Incorporation of Noncomplementary Nucleotides at High Frequencies by Ribodeoxyvirus DNA Polymerases and *Escherichia coli* DNA Polymerase I†

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ABSTRACT: The fidelity of DNA synthesis with synthetic homopolymer templates by two ribodeoxyvirus DNA polymerases and *E. coli* DNA polymerase I was examined by nearest neighbor frequency analyses. The experiments were designed to favor the incorporation of noncomplementary nucleotides, and the reactions were carried out only for short periods of time. All incorporations were template dependent. The frequency of incorporation of noncomplementary nucleotides next to complementary nucleotides by all three of these DNA polymerases significantly varied, depending on the conditions, from almost none (less than 0.2%) to 100% of the incorporation of the complementary nucleotide. In general, there was more incorporation of noncomplementary nucleotides in the presence of Mn^{2+} than in the presence of Mg^{2+} and with ribohomopolymer templates than with deoxyribohomopolymer templates. With a heteropolymer RNA template, the infidelity of DNA synthesis by one of the ribodeoxyvirus DNA polymerases was also increased by the presence of Mn^{2+} in the DNA synthesizing reaction. These findings indicate that, under appropriate conditions, any DNA polymerase has the potential to make a significant number of errors in DNA synthesis and that altered conditions for DNA polymerase action might play a role in spontaneous mutation.

Ribodeoxyviruses, viruses whose virions contain RNA[1] and a DNA polymerase, replicate through a DNA intermediate. The virion DNA polymerase apparently synthesizes the viral DNA intermediate using the virion RNA as a template. This mechanism for ribodeoxyvirus replication is supported by the existence of a noninfectious strain of Rous sarcoma virus, Bryan high-titer RSVα, lacking a virion DNA polymerase (Hanafusa and Hanafusa, 1971) and of temperature-sensitive mutants of Rous sarcoma virus with temperature-sensitive DNA polymerases (Linial and Mason, 1973).

Spleen necrosis virus (SNV) is a member of the reticuloendotheliosis group of avian ribodeoxyviruses; that is, SNV virions contain RNA and a DNA polymerase, and SNV replicates through a DNA intermediate (Temin, 1974). However, we found that the DNA product synthesized by purified SNV DNA polymerase using SNV RNA as a template·primer did not hybridize to SNV RNA (Mizutani and Temin, 1975).

One possible hypothesis to explain the failure of the SNV DNA product to hybridize to SNV RNA, the apparent template, is massive infidelity of DNA synthesis by SNV DNA polymerase.

Studies of the fidelity of DNA synthesis by avian myeloblastosis and Rauscher murine leukemia virus DNA polymerases (Springgate et al., 1973; Sirover and Loeb, 1974) indicated that these DNA polymerases incorporated one noncomplementary nucleotide (dCMP) for every 500–1000 complementary nucleotides (dTMP) polymerized on an $(rA)_n$·$(dT)_{12-18}$ template·primer. Under similar conditions *E. coli* (*Escherichia coli*) DNA polymerase I incorporated only one noncomplementary nucleotide for every 100 000 nucleotides polymerized (Springgate et al., 1973). Although the presence of one noncomplementary nucleotide for every 500–1000 complementary nucleotides would not affect nucleic acid hybridization, a much greater frequency of incorporation of noncomplementary nucleotides would. Therefore, we studied the infidelity of DNA synthesis by SNV DNA polymerase to investigate further the hypothesis that there was massive infidelity in DNA synthesis by SNV DNA polymerase.

Materials and Methods

Chemicals. The synthetic homopolymer·oligomer combinations, $(dC)_n$·$(dG)_{12-18}$, $(rC)_n$·$(dG)_{12-18}$, and $(rA)_n$·$(dT)_{12-18}$ (n = 700–800), and all 2′-deoxyribonucleoside 5′-triphosphates and 2′-deoxyribonucleoside 3′-monophosphates were purchased from P-L Biochemicals Inc., Milwaukee, Wis. A single set of homopolymer·oligomers was used in all of the experiments in this paper. The purity of each homopolymer was tested by direct digestion of the homopolymers and identification of the resultant nucleotides by two dimensional poly(ethylenimine) (PEI)cellulose thin-layer chromatography as described below. No detectable (less than 1%) contamination was observed in each case. The purity of each of the homopolymers was also shown by determining the amount of incorporation of noncomplemen-

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[1] Abbreviations used: DNA, deoxyribonucleic acid; RNA, ribonucleic acid; SNV, spleen necrosis virus; CZAV, Carr-Zilber associated virus; dATP, 2′-deoxyadenosine 5′-triphosphate; dCTP, 2′-deoxycytidine 5′-triphosphate; dGTP, 2′-deoxyguanosine 5′-triphosphate; dTTP, 2′-deoxythymidine 5′-triphosphate; dAp or dAMP, 2-deoxyadenosine 3′-monophosphate; dCp or dCMP, 2′-deoxycytidine 3′-monophosphate; dGp or dGMP, 2′-deoxyguanosine 3′-monophosphate; dTp or dTMP, 2′-deoxythymidine 3′-monophosphate; $(dC)_n$·$(dG)_{12-18}$, poly(deoxyribocytidylic acid)·oligo(deoxyriboguanylic acid); $(rC)_n$·$(dG)_{12-18}$, poly(ribocytidylic acid)·oligo(deoxyriboguanylic acid); $(rA)_n$·$(dT)_{12-18}$, poly(riboadenylic acid)·oligo(thymidylic acid); EDTA, ethylenediaminetetraacetate; PEI, poly(ethylenimine); uv, ultraviolet light; Cl_3CCOOH, trichloroacetic acid.

tary nucleotides next to the complementary nucleotide by the nearest neighbor frequency analysis described below under conditions of high fidelity DNA synthesis (see later).

