AN UNUSUAL EXONUCLEASE ACTIVITY ASSOCIATED WITH MICROCOCCUS LUTEUS DNA POLYMERASE

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SUMMARY. A trace of an unusual exonucleolytic activity is present in highly purified preparations of Micrococcus luteus DNA polymerase. With poly dAT, about 500 times as much polymerase as nuclease activity is obtained. Nuclease activity is higher in Mn^{++} than in Mg^{++}, but in the presence of deoxynucleoside triphosphates, rates are stimulated in Mg^{++} but suppressed in Mn^{++}. The products of degradation of several DNA-like polymers are largely (90%) mono- and dinucleotides with traces of oligonucleotides. With poly dAT, mainly dinucleotides are produced, but with poly dAdT and dGdC, the ratio of mono- to dinucleotides is different from that of poly dAT and different on each of the two strands.

The Micrococcus luteus DNA polymerase as purified by the DNA-cellulose procedure possesses a trace of exonuclease activity but little or no endonuclease (1). These polymerase preparations yield upon acrylamide gel electrophoresis at pH 8.5 essentially one band of protein which contains both polymerase and nuclease activity (Litman, in preparation). Recently Harwood et al (2) reported that their less highly purified preparations of M. luteus DNA polymerase also contain nuclease activity, which when reacting with the alternating copolymer, poly dAT, produces as the major breakdown products dinucleotides of A and T. In our work with poly dAT, we find, in agreement with these authors, dinucleotides as the major products, with lesser amounts of mononucleotides and small oligonucleotides. However, since with other DNA-like polymers, a different distribution of mono-, di-, and oligonucleotides is obtained, we conclude that the sequences of bases along a DNA chain is the major factor determining the identity of the products of this nuclease reaction.

MATERIALS AND METHODS. DNA polymerase was purified from M. luteus as previously described (1). All nucleotides, including radioactive ones, were purchased from Schwarz BioResearch and were periodically checked for purity by chromatography in system II (see Fig. 2). Poly dAT and poly dGdC

labeled with ^{14}C or ^{3}H in one or the other of the bases were synthesized with the M. luteus DNA polymerase (Litman, submitted for publication). Poly dAdT was formed by annealing equal concentrations of poly dA and dT, both kindly provided by Dr. F. J. Bollum, and used to synthesize labeled polymer as for poly dGdC. All concentrations of polymers are given in nucleotide equivalents.

All measurements of radioactivity were made on Packard scintillation counters, calibrated for isotope overlap in double-isotope experiments. Samples were placed on a variety of papers which differentially affect ^{14}C or ^{3}H counting efficiencies; appropriate standards were always included to enable a correction of all counts reported in this work to their respective values on Whatman GF/C glass fiber circles.

RESULTS. As shown in Table 1, when tested at the pH optimum for polymerase activity and starting with equal amounts of poly dAT, several different preparations of M. luteus DNA polymerase exhibit ratios of polymerase to

Table 1

Comparison of synthetic to degradative rates of poly dAT by M. luteus DNA polymerase

Polymerase preparation	nmoles poly dAT/30 min/μg protein		
	polymerized	degraded	ratio
1	148.6	0.25	594
2	155.5	0.22	707
3	135.7	0.233	582

Synthesis: each reaction contained in 0.2 ml, 20 μmoles Tris-HCl pH 7.7, 0.4 μmoles $MgCl_2$, 5.6 nmoles poly dAT, 20 nmoles each dTTP and dATP-8 ^{14}C(spec. act. 0.2 μc/μmole), and 0.05 to 0.13 μg polymerase protein. After incubation at 37° for 30 min, the polymerization of dAMP-^{14}C was measured after acid precipitation and filtration on glass fiber circles as previously described (1). Degradation: each reaction contained in 0.2 ml, 20 μmoles Tris-HCl pH 7.7, 50 nmoles $MnCl_2$, 5.1 nmoles poly dA(^{14}C)T (about 2,000 cpm), and 0.15 to 0.3 μg polymerase protein. After incubation for 16.5 hours at 37°, the amount of polymer rendered acid-soluble was measured as previously described (1).

nuclease activity of over 500:1 (nmoles poly dAT/30 min/μg protein, about 150 synthesized to 0.25 degraded). Polymerase activity was measured after 30 min of reaction and nuclease after 16.5 hours. Both reactions are linear during these time periods and generally continue to completion (Fig. 1). In the reactions of Table 1, synthesis was measured in the presence of Mg^{++} and

