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O^4-Methyldeoxythymidine replacing deoxythymidine in poly[d(A−T)] renders the polymer resistant to the 3′ → 5′ exonuclease activity of the Klenow and T_4 DNA polymerases

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ABSTRACT

We previously reported that O^4-alkyl dTTPs could replace, for short times, dTTP in polymer synthesis [Singer et al., PNAS 83, 26-32, 1986]. The reasons for such early termination of synthesis could be either proofreading or the eventual formation of weakly paired primer termini. Utilizing the known 3' → 5' exonucleolytic activity of polymerases, in the absence of dNTPs, enabled us to conclude that, in contrast to the digestibility of poly[d(A-T)] which yielded the expected 3'-mononucleotides, the polymerizing enzymes did not digest O^4-methyl dT or its neighbors. The presence of the resistant α-phosphorothionate linkage did not prevent measurable digestion of poly[d(A-T)] by the Klenow fragment. This, together with evidence that polymerization of O^4-methyl dTTP is favored at low temperatures, supports the model proposed by Ollis et al. [Nature 313, 762-766, 1985] showing independent domains for the two activities in the Klenow fragment.

INTRODUCTION

O^4-methyldeoxythymidine (m^4dT) and O^4-ethyldeoxythymidine (e^4dT) are mutagenic derivatives (1-4) which are strongly implicated in N-nitrosamine and N-nitrosourea-induced carcinogenesis (5-8). The kinetics of the incorporation of O^4-alkyl dTTPs as analogues of dTTP indicate that they are recognized by polymerases but, in their presence, synthesis on polymer or DNA templates terminates within a short period (9). Relatively more synthesis, compared to that using unmodified dTTP, occurs as the temperature of polymerization is decreased (9). This we interpret as evidence that the newly synthesized primer end is not well enough hydrogen-bonded, at normal polymerization temperatures, to utilize the 3'-OH end. Alternatively, it was not excluded that termination of synthesis was due to idling or proofreading. One approach to discriminating between these possibilities was to study the effect on these polymers of the 3' → 5' exonuclease activity of polymerases known to degrade poly[d(A-T)], in the absence of dNTPs. Contrary to expectation, polymers containing m^4dT prevented the release of any mononucleotide. Resistant phosphodiesterase linkages have been known, e.g., α-S linkages replacing normal

phosphates (10,11). However, polymers containing this type of linkage can be digested to oligomers wherever the normal linkage occurs (11). In other cases where modified nucleotides have been incorporated into polymers, the high turnover observed could not be ascribed solely to proofreading (12).

MATERIALS AND METHODS

Materials

The Klenow fragment of E. coli DNA polymerase I (Pol I) was obtained from two sources. Commercial Klenow from Pharmacia P-L Biochemicals had a specific activity of 7500 U/mg. Dr. L. A. Loeb generously provided highly purified Pol I and the Klenow fragment from this preparation which had a specific activity of 23000 U/mg and was free of contaminating endonuclease or 5' → 3' exonuclease. T_4 DNA polymerase from Pharmacia P-L Biochemicals had a specific activity of 20,000 U/mg.

[^{3}H]dATP, [^{3}H]dTTP, ^{35}S-(Sp)-dATPαS and nonradioactive (Sp)-dATPαS were purchased from New England Nuclear. Unlabeled dATP, dTTP, dCTP, dGTP and poly[d(A-T)] were from Pharmacia P-L Biochemicals. O^4-Methyldeoxythymidine triphosphate (m^4dTTP) was prepared as previously described.

Methods

Synthesis of poly[d(A-T)] with [^{3}H]dATP or [^{35}S]-dATPαS and dTTP. Elongation of poly[d(A-T)] was performed as follows. A 100 µl reaction mixture contained 20 µmoles Tris·HCl (pH 7.8), 200 nmoles $MgCl_2$, 0.1 A_{260} poly [d(A-T)] (5 µg), 10 nmoles dTTP and 10 nmoles [^{3}H]dATP (1 Ci/mmol) or [^{35}S]dATPαS (160 mCi/µmol) and 0.45 units Klenow fragment. After incubation for 10 min at 37° the reaction mixture was applied to a Biogel P-150 column (0.9 x 20 cm) and eluted with water. 0.5 ml fractions were collected. The polymer peak fractions (#6-9) were well separated from monomeric material (#13-23). No labeled nucleoside mono- or triphosphate was detectable in polymer peak fractions after thin layer chromatography on Eastman #6065 cellulose developed in 75 ethanol/30 ammonium acetate (pH 7). Incubation of these polymers for 18 h, 37° in the same reaction mixture as used for synthesis did not release nucleoside monophosphates. Thus no detectable exonuclease activity remained associated with the polymers.