[^{3}H]dCTP, [^{3}H]dGTP, [^{3}H]dTTP, [α-^{32}P]dGTP, and [α-^{32}P]dTTP were purchased from New England Nuclear, Boston, Mass. The purity of the labeled deoxyribonucleoside triphosphates was tested by two-dimensional poly(ethylenimine)-cellulose thin-layer chromatography using the solvent system of Cashel et al. (1969). No detectable contamination (<0.1%) of the labeled deoxyribonucleoside triphosphates was found. Micrococcal nuclease [EC 3.1.4.7] and spleen phosphodiesterase [EC 3.1.4.1] were purchased from Worthington Biochemical Co., Freehold, N.J.

Spleen necrosis virus (SNV) and Carr-Zilber associated virus (CZAV) DNA polymerases were purified as described previously (Mizutani and Temin, 1975). *E. coli* DNA polymerase I was a kind gift of Dr. R. D. Wells, University of Wisconsin. It was purified to step 6 of the procedure described in Jovin et al. (1969).

^{32}P-Transfer Experiments (Nearest Neighbor Frequency Analysis). Product Preparation. The reaction mixtures (625 μl) contained 0.02 M Tris-HCl, pH 8.0, 0.005 M dithiothreitol, 0.025 A_{260} $(dC)_n \cdot (dG)_{12-18}$, $(rC)_n \cdot (dG)_{12-18}$, or $(rA)_n \cdot (dT)_{12-18}$ template·primer, 40 μM each dATP, dCTP, and dTTP, and 20 μM or 6.6 μM [α-^{32}P]dGTP for the former two template·primers or 40 μM each dATP, dCTP, and dGTP and 6.6 μM [α-^{32}P]dTTP for the latter template·primer, the optimum concentration of $MgCl_2$ or $MnCl_2$ (which was determined for each template and each DNA polymerase and is indicated for each experiment), and 1 μg of CZAV or SNV DNA polymerase or 0.3 μg of *E. coli* DNA polymerase I. The reactions were carried out at 37 °C for 20 min for spleen necrosis virus and Carr-Zilber associated virus DNA polymerases and for 10 min for *E. coli* DNA polymerase I and were terminated by adding 0.5 M EDTA to a final concentration of 0.04 M. The reaction mixtures were extracted with equal volumes of chloroform–isoamyl alcohol (24:1) and then chromatographed on Sephadex G-75 columns (0.5 × 40 cm) equilibrated with 0.01 M Tris-HCl, 0.05 M NaCl, 0.001 M EDTA, pH 7.5. The fractions containing radioactivity were pooled. These pooled fractions, containing 80–90% of the Cl_3CCOOH-insoluble ^{32}P counts, were used for further analysis.

To see all of the incorporation of noncomplementary nucleotides next to the complementary nucleotide all of the DNA polymerase reactions were carried out in the presence of all four deoxyribonucleoside triphosphates. This method made negligible effects of any possible small amounts of contamination of labeled deoxyribonucleoside triphosphates and the effects of any terminal addition of nucleotides.

All incorporations with spleen necrosis virus, Carr-Zilber associated virus, and *E. coli* DNA polymerases were absolutely dependent on the presence of a template·primer under all reaction conditions used (less than 0.1% of the incorporation in the presence of template·primers). For *E. coli* DNA polymerase I there was some $(dC)_{12-18}$- or $(dT)_{12-18}$-primed incorporation of [^{3}H]dTMP or [^{3}H]dCMP, respectively, into Cl_3CCOOH-insoluble material in the presence of $MgCl_2$ (there was less incorporation in the presence of $MnCl_2$). The total amount of this incorporation was less than 1% of that of a template-directed reaction.

Analysis of DNA Products. DNA products were hydrolyzed with micrococcal nuclease in 0.01 M Tris-HCl, pH 8.5, containing 10 mM $CaCl_2$, until more than 95% of the incorporated ^{32}P became Cl_3CCOOH-soluble, and then were hydrolyzed further with spleen phosphodiesterase in 0.03 M potassium phosphate buffer, pH 6.5 (Josse et al., 1961). The liberated nucleotides were adsorbed to 100 mg of partially inactivated Norit (Josse and Moffatt, 1965) and then, after washing with 4 ml of 0.001 M formic acid which completely removed inorganic ^{32}P, were eluted with 2 ml of a 1% ammonium–50% ethanol mixture. The ammonium–ethanol mixtures were evaporated under vacuum. The recovery of ^{32}P in nucleotides was 50–80%. The rest of the ^{32}P was recovered as inorganic ^{32}P. For all experiments the nucleotides were dissolved in 15 to 25 μl of double-distilled water, an aliquot (5000–150 000 cpm) and the four 2′-deoxyribonucleoside 3′-monophosphates as markers were spotted on each plate, and the nucleotides were developed by two dimensional poly(ethylenimine) (PEI)-cellulose thin-layer chromatography in two different solvent systems. System I was: (a) 1 N acetic acid, 1 N acetic acid–3.0 M LiCl (9:1, v/v) and (b) a solution of 6 g of $Na_2B_4O_7 \cdot 10H_2O$, 3 g of H_3BO_3, and 25 ml of ethylene glycol in 70 ml of H_2O (Randerath and Randerath, 1965). System II was: (a) a mixture of 100 ml of isobutyric acid, 588 ml of H_2O, 42 ml of concentrated NH_4OH, and 16 ml of 0.1 M EDTA, pH 4.6, and (b) a mixture of 1600 ml of saturated $(NH_4)_2SO_4$, 360 ml of CH_3COONa (8.2 g/100 ml), and 40 ml of 2-propanol. In both systems the plates were washed in methanol after the first dimension of development. Inorganic ^{32}P moved with dGp in solvent system I, but no inorganic ^{32}P was detected in the samples using solvent system II. The nucleotides on the thin-layer plates were detected with the aid of a uv lamp. The uv-absorbing spots were scraped from the plates and soaked for about 30 min in 1 ml of double-distilled water in scintillation vials, and then 10 ml of Scintisol (Isolab, Inc., Akron, Ohio) was added. The radioactivity was measured in a Nuclear Chicago liquid scintillation spectrometer. The recovery of radioactivity from the thin-layer plates was 90–100%.