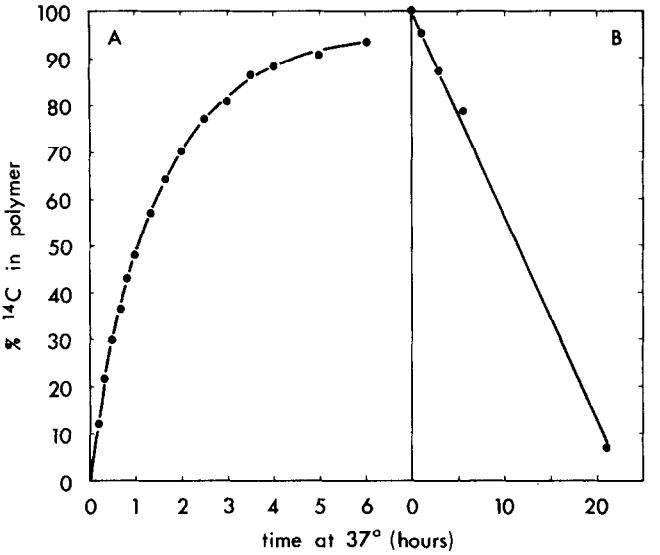

Fig. 1. Kinetics of synthesis and degradation of poly dAT by M. luteus DNA polymerase. A. Synthesis: the reaction containing in 0.5 ml, 50 µmoles Tris-HCl pH 7.7, 1 µmole $MgCl_2$, 14 nmoles poly dAT, 50 nmoles each dTTP and dATP-8^{14}C (0.2 µc/µmole) and 0.22 µg polymerase protein was incubated at 37°. At indicated times, the % dAMP-^{14}C polymerized (ordinate) was measured in aliquots by the DEAE binding technique (3). B. Degradation: the reaction mixture containing in 0.12 ml, 10 µmoles Tris-HCl pH 7.7, 70 nmoles $MnCl_2$, 27 nmoles poly dA(^{14}C)T (about 50,000 cpm), and 0.72 µg polymerase protein was incubated at 37°. At indicated times, an aliquot was spotted and chromatographed in system II (see Fig. 2). The ordinate presents the % of total counts on each chromatogram which remained at the origin (undegraded polymer).

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breakdown with Mn^{++}. The substitution of optimal concentrations of Mn^{++} for Mg^{++} has little effect on synthetic rates of poly dAT (Litman, submitted for publication), but degradation rates are faster in Mn^{++} than in Mg^{++} (Table 2). Furthermore, if dATP and dTTP are also added (Table 2), allowing for concomittant synthesis, it is found with Mg^{++}, in agreement with the results of Harwood et al (2), that the rate of degradation is stimulated; with Mn^{++}, however, the degradation rate is suppressed to about 20% that in the absence of synthesis. Neither poly dAdT nor dGdC are degraded as extensively as poly dAT (see Table 3), but with these polymers also, in the absence of synthesis, more degradation is obtained with Mn^{++} than with Mg^{++}.

Some typical results of chromatographic analyses of the degradation

Table 2

Effect of Mn^{++} and Mg^{++} and of deoxynucleoside triphosphates on the degradation of poly dAT

	nmoles added				% ^{14}C acid-soluble
	$MnCl_2$	$MgCl_2$	dATP	dTTP	
1.	-	-	-	-	< 0.5
2.	50	-	-	-	17.7
3.	-	1,000	-	-	9.2
4.	50	-	30	30	3.0
5.	-	1,000	30	30	14.9

Each reaction mixture contained in 0.2 ml, 20 µmoles Tris-HCl pH 7.7, 2.8 nmoles poly dA(^{14}C)T (about 3,000 cpm) and 0.6 µg polymerase protein. The other indicated components were added, and after incubation at 37° for 3 hours, the % ^{14}C rendered acid-soluble was measured as previously described (1).

