been well-characterized by numerous controls for reagent and enzyme catalytic purity (see Experimental Procedures). In addition, the products of misincorporation by fractions from human cells have been examined by isopycnic centrifugation (Linn et al., 1976) and nearest-neighbor analysis (Seal et al., 1979; Murray & Holliday, 1981), and appropriate mixing controls between faithful and unfaithful enzyme fractions have been performed (Linn et al., 1976; Murray & Holliday, 1981). Finally, polymerase fidelity estimates with primed $\phi X174$ DNA correlate well with those obtained by using the synthetic polymers (Loeb, 1978; Kunkel & Loeb, 1981).

Although the general mechanisms that ensure fidelity of eukaryotic polymerases remain unknown, several possible explanations for the loss of fidelity associated with increased passaging of cells of limited replicative life span seem unlikely. First, the changes do not appear to result from the appearance of a unique "aging" polymerase species, since all cellular polymerases show losses of fidelity (Table I). Second, the decreased fidelity does not appear to be due to the loss of an associated 3′ → 5′ exonuclease activity, since human placental DNA polymerases can exhibit high fidelities in the absence of detectable 3′ → 5′ exonuclease (Krauss & Linn, 1980). Third, the observation that both β_1 and β_2 from PDL = 37 extracts had decreased fidelity indicates that the fidelity loss observed for β-polymerase in the preceding DEAE-cellulose fraction was not due to a simple conversion of the relatively faithful β_2 form to the less faithful β_1 form. Hence, fidelity changes in β-polymerase can occur through some alteration peculiar to late passages. Fourth, although changes in polymerase fidelity could result from alterations or appearances of extrinsic factor(s), the ubiquitous change among so many species and subspecies of DNA polymerases would predict that such factors probably act by modification of the polymerases, as the factors would be unlikely to be present in each of the large number of species isolated. In essence, the experimental results provide no simple explanation for the loss of fidelity of each of the many forms of α-, β-, and γ-polymerase as the cells lose their replicative potential.

The biological functions of the various polymerase species have not been precisely defined, but levels of α activity correlate positively with cellular replicative function during de-

velopment and during the cell cycle. In general, α levels declined with continued passaging, but there was a relatively greater loss of α_2 and α_3 [or they became unresolvable from α_1 upon DEAE-cellulose chromatography (Figure 1)]. In addition, polymerase activity appeared in the α_0 region. In this context, antibody directed toward α-polymerase would be useful for studying the relationship between the α-polymerase species and for determining whether enzyme fractions from various preparations which elute at similar salt concentrations do in fact contain the same polymerase protein species. In addition, the possible presence of catalytically inactive cross-reacting material could be measured in order to distinguish between polymerase inactivation or degradation.

The levels of β-polymerase activity remain relatively constant throughout the replicative life span. Since indirect evidence supports a major role for β-polymerase in DNA repair processes, this finding is consistent with reports that cultured cells maintain their DNA repair replicative capacity, except perhaps in phase III cultures (Clarkson & Painter, 1974; Painter et al., 1973; Hart & Setlow, 1976; Mattern & Cerrutti, 1975). Indeed, when an open-cell assay system was used to measure DNA repair replication (Ciarrocchi & Linn, 1978), IMR-90 fibroblasts of PDL = 21, 40, and 43.5 were observed to respond similarly to ultraviolet light induced incorporation of [^{3}H]dTTP (unpublished experiments). However, if β-polymerase fidelity were to be decreasing in vivo as cells are passaged (as predicted from the in vitro assays), yet DNA repair replicative capacity is not affected, one might predict an increase in mutation frequency in progeny from late-passage cells which had been stimulated to perform DNA repair synthesis. Similarly, the apparent reduction in γ-polymerase fidelity suggests that possible heterogeneity might exist in DNA sequences of mitochondria obtained from late-passage cells or from cells of old individuals.

Detailed observations of the lineages of individual cells in early- as well as in late-passage cultures indicate that there is heterogeneity both in interdivision times and in the number of divisions completed before cycling ceases and cells become refractive to further mitotic stimulation (Bell et al., 1978, Smith & Whitney, 1980). In addition, cultured diploid fibroblast cell populations at late passages have increased proportions of noncycling cells (Merz & Ross, 1969; Cristofalo & Sharf, 1973; Smith & Hayflick, 1974; Bell et al., 1978). Polymerase activities present in nonmitotic cell populations from early-passage cells were analyzed to aid in interpreting the observations with late-passage cells. Cells were put into a postconfluent state to obtain a quiescent population with reasonably unperturbed metabolic processes. Five-day-postconfluent fibroblasts have negligible replication activity by an open-cell replication assay or by autoradiography (Ciarrocchi et al., 1979), but they can resume cell division (Augenlicht & Baserga, 1974; Krauss & Linn, 1980). In extracts of such postconfluent cells, total α activity was markedly decreased, α_1-polymerase appeared as a broadened peak (Figure 2), and each of the polymerases exhibited decreased fidelity (Table III). A decrease in α levels does not appear to be obligatorily coupled to changes in polymerase properties detected by DEAE-cellulose chromatography, however, because after exposure of subconfluent fibroblasts to sodium butyrate cellular replication is inhibited and α_1-polymerase region broadens but α-polymerase activity levels do not decrease substantially (unpublished experiments).