This method of analysis only detects minimal amounts of incorporation of noncomplementary nucleotides, because only noncomplementary nucleotides next to complementary nucleotides are detected. Noncomplementary nucleotides next to noncomplementary nucleotides are not detected.

Results

Infidelity of DNA Synthesis by SNV DNA Polymerase. The frequency of incorporation of noncomplementary nucleotides during DNA synthesis by SNV DNA polymerase was first determined by measuring the rates of incorporation of [^{3}H]dTMP and [α-^{32}P]dGMP with an $(rA)_n$ template and the rates of incorporation of [^{3}H]dGMP and [α-^{32}P]dTTP with an $(rC)_n$ template. In both cases approximately 1 noncomplementary nucleotide was incorporated per 50 complementary nucleotides (data not shown). Furthermore, the incorporation of the noncomplementary nucleotide was almost completely dependent upon the presence of the complementary nucleotide. For example, SNV DNA polymerase incorporated 0.13 pmol of [^{3}H]dTMP in 10 min with a $(dC)_n$ template in the presence of dGTP, but incorporated only 0.02 pmol in the absence of dGTP (data not shown).

Nearest neighbor frequency analyses were used then to confirm and extend these results. By this method only incorporation of noncomplementary nucleotides next to complementary nucleotides can be seen. It may, therefore, have

Table I: Comparison of the Frequency of Incorporation of Noncomplementary Nucleotides next to Complementary Nucleotides by Three DNA Polymerases.[a]

Nearest Neighbor	DNA Polymerase		
	SNV	CZAV	*E. coli* I
dGp	80.7	97.7	98.7
dAp	18.3	2.2	0.3
dCp	0	0.1	0.9
dTp	0.9	0.1	0.1

[a] DNAs were prepared in standard DNA polymerase reactions with a $(dC)_n \cdot (dG)_{12-18}$ template·primer, 40 μM each of dATP, dCTP, and dTTP, and 6.6 μM [α-^{32}P]dGTP. 1, 4, and 10 mM $MgCl_2$ were used for SNV, CZAV, and *E. coli* DNA polymerases, respectively. Total incorporation of [α-^{32}P]dGMP was 162, 112, and 294 pmol, respectively. DNAs were purified and hydrolyzed, and the nucleotides were separated by two-dimensional PEI-cellulose thin-layer chromatography as described in Materials and Methods. For each DNA polymerase, the distribution of ^{32}P in nucleoside monophosphates is expressed as the percent of ^{32}P in all nucleotides. One hundred percent ^{32}P was 62 000 cpm (3.2 pmol) for the SNV DNA polymerase, 140 000 cpm (7.2 pmol) for the CZAV DNA polymerase, and 64 000 cpm (8 pmol) for the *E. coli* DNA polymerase I.

Table II: Effects of $MnCl_2$ and $MgCl_2$ on the Frequency of Incorporation of Noncomplementary Nucleotides with a $(dC)_n \cdot (dG)_{12-18}$ Template·Primer.[a]

Nearest Neighbor	DNA Polymerase					
	SNV		CZAV		*E. coli* I	
	$MnCl_2$	$MgCl_2$	$MnCl_2$	$MgCl_2$	$MnCl_2$	$MgCl_2$
dGp	44.4	96.9	95.7	93.8	97.2	99.4
dAp	1.2	1.5	2.5	4.2	0.4	0.1
dCp	1.2	0.6	0.6	0.7	0.2	0.1
dTp	53.2	1.0	1.3	1.4	2.2	0.3

[a] DNAs were prepared in standard DNA polymerase reactions with a $(dC)_n \cdot (dG)_{12-18}$ template·primer, 40 μM each of dATP, dCTP, and dTTP, and 20 μM [α-^{32}P]dGTP. The $MgCl_2$ concentration for each DNA polymerase was the same as in the experiment described in the legend of Table I. The $MnCl_2$ concentration was 1 mM for all three DNA polymerases. Total incorporation of [α-^{32}P]dGMP was: 75 pmol in the presence of $MnCl_2$ and 140 pmol in the presence of $MgCl_2$ with SNV DNA polymerase; 60 pmol in the presence of $MnCl_2$ and 190 pmol in the presence of $MgCl_2$ with CZAV DNA polymerase; and 14 nmol in the presence of $MnCl_2$ and 18 nmol in the presence of $MgCl_2$ with *E. coli* DNA polymerase I. One hundred percent ^{32}P was: 30 000 cpm (8 pmol) for the SNV DNA polymerase in the presence of $MnCl_2$ and 25 000 cpm (6.7 pmol) for the SNV DNA polymerase in the presence of $MgCl_2$; 11 000 cpm (3 pmol) and 40 000 cpm (10.7 pmol) for the CZAV DNA polymerase; and 150 000 cpm (35.9 pmol) and 140 000 cpm (33.5 pmol) for the *E. coli* polymerase I.

underestimated the total infidelity. But since the templates were homogeneous, it assured that only real infidelity was measured.