products of poly dAT, dAdT and dGdC (with ^{14}C in the purine and ^{3}H in the pyrimidine) are shown in Fig. 2. The degradation reactions were carried out with Mn^{++}, but in the absence of any nucleoside triphosphates. It can be seen that the radioisotopes either remained at the origin of the chromatogram or migrated as mononucleotides or small oligonucleotides, and that the ratio of these products was different for the three polymers. With poly dAT (Fig. 2A), some of the ^{14}C and ^{3}H counts coincided respectively with dAMP or dTMP. The major migrating material, marked X, however, contained the same $^{3}H/^{14}C$ ratio as the original polymer (1.15). It had furthermore migrated to a position relative to either dAMP or dTMP which Aposhian et al (4) found to be that of dpTpA in the solvent employed. We therefore conclude that X is a dinucleotide containing A and T, but we have made no attempt to determine the distribution of the two possible sequences within this spot. In the material remaining at the origin and in dTMP to dAMP, $^{3}H/^{14}C$ was also 1.15. Between the origin and X, there was a small amount of overlapping material containing both isotopes, which are probably tri-,

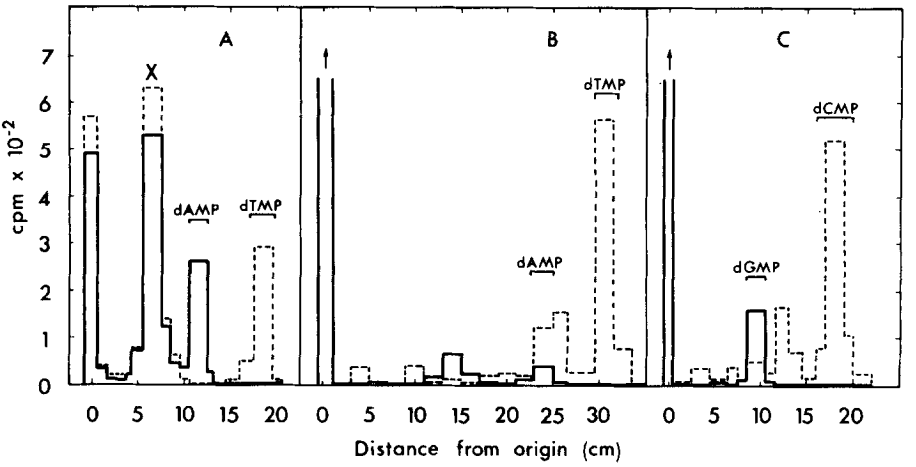

Fig. 2. Chromatography of degradation products of poly dAT, dAdT, and dGdC. Degradation reactions were carried in 0.1 M Tris-HCl pH 7.7. In addition, the poly dAT reaction (A) contained in 0.3 ml, 0.5 µmoles $MnCl_2$, 72 nmoles poly dA(^{14}C)T(^{3}H) (about 10,500 cpm ^{14}C and 12,000 cpm ^{3}H), and 1.5 µg polymerase protein. The poly dAdT reaction (B) contained in 0.5 ml, 0.25 µmoles $MnCl_2$, 27.5 nmoles poly dA(^{14}C)dT(^{3}H) (about 5,000 cpm ^{14}C and 12,500 cpm ^{3}H), and 0.65 µg polymerase protein. The poly dGdC reaction (C) contained in 0.6 ml, 100 nmoles $MnCl_2$, 64 nmoles poly dG(^{14}C)dC(^{3}H) (about 4,000 cpm ^{14}C and 8,000 cpm ^{3}H), and 7.2 µg polymerase protein. The three reactions were incubated at 37° for 3 (A), 24 (B), and 40 (C) hours, at which time samples were spotted and chromatographed, along with known nucleotides whose positions are shown by the inverted brackets. In A and B, system I, descending chromatography on Whatman # 3 paper in 100% ethanol : 1 M ammonium acetate pH 7.5, 70:30 for 20 hours or more (4) was employed; in C, system II, descending chromatography on Whatman # 40 paper in Isobutyric acid : concentrated NH_4OH: 0.2 M EDTA (pH 8.1), 100:60:0.8 for 20 hours. The chromatograms were dried, cut at the distances indicated, immersed in scintillation fluid and counted. In B, there were at the origin 1040 cpm ^{14}C and 1950 cpm ^{3}H, and in C, 890 cpm ^{14}C and 2000 cpm ^{3}H. No counts were found elsewhere on the chromatograms other than those shown. The position of the front in A was at 29 cm, in B at 47.5 cm, and in C at 40.5 cm. Solid lines ^{14}C counts and dashed lines ^{3}H counts.

tetra- and pentamers containing A and T.