Clearly the presence of heterogeneous subpopulations in late-passage cell cultures complicates interpretation of data from mass cell populations, but it appears at this time that DNA polymerase activities from cells which have not lost cell division potential yet are not engaged in traversing the cell cycle have some properties that are characteristic of activities present in late-passage cell populations. Therefore, at least some characteristics of DNA polymerase from late-passage cultures could be a consequence simply of the cessation of DNA replication by an increased proportion of the cell population. In fact, this interpretation would be consistent with the observation that polymerase activities isolated from lymphocytes of old individuals, when stimulated by phytohemagglutinin to divide, did not exhibit losses of fidelity (Agarwal et al., 1978). In this context then, one must evaluate carefully the use of the cessation of replication capacity of cultured fibroblasts as a model system for studying the relationship between altered DNA polymerase fidelity and "aging" sequelae (Hayflick, 1979; Bell et al., 1978; Smith & Whitney, 1980), since the effects on polymerase may be a consequence of a generalized cellular response to entry into a nonreplicative state. Clearly an understanding of the exact molecular basis of the altered polymerase properties in each cell state is necessary before implicating similar biochemical processes.

Acknowledgments

We thank T. Mullenbach for the culturing of cells used for these studies and Dr. R. Snapka for advice and assistance in experiments measuring repair replication and with sodium butyrate (unpublished experiments).

References

Agarwal, S. S., Tuffner, M., & Loeb, L. A. (1978) *J. Cell. Physiol. 96*, 235–244.

Augenlicht, L. H., & Baserga, R. (1974) *Exp. Cell Res. 89*, 255–262.

Barton, R. W., Waters, L. C., & Young, W. K. (1974) *Fed. Proc. Fed. Am. Soc. Exp. Biol. 34*, 1419.

Bell, E., Marek, F., Levinstone, D. S., Merrill, C., Sher, S., Young, I. T., & Eden, M. (1978) *Science (Washington, D.C.) 202*, 1158–1163.

Bozhkov, V. M., Barskaya, T. V., Fridlyanskaya, I. I., & Tomilin, N. V. (1978) *FEBS Lett. 86*, 205–208.

Burnet, Mc. (1974) *Intrinsic Mutagenesis, a Genetic Approach to Aging*, Medical and Technical Publishing Co., Ltd., Lancaster, Great Britain.

Chan, J. Y., & Becker, F. F. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 814–818.

Ciarrocchi, G., & Linn, S. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 1887–1891.

Ciarrocchi, G., Jose, J., & Linn, S. (1979) *Nucleic Acids Res. 7*, 1205–1219.

Clarkson, J. M., & Painter, R. B. (1974) *Mutat. Res. 23*, 107–112.

Cristofalo, V., & Sharf, B. (1973) *Exp. Cell Res. 76*, 419–427.

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

Hall, Z., & Lehman, I. (1968) *J. Mol. Biol. 36*, 321–331.

Hart, R. W., & Setlow, R. B. (1976) *Mech. Ageing Dev. 5*, 67–77.

Hayflick, L. (1979) *Fed. Proc. Fed. Am. Soc. Exp. Biol. 38*, 1847–1849.

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

Krauss, S. W., & Linn, S. (1980) *Biochemistry 19*, 220–228.

Kunkel, T. A., & Loeb, L. A. (1981) *Science (Washington, D.C.) 213*, 765–767.

Linn, S., Kairis, M., & Holliday, R. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2818–2822.

Loeb, L. (1978) *J. Supramol. Struct., Suppl. 2*, 284.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Mattern, M. R., & Cerrutti, P. A. (1975) *Nature (London) 254*, 450–456.
Medvedev, Zh. A. (1972a) *Nature (London) 237*, 453–454.
Medvedev, Zh. A. (1972b) *Exp. Gerontol. 7*, 227–238.
Merz, G. S., & Ross, J. D. (1969) *J. Cell. Physiol. 74*, 219–224.
Murray, V., & Holliday, R. (1981) *J. Mol. Biol. 146*, 55–76.
Muzyczka, N., Poland, R., & Bessman, M. (1972) *J. Biol. Chem. 247*, 7116–7122.
Painter, R. B., Clarkson, J. M., & Young, B. R. (1973) *Radiat. Res. 56*, 560–564.
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.
Smith, J. R., & Hayflick, L. (1974) *J. Cell Biol. 62*, 48–53.
Smith, J. R., & Whitney, R. G. (1980) *Science (Washington, D.C.) 207*, 82–84.
Speyer, J., Karam, J., & Lenny, A. (1966) *Cold Spring Harbor Symp. Quant. Biol. 31*, 693–697.
Szilard, L. (1959) *Proc. Natl. Acad. Sci. U.S.A. 45*, 30–45.