Comparison of Infidelity of Three DNA Polymerases. To compare the frequency of incorporation of noncomplementary nucleotides next to complementary nucleotides by spleen necrosis virus DNA polymerase with that by Carr-Zilber associated virus DNA polymerase and *E. coli* DNA polymerase I, [^{32}P]DNA products were synthesized with a $(dC)_n \cdot (dG)_{12-18}$ template·primer, $MgCl_2$, [α-^{32}P]dGTP, and the other three unlabeled deoxyribonucleoside triphosphates. The results of a nearest neighbor frequency analysis of the three DNA products are shown in Table I. Under these conditions spleen necrosis virus DNA polymerase incorporated a noncomplementary nucleotide next to the complementary nucleotide at least 7 times more frequently than did Carr-Zilber associated virus DNA polymerase and at least 15 times more frequently than did *E. coli* DNA polymerase. Therefore, with a $(dC)_n \cdot (dG)_{12-18}$ template·primer and in the presence of $MgCl_2$, SNV DNA polymerase incorporated more noncomplementary nucleotides next to the complementary nucleotide than did the other two DNA polymerases. This high frequency of incorporation of noncomplementary nucleotides was not the result of an impure template as shown by the results with *E. coli* DNA polymerase and the direct analysis of the template described in Materials and Methods.

Effects of $MnCl_2$ and $MgCl_2$ on the Frequencies of Incorporation of Noncomplementary Nucleotides next to Complementary Nucleotides. Both wild-type and mutant T4 DNA polymerases were reported to incorporate more noncomplementary nucleotides in the presence of $MnCl_2$ than in the presence of $MgCl_2$ (Hall and Lehman, 1968). Therefore, the frequencies of incorporation of noncomplementary nucleotides next to complementary nucleotides in the presence of $MnCl_2$ or $MgCl_2$ with $(dC)_n \cdot (dG)_{12-18}$ and $(rA)_n \cdot (dT)_{12-18}$ template·primers were determined for spleen necrosis virus, Carr-Zilber associated virus, and *E. coli* DNA polymerases.

$MnCl_2$ only caused a slight increase in the frequency of noncomplementary nucleotides incorporated next to the complementary nucleotide by *E. coli* DNA polymerase with a $(dC)_n$ template (Table II). $MnCl_2$ did not increase the frequency of incorporation of noncomplementary nucleotides next to the complementary nucleotide by Carr-Zilber associated virus DNA polymerase with a $(dC)_n$ template (Table II). However, with a $(dC)_n$ template, $MnCl_2$ increased the frequency of incorporation of noncomplementary nucleotides by spleen necrosis virus DNA polymerase to more than 50% noncomplementary deoxyribonucleotides next to the complementary nucleotide, while only about 3% were incorporated in the presence of $MgCl_2$. With a $(rA)_n$ template, $MnCl_2$ increased the frequency of incorporation of noncomplementary nucleotides next to the complementary nucleotide with both SNV and CZAV DNA polymerases to over 30–60% (Table III). Therefore, $MnCl_2$ caused DNA polymerases to incorporate more noncomplementary nucleotides next to complementary nucleotides.

Effect of Different Template·Primers on the Frequency of Incorporation of Noncomplementary Nucleotides next to Complementary Nucleotides. The frequency of incorporation of noncomplementary nucleotides next to complementary nucleotides by DNA polymerases seems also to be affected by the template·primer. For example, in the experiments described in Tables II and III Carr-Zilber associated virus DNA polymerase incorporated approximately the same frequency of noncomplementary nucleotides next to complementary nucleotides with a $(dC)_n \cdot (dG)_{12-18}$ template·primer in the presence of $MgCl_2$ or $MnCl_2$. However, with an $(rA)_n \cdot (dT)_{12-18}$ template·primer, the Carr-Zilber associated virus DNA polymerase had a significantly greater frequency of incorporation of noncomplementary nucleotides next to the complementary nucleotide in the presence of $MnCl_2$.

To compare the fidelity of DNA synthesis with compara-

Table III: Effects of $MnCl_2$ and $MgCl_2$ on the Frequency of Incorporation of Noncomplementary Nucleotides with an $(rA)_n \cdot (dT)_{12-18}$ Template·Primer.[a]

	DNA Polymerase			
	SNV		CZAV	
Nearest Neighbor	$MnCl_2$	$MgCl_2$	$MnCl_2$	$MgCl_2$
dGp	55.3	0.3	32.5	0.2
dAp	0	0.9	0	0
dCp	0	0	0	0
dTp	44.7	98.8	67.5	99.8

[a] DNAs were prepared in standard DNA polymerase reactions with an $(rA)_n \cdot (dT)_{12-18}$ template·primer, 40 μM each of dATP, dCTP, and dGTP, and 6.6 μM [α-^{32}P]dTTP. $MnCl_2$ and $MgCl_2$ concentrations were 0.5 and 2 mM, respectively, for the SNV DNA polymerase and 1 and 4 mM, respectively, for the CZAV DNA polymerase. Total incorporation of [α-^{32}P]dTMP was: 139 pmol in the presence of $MnCl_2$ and 3086 pmol in the presence of $MgCl_2$ with SNV DNA polymerase; and 105 pmol in the presence of $MnCl_2$ and 2974 pmol in the presence of $MgCl_2$ with CZAV DNA polymerase. One hundred percent ^{32}P was: 5100 cpm (0.4 pmol) and 52 000 cpm (3 pmol) for the SNV DNA polymerase in the presence of $MnCl_2$ and $MgCl_2$, respectively; and 5000 cpm (0.4 pmol) and 19 000 cpm (1.1 pmol) for the CZAV DNA polymerase in the presence of $MnCl_2$ and $MgCl_2$, respectively.