With poly dAdT (Fig. 2B), the bulk of the migrated ^{3}H counts coincided with dTMP. A smaller peak was found at the position expected for dpTpT (4), and the few counts distributed between the latter peak and the origin are likely to be contained in trimers and tetramers of dpT. The dA strand, on the other hand, was preferentially broken down to material which migrated with an R_f relative to dAMP equal to that of dpApA (4). With poly dGdC (Fig. 2C), again the bulk of the migrating ^{3}H label coincided with the mono-

nucleotide (dCMP), and most of the remainder was in a peak with an R_f reasonable for dpCpC. There were also some counts in material likely to be tri-, tetra-, and pentamers of dpC. The dG strand breakdown products, however, coincided almost entirely with dGMP.

The distribution of degradation products of the three polymers after exonucleolytic attack in the presence of Mn^{++} for two reaction times are listed in Table 3. It can be seen that of the poly dAT which was degraded, 25% is in dTMP and dAMP, 70% in dinucleotides of A and T, and the rest in small oligonucleotides. This distribution is obtained at all times during nucleolytic breakdown, in either Mn^{++} or Mg^{++} and in the presence or absence of synthesis. It is identical to that found by Harwood et al (2) with Mg^{++}.

Table 3

Degradation products of poly dAT, poly dAdT, and poly dGdC

Polymer	Hours of reaction	Base	% of total counts at origin	% of total migrated counts		
				Mononucleotide	Dinucleotide	Small oligonucleotide*
dAT	3	A	29.8	27.7	67.4	4.8
		T	28.9	26.5	67.8	5.7
dAT	6	A	11.4	25.8	68.7	5.5
		T	11.1	26.2	68.6	5.2
dAdT	4	A	90.5	30.2	63.6	8.5
		T	74.6	58.8	30.7	10.5
dAdT	24	A	86.0	31.5	60.3	8.2
		T	61.9	59.5	28.4	12.1
dGdC	5	G	89.7	93.6	6.4	-
		C	81.7	62.6	25.2	12.2
dGdC	27	G	76.2	93.0	7.0	-
		C	63.5	55.2	29.1	15.7

*refers to trimers, tetramers and pentamers
Degradation reactions and chromatographic analyses were carried out as in the legend to Figure 2.

With the homopolymer duplexes, however, it is obvious that a different reaction is taking place on the two strand types. With both poly dAdT and dGdC, the pyrimidine strand was more extensively degraded than the purine strand, and in both was degraded to 60% mononucleotides, 25-30% dinucleotides and about 10% small oligonucleotides. The dA strand of poly dAdT was simultaneously broken down to the opposite distribution, 30% mono-, 60% di-, and 10% small oligonucleotides. The dG strand of poly dGdC, however, seems to be degraded almost exclusively to dGMP.

DISCUSSION. M. luteus DNA polymerase preparations contain traces of nuclease activity, which while not that of an endonuclease is also not typical of that of an exonuclease. We have not identified the direction of breakdown by this enzyme, but in view of the identical results obtained by ourselves and by Harwood et al (2) on the breakdown of poly dAT, their finding that their preparations act from the 5' end of the polymer undoubtedly applies to our enzyme as well.

The nuclease reacts differently on polymers of different sequences. Clearly in homopolymer duplexes, the pyrimidine strands are cleaved largely to mono- and dinucleotides in the ratio of 2:1, whereas on poly dA, the reverse is true. From these results, it can be predicted that in poly dAT from which dinucleotides are primarily produced that they should contain a preponderance of dpApT over dpTpA. Harwood et al (2) in their investigations have shown that this is indeed the case. It would also be expected that in DNA, mixtures of mono- and dinucleotides should be the principal products. In preliminary experiments with T7 DNA (50% G+C) uniformly labeled with ^{32}P, it was found that the breakdown products consisted of about 60% mononucleotides and the remainder primarily dinucleotides. It seems likely, therefore, that the apparent multiple of activities observed on polymers of different sequences is the result of the action of the same enzyme.

Possibly similar nuclease activity is also present in preparations of E. coli DNA polymerase. Kelly et al (5,6) found that the dT strand of poly

dAdT is cut primarily to mononucleotides, but that dinucleotides and lesser amounts of oligonucleotides are also obtained. Kadohoma and McCarter (7) report the production by the same enzyme from poly dAT of dinucleotides containing A and T. Both sets of workers also find that nuclease activity in Mg^{++} is stimulated by the presence of the deoxynucleoside triphosphates. In the work with the E. coli polymerase, the relative production of dinucleotides is less than that obtained with the M. luteus polymerase, but the E. coli preparations also contain a 3' exonuclease which cleaves almost exclusively to mononucleotides (8).

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