Chemical Synthesis of a Messenger Ribonucleic Acid Fragment: AUGUUCUUCUUCUUCUUC†

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ABSTRACT: The synthesis via a phosphotriester method of the octadecaribonucleotide AUG(UUC)$_5$ (**19**) is reported. The octadecanucleotide is meant to serve as a synthetic messenger in a ribosomal protein synthesizing system. A fully protected octadecamer intermediate (**18a**) was prepared by a block coupling procedure. For the introduction of the desired 3′–5′-internucleotide bonds a 3′-*O*-(2,2,2-trichloroethyl 2-chlorophenyl phosphate) function was incorporated into the monomeric building blocks which were applied in the synthesis of **18a**. Monomeric and oligomeric compounds with a thus protected 3′-*O*-phosphotriester function can be selectively deblocked to give 3′-*O*-phosphodiester derivatives suitable for condensation with 5′-hydroxyl (oligo)nucleotides. Conversion of fully protected oligomers to 5′-hydroxyl derivatives, suitable for further coupling at the 5′ end, was effected by selective removal of the levulinyl function at the 5′ end. The fully protected octadecanucleotide **18a** was deblocked with fluoride ions, followed by ammonia and acid to give the required octadecamer **19**. The condensing agent 1-(2,4,6-triisopropylphenylsulfonyl)-3-nitro-1,2,4-triazole, which was applied to effect the formation of fully protected 3′–5′-internucleotide phosphotriester functions, may give rise to side reactions with the heterocyclic bases uracil and guanine. The consequences of these side reactions for the synthesis of octadecamer **19** are reported.

The first requirement for obtaining better insight into protein synthesis of prokaryotic and eukaryotic cells is a knowledge of the precise structure and function of the ribosome. The complex system of the ribosome supplies the environment in which the correct codon–anticodon interactions between messenger RNA and the various transfer RNAs are selected, thereby assuring an ordered translation of the messenger. The complexity of the ribosome structure has restrained efforts to unravel the precise molecular mechanism of protein synthesis (Chambliss et al., 1979). Many studies on protein biosynthesis have been performed with enzymatically prepared messenger RNAs. Due to recent improvements in the chemical synthesis of RNA fragments, biochemical investigations using well-defined synthetic messengers have come within reach. We therefore decided to synthesize a messenger RNA fragment containing the initiation codon AUG followed by five successive UUC codons (see Figure 1). In translation studies with the synthetic octadecamer AUG(UUC)$_5$ (**19**), we hope to correlate errors in translation with well-defined structural alterations of the ribosome. Since UUC codes for the amino acid phenylalanine (Phe), UCU for serine (Ser), and CUU for leucine (Leu), slippage of the messenger should be detected by scoring the frequency of Ser and Leu incorporation relative to Phe.

The availability of well-defined oligonucleotide fragments is of crucial importance for studies of cellular processes. In the field of oligodeoxyribonucleotides (DNA fragments) striking examples of the application of chemically prepared oligomers have been reported. Thus, the synthesis of the structural gene for yeast alanine transfer RNA (Khorana et al., 1972), the *Escherichia coli* tyrosine transfer RNA suppressor gene (Khorana, 1978), and genes for the peptide hormones somatostatin (Itakura et al., 1977) and insulin (Crea et al., 1978) was successfully achieved by enzymatic assemblage of chemically prepared DNA fragments. The development of synthetic methods for the preparation of oligoribonucleotides (RNA fragments) has taken a much slower course. However, in the last few years considerable progress, in particular by application of phosphotriester methods, has been made in the field of oligoribonucleotide synthesis (Reese, 1978), and syntheses of several relatively large RNA fragments with heterosequences of up to 17 ribonucleoside units have been reported (Werstiuk & Neilson, 1976; Adamiak et al., 1978; van Boom et al., 1978; van Boom & Burgers, 1978; Ogilvie & Theriault, 1979; Ohtsuka et al., 1980; Jones et al., 1980). In the first part of this paper we present the synthesis of the octadecaribonucleotide AUG(UUC)$_5$ via a phosphotriester method. This octadecamer represents, up to now, the largest

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0006-2960/82/0421-1009$01.25/0