Table IV: Effect of Deoxyribo- and Ribohomopolymers on the Frequency of Incorporation of Noncomplementary Nucleotides next to Complementary Nucleotides by SNV DNA Polymerase.[a]

	Template·Primer	
Nearest Neighbor	$(dC)_n \cdot (dG)_{12-18}$	$(rC)_n \cdot (dG)_{12-18}$
dGp	59.5	0.6
dAp	7.8	0
dCp	0	0
dTp	32.5	99.2

[a] DNAs were prepared in standard DNA polymerase reactions with $(dC)_n \cdot (dG)_{12-18}$ or $(rC)_n \cdot (dG)_{12-18}$ template·primers, 40 μM each of dATP, dCTP, and dTTP, and 6.6 μM [α-^{32}P]dGTP. The $MnCl_2$ concentrations were 1 mM for the $(dC)_n \cdot (dG)_{12-18}$ template·primer and 0.5 mM for the $(rC)_n \cdot (dG)_{12-18}$ template·primer. Total incorporation of [α-^{32}P]dGMP was 72 pmol with $(dC)_n \cdot (dG)_{12-18}$ and 105 pmol with $(rC)_n \cdot (dG)_{12-18}$ template·primer. One hundred percent ^{32}P was 160 000 cpm (8.1 pmol) with the $(dC)_n \cdot (dG)_{12-18}$ template·primer and 22 000 cpm (1.6 pmol) with the $(rC)_n \cdot (dG)_{12-18}$ template·primer.

Table V: Effect of Deoxyribo- and Ribohomopolymers on the Frequency of Incorporation of Noncomplementary Nucleotides next to Complementary Nucleotides by CZAV DNA Polymerase.[a]

	Template·Primer	
Nearest Neighbor	$(dC)_n \cdot (dG)_{12-18}$[b]	$(rC)_n \cdot (dG)_{12-18}$
dGp	95.7	24.7
dAp	2.5	0.9
dCp	0.6	0
dTp	1.3	74.4

[a] DNAs were prepared in standard DNA polymerase reactions with $(dC)_n \cdot (dG)_{12-18}$ or $(rC)_n \cdot (dG)_{12-18}$ template·primers, 40 μM each of dATP, dCTP, and dTTP, and 20 μM [α-^{32}P]dGTP. Total incorporation of [α-^{32}P]dGMP was 60 pmol with $(dC)_n \cdot (dG)_{12-18}$ and 50 pmol with $(rC)_n \cdot (dG)_{12-18}$ template·primer. The $MnCl_2$ concentration was 1 mM for both templates. One hundred percent ^{32}P was 11 000 cpm (3 pmol) with the $(dC)_n \cdot (dG)_{12-18}$ template·primer and 6700 cpm (4.2 pmol) with the $(rC)_n \cdot (dG)_{12-18}$ template·primer. [b] Data from Table II.

ble deoxyribo- and ribohomopolymer templates, the frequency of incorporation of noncomplementary nucleotides next to complementary nucleotides with $(dC)_n \cdot (dG)_{12-18}$ and $(rC)_n \cdot (dG)_{12-18}$ template·primers was compared in the presence of $MnCl_2$ (Table IV). (The purity of the $(rC)_n$ template was shown by the finding of less than 0.5% incorporation of noncomplementary nucleotides next to dGMP by spleen necrosis virus DNA polymerase in the presence of $MgCl_2$.) Spleen necrosis virus DNA polymerase incorporated approximately 30% noncomplementary nucleotides next to the complementary nucleotide with the deoxyribohomopolymer template, $(dC)_n$, while under the same conditions with a ribohomopolymer template, $(rC)_n$, the spleen necrosis virus DNA polymerase synthesized a polymer with almost all the dGMPs next to dTMP.

A similar effect of deoxyribo- vs. ribohomopolymer templates was observed with Carr-Zilber associated virus DNA polymerase (Table V). Carr-Zilber associated virus DNA polymerase in the presence of $MnCl_2$ incorporated with a $(dC)_n \cdot (dG)_{12-18}$ template·primer only a few percent of the noncomplementary nucleotide (dTMP) next to the complementary nucleotide (dGMP). Under the same conditions with an $(rC)_n \cdot (dG)_{12-18}$ template·primer about 75% of dTMP was incorporated next to dGMP.

A difference in the frequency of incorporation of noncomplementary nucleotides next to complementary nucleotides with deoxyribo- and ribohomopolymer templates was also observed with *E. coli* DNA polymerase I (Table VI). There was little incorporation of noncomplementary nucleotides next to dGMP with a $(dC)_n \cdot (dG)_{12-18}$ template·primer. However, *E. coli* DNA polymerase I incorporated a high frequency of dAMP, dCMP, and dTMP next to dGMP with an $(rC)_n \cdot (dG)_{12-18}$ template·primer. Incorporation of all four deoxyribonucleotides in significant amounts by *E. coli* DNA polymerase I also occurred in the presence of $MgCl_2$ with an $(rC)_n \cdot (dG)_{12-18}$ template·primer (Table VI).

The incorporation of noncomplementary nucleotides by *E. coli* DNA polymerase I with an $(rC)_n \cdot (dG)_{12-18}$ template·primer was also examined in a double label experiment (Figure 1). Both the complementary, [^{3}H]dGMP, and the noncomplementary, [^{32}P]dTMP, nucleotides were incorporated in the presence of $MnCl_2$ or $MgCl_2$ at rates that were similar within a factor of 1.5–2.0. These results confirm those of the experiment shown in Table VI. This incorporation of noncomplementary nucleotides was not the result of contaminating DNA or RNA in the *E. coli* DNA polymerase, because no such incorporation was observed in the absence of any template.

Effect of Mn^{2+} on Fidelity of DNA Synthesis with a Spleen Necrosis Virus RNA Template. To determine whether or not there was a high enough frequency of incorporation of noncomplementary nucleotides with a heteropolymer RNA template to affect nucleic acid hybridization, spleen necrosis virus RNA was used as a template for DNA synthesis by Carr-Zilber associated virus DNA polymerase in the presence of $MnCl_2$ and $MgCl_2$. The spleen necrosis virus [^{3}H]DNAs prepared in the presence of $MnCl_2$ or $MgCl_2$ were both 4S in size and hybridized 80 and 90%, respectively, to spleen necrosis virus RNA (Mizutani and

Table VI: Effect of Deoxyribo- and Ribohomopolymers on the Frequency of Incorporation of Noncomplementary Nucleotides next to Complementary Nucleotides by *E. coli* DNA Polymerase I.[a]

Nearest Neighbor	Template·Primer $(dC)_n$·$(dG)_{12-18}$[b] $MnCl_2$	$(rC)_n$·$(dG)_{12-18}$ $MnCl_2$	$(rC)_n$·$(dG)_{12-18}$ $MgCl_2$
dGp	97.2	34.0	27.9
dAp	0.4	17.6	17.7
dCp	0.2	24.4	29.8
dTp	2.2	23.9	24.6

[a] DNAs were prepared in standard DNA polymerase reactions with $(dC)_n$·$(dG)_{12-18}$ or $(rC)_n$·$(dG)_{12-18}$ template·primers, 40 μM each of dATP, dCTP, and dTTP, and 20 μM [α-^{32}P]dGTP. The $MnCl_2$ concentrations were 1 mM for the $(dC)_n$·$(dG)_{12-18}$ template·primer and 0.75 mM for the $(rC)_n$·$(dG)_{12-18}$ template·primer. The optimum $MgCl_2$ concentration was 2 mM for the $(rC)_n$·$(dG)_{12-18}$ template·primer. Total incorporation of [α-^{32}P]dGMP was 14 nmol with $(dC)_n$·$(dG)_{12-18}$; 162 pmol in the presence of $MnCl_2$ and 154 pmol in the presence of $MgCl_2$ with $(rC)_n$·$(dG)_{12-18}$ template·primer. One hundred percent ^{32}P was 150 000 cpm (35.9 pmol) with the $(dC)_n$·$(dG)_{12-18}$ template·primer, 23 000 cpm (5.5 pmol) with the $(rC)_n$·$(dG)_{12-18}$ template·primer and $MnCl_2$, and 17 500 cpm (4.2 pmol) with the $(rC)_n$·$(dG)_{12-18}$ and $MgCl_2$. [b] Data from Table II.

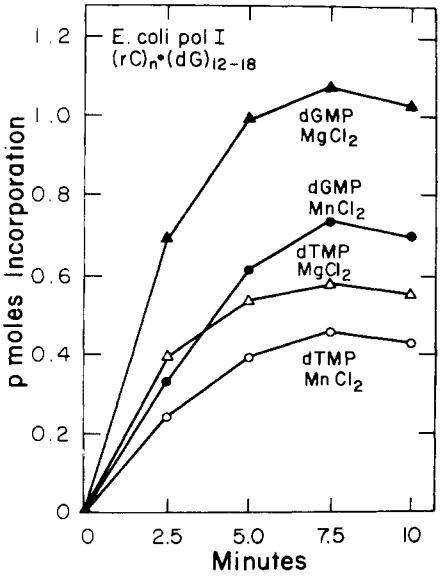

FIGURE 1: Incorporation of dTMP and dGMP by *E. coli* DNA polymerase I with an $(rC)_n$·$(dG)_{12-18}$ template·primer. Standard DNA polymerase reactions were carried out with an $(rC)_n$·$(dG)_{12-18}$ template·primer, 40 μM each of [^{32}P]dTTP, dATP, and dCTP, 20 μM [^{3}H]dGTP, and 0.03 μg of *E. coli* DNA polymerase I in the presence of 2 mM $MgCl_2$ or 0.75 mM $MnCl_2$. The reactions were carried out at 37 °C, and aliquots (20 μl) were withdrawn at the indicated time intervals. The specific activities of [^{3}H]dGTP and [α-^{32}P]dTTP were 33.2 and 6.4 Ci/mmol, respectively. dGMP incorporation in the presence of $MgCl_2$ (▲); dGMP incorporation in the presence of $MnCl_2$ (●); dTMP incorporation in the presence of $MgCl_2$ (△); dTMP incorporation in the presence of $MnCl_2$ (○).

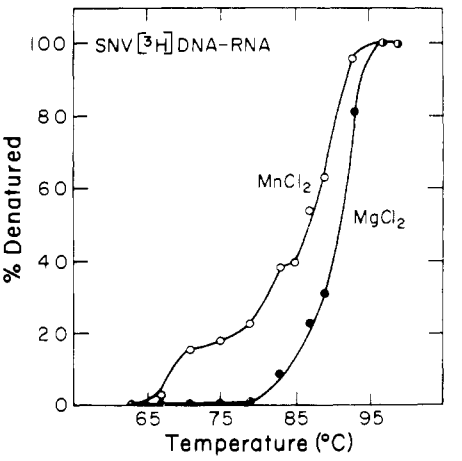

FIGURE 2: Thermostability of SNV RNA-[^{3}H]DNA hybrids. SNV [^{3}H]DNAs were prepared in the presence of $MgCl_2$ or $MnCl_2$ and were hybridized to SNV RNA as described previously (Mizutani and Temin, 1975). Aliquots (25 μl:4000 dpm) were placed in 25-μl disposable pipets, and both ends of the pipets were sealed. The pipets were heated for 15 min at the indicated temperatures and cooled quickly in ice-cold water. The contents were digested with S1 nuclease as described previously (Mizutani and Temin, 1975). The percent hybrid denatured was calculated by subtracting the percent resistant to S1 nuclease from 100.

Temin, 1975). The thermostabilities of the hybrids of the two spleen necrosis virus [^{3}H]DNAs and spleen necrosis virus RNA were compared (Figure 2). Comparison of the shapes and midpoints (T_m) of the melting curves of these hybrid molecules (T_m of 87 and 91 °C for the $MnCl_2$ and the $MgCl_2$ products, respectively) indicates that spleen necrosis virus [^{3}H]DNA prepared in the presence of $MnCl_2$ had more mismatched nucleotides than spleen necrosis virus [^{3}H]DNA prepared in the presence of $MgCl_2$. Therefore, the infidelity of DNA synthesis by Carr-Zilber associated virus DNA polymerase with a spleen necrosis virus RNA template·primer was greater in the presence of $MnCl_2$ than in the presence of $MgCl_2$ and was enough to affect nucleic acid hybridization.

Discussion

We previously reported the lack of nucleic acid hybridization between spleen necrosis virus RNA and the DNA product synthesized by spleen necrosis virus DNA polymerase with a spleen necrosis virus RNA template (Mizutani and Temin, 1975). To test the possibility that SNV DNA polymerase had a high degree of infidelity in DNA synthesis, a nearest neighbor frequency analysis was made of DNA products synthesized by SNV DNA polymerase with synthetic homopolymer templates in the presence of the α-^{32}P-labeled complementary nucleoside triphosphate and an excess of the three noncomplementary nucleoside triphosphates. The frequencies of incorporation of the noncomplementary nucleotides were compared with those by Carr-Zilber associated virus DNA polymerase and *E. coli* DNA polymerase I under the same conditions.

The experiments described here were designed to detect all incorporation of noncomplementary nucleotides next to complementary nucleotides. Further, the experiments were designed to favor the incorporation of noncomplementary nucleotides. All incorporation of nucleotides were template dependent, and all reactions were short (10 or 20 min). The incorporation of noncomplementary nucleotides was a result of infidelity of DNA synthesis because no synthetic homopolymer templates, labeled deoxyribonucleoside triphosphates, or DNA polymerases contained detectable contamination.

The concentrations of the noncomplementary nucleoside triphosphates were 40 μM, while the concentrations of the labeled complementary deoxyribonucleoside triphosphates were 20 or 6.6 μM. Since the K_m's of most DNA polymerases for the substrates are 15 to 50 μM (Loeb, 1974; Temin

and Mizutani, 1974), the concentrations of complementary deoxyribonucleoside triphosphates were usually slightly below the K_m's.

With a single DNA polymerase, the frequency of incorporation of the noncomplementary nucleotides next to the complementary nucleotide varied according to the conditions from almost none to up to 100% of noncomplementary nucleotides incorporated next to the complementary nucleotides. In general, the frequency of misincorporation was greater in the presence of Mn^{2+} than in the presence of Mg^{2+} and with a ribohomopolymer template rather than with a deoxyribohomopolymer. In several cases these results were confirmed by directly measuring the incorporation of labeled noncomplementary nucleotides. In addition, it was shown by comparison of melting temperatures with a DNA product synthesized with a heteropolymer RNA template that the presence of Mn^{2+} during DNA synthesis decreased the melting temperature of the product below that of the product synthesized in the presence of Mg^{2+}, again indicating an increased infidelity of DNA synthesis in the presence of Mn^{2+}.

When the frequency of incorporation of noncomplementary nucleotides next to complementary nucleotides by the three DNA polymerases was compared under one set of conditions, clear differences were observed. However, under another set of conditions the relative frequencies of misincorporation varied. Therefore, it could not be concluded that one DNA polymerase always made more errors in DNA synthesis than the others. The effects of Mn^{2+} rather than Mg^{2+} and ribohomopolymer rather than deoxyribohomopolymer templates were greater than any differences among the three DNA polymerases.

The results of the present studies are consistent with our hypothesis that the failure of hybridization between SNV RNA and SNV DNA synthesized by the SNV DNA polymerase with an SNV RNA template in a cell-free reaction is the result of a high frequency of infidelity of DNA synthesis. Therefore, in SNV infection, some presently unknown mechanisms must maintain the fidelity of SNV DNA synthesis.

Previously, the fidelity of DNA synthesis by purified avian myeloblastosis virus DNA polymerase was studied by other workers. Wells et al. (1972) reported no infidelity in copying synthetic polynucleotides, but Springgate et al. (1973) and Battula and Loeb (1974) reported that there were approximately 1/600 errors in copying synthetic polynucleotides. Although these workers also used homopolymer templates, their reactions were carried out in the presence of Mg^{2+} and only some of the noncomplementary nucleoside triphosphates, which were at lower concentrations than we used.

Furthermore, Hall and Lehman (1968) have shown with T4 DNA polymerase that $MnCl_2$ caused a 20-fold higher infidelity than $MgCl_2$ and that the amount of infidelity increased when the concentrations of the noncomplementary nucleoside triphosphates were increased relative to that of the complementary one. Battula et al. (1975) also found with avian myeloblastosis virus DNA polymerase an increased incorporation of noncomplementary nucleotides with an increased concentration of noncomplementary nucleotides.

Therefore, the results in the present paper are consistent with those of previous workers once the changes in conditions are recognized.

The present findings may have both practical and theoretical significance. Ribonucleoside substitution by *E. coli* DNA polymerase in the presence of $MnCl_2$ (Berg et al., 1962) is being used in DNA sequencing (Salser et al., 1972; de Sande et al., 1972). The high frequency of incorporation of noncomplementary nucleotides by *E. coli* DNA polymerase I in the presence of $MnCl_2$ and some templates reported here indicates that care must be used with this technique.

Previous studies with cell-free systems have tried to define error-prone DNA polymerases (Springgate et al., 1973; Sirover and Loeb, 1974). However, we have shown that differences in conditions of reactions are of more significance for determining error rates in cell-free studies than differences between viral and cellular DNA polymerases.

However, the high frequency of misincorporation seen under certain conditions in this paper might reflect conditions in cells. $MnCl_2$ is a mutagen for *E. coli* (Demerec and Hanson, 1951), and mutant DNA polymerases have been implicated in the high mutation rate of some T4 mutants (Speyer, 1965; Bessman et al., 1974; Goodman et al., 1974).

Ribodeoxyviruses have an unusually high rate (1 per 100 replications) of spontaneous mutation in certain characters (Zarling and Temin, 1976). Changes in the physiological environment or altered DNA polymerases may be responsible for this high mutation rate. Similar processes may also result in the formation of new nucleotide sequences (Temin, 1971, 1974).

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The Monosaccharide Composition and Sequence of the Carbohydrate Moiety of Human Serum Low Density Lipoproteins†

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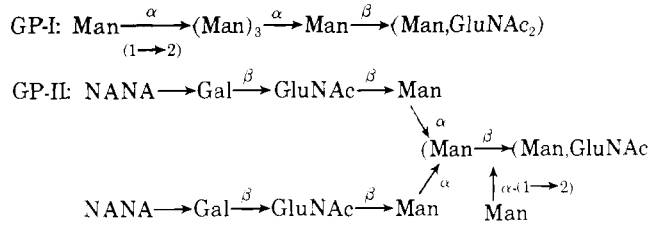
ABSTRACT: Human serum low density lipoprotein (d = 1.027–1.045) was delipidated with organic solvents and the apoprotein digested with thermolysin. The digest was fractionated by gel filtration and DEAE-cellulose chromatography. Two glycopeptides were obtained. One of the glycopeptides (GP-I) contained 2 residues of *N*-acetylglucosamine and 6 residues of mannose per mole of the glycopeptide, while the other contained 2 sialic acid, 5 mannose, 2 galactose, and 3 *N*-acetylglucosamine residues per mole of glycopeptide. The results of sequential enzymatic digestion with purified glycosidases, periodate oxidation, and partial acid hydrolysis lead us to propose the following structures for the two glycopeptides:



These glycopeptides represent at least 50% of the carbohydrate moiety of LDL.

The protein moiety of low density lipoproteins has been shown to contain 5–9% carbohydrate consisting of galactose, mannose, glucosamine, and sialic acid (Schultze and Heide, 1960; Ayrault-Jarrier, 1961; Marshall and Kummerow, 1962; Swaminathan and Aladjem, 1974; Kwiterovich et al., 1974). Shore and Shore (1973) demonstrated that serum VLDL[1] interacts with concanavalin-A and McConathy and Alaupovic (1974) showed that LDL interacts with concanavalin-A while HDL does not, both studies indicating the presence of terminal glucopyranosyl, mannopyranosyl, or sterically related residues in LDL. Margolis and Langdon (1966) found that sialic acid residues could be removed from native LDL with sialidase treatment without affecting lipid binding. The present paper describes the results of our studies to define the number, nature, and the monosaccharide sequence of the carbohydrate units in LDL. We have prepared glycopeptides from LDL and succinylated LDL using a specific protease, thermolysin. The monosaccharide sequence was deduced from the combined application of acid hydrolysis, periodate oxidation, and digestion with glycosidases of demonstrated specificity.

Materials and Methods

Low density lipoprotein (d = 1.027–0.045 gm/cc) was isolated from pooled normal human serum containing 0.1% EDTA as previously reported (Aladjem et al., 1957). Approximately 100 mg of protein was obtained from 1 l. of serum. The purity of preparation was tested by immunochemical methods using specific antisera. Within the detectability of these methods, no contamination of HDL, VLDL, or of other serum proteins was found.

Delipidation was performed using ether–ethanol mixtures. To a preparation of native LDL (or succinylated native LDL) dialyzed against 0.15 M NaCl overnight and diluted to a protein concentration of 3 to 4 mg/ml, 20 volumes of a mixture of ethanol–ether (3:1 v/v) was added dropwise with continuous stirring. The solution was stirred for 4 to 6 h and centrifuged at 1500g for 20 min. The precipitated protein was extracted with ethanol–ether (3:2 v/v) and centrifuged. The white precipitate was washed with ether and then dried in a current of N_2.

Succinylation was performed on native LDL as described by Scanu et al. (1968) followed by delipidation as described above. Completion of the succinylation reaction was monitored by ninhydrin reaction (Frankel-Conrat, 1957).

Preparation of Glycopeptides from LDL. Thermolysin

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[1] Abbreviations used: VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; S-LDL, succinylated low density lipoproteins; Gal, galactose; Man, mannose; GluNAc, *N*-acetylglucosamine; NANA, *N*-acetylneuraminic acid.