The specific radioactivity of the purified polymers indicated that 8-12% net synthesis occurred. As previously reported by Kunkel et al. (13), dATPαS was utilized by the Klenow fragment to a similar extent as was dATP.

Synthesis of Poly[d(A-T,m^4T)]. The reaction conditions were the same as used for elongating poly[d(A-T)] with [^{3}H]dATP except that 9 nmoles m^4dTTP and

1 nmole dTTP were used, and synthesis continued for 30 min. This ratio of m^4dTTP and dTTP was earlier found to yield polymers with 5-10% of the pyrimidines as m^4dT (1, and unpublished data). The specific radioactivity indicated that 4-7% net synthesis occurred.

3'-Terminal Labeling of Poly[d(A-T)] and DNA. Poly[d(A-T)] was 3'-end labeled with either [^{3}H]dATP or [^{3}H]dTTP using the same reaction conditions as for extended elongation, with the exception that 1.5 A_{260} polymer (75 µg) was used as primer and only dATP or dTTP was present at a specific radioactivity of 15 Ci/mmol. Assuming that end-labeling is quantitative, the chain length was calculated to be 1200 nucleotides. Gupta et al. (14) reported a chain length of ∿900 using terminal labeling but slightly different conditions.

Activated salmon sperm DNA was similarly end labeled using [^{3}H]dATP, dCTP, and dGTP. Assuming a random nucleotide sequence, there was 1 dA/2100 nucleotides.

Exonuclease Hydrolysis. Substrates elongated 4-12% were generally tested for the rate and extent of hydrolysis under the following conditions. A 100 µl reaction mixture containing 20 µmoles Tris·HCl (pH 7.8), 0.6 µmole $MgCl_2$, 0.002 A_{260} polymer (0.1 µg) and 9 units commercial Klenow fragment or 10 units Loeb Klenow fragment or 5 units T4 polymerase, was incubated at 37° for 0.5-18 h. Methods for determining nucleotide release are in the following section. Other conditions are in the Figure Legends.

3'-end labeled substrates were treated under the same conditions except that the amount of polymer was increased to 0.1 A_{260} and times of reaction were decreased to 2-30 min. Aliquots were applied to DEAE discs which were immediately washed with 7% Na_2HPO_4 to stop the reaction. After further washing with phosphate, followed by ethanol, the discs were dried and the radioactivity determined in a toluene-based scintillant.

Quantitation of Exonuclease Action. The extent and rate of exonuclease action were determined using three methods. Exonuclease-treated polymers were applied to a small Biogel P-150 column (0.4 x 5 cm), fractions collected and counted in Aquasol. This column differentiates polymer from monomers. Further evidence of the nature of the products was obtained using the cellulose TLC system (described in the section on Synthesis of Polymers) which separates polymer, or di- or trinucleotides from mononucleotides. The R_F values are poly[d(A-T)], 0.0; $d(T_p)_3$, 0.10; 5'-dAMP, 0.35; 5'-dTTP, 0.54. TLC was used for unfractionated samples as well as those from the Biogel P-150 column. The third method, described above, is a filter assay which discriminates between monomers or small oligomers and higher molecular weight polymers which are retained on the filter.

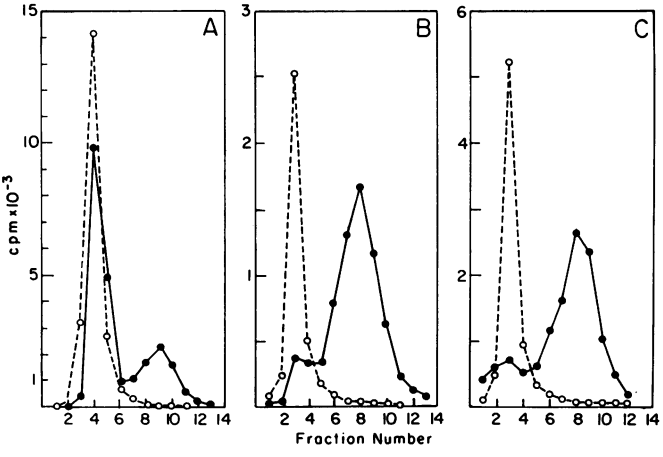

Figure 1. Products from DNA polymerase-associated exonuclease digestion of poly[d(A-T)] (●—●) and poly[d(A-T,m⁴T)] (o---o) containing [³H]-labeled dA. Specific radioactivity of various preparations ranged from 3-6 x 10^5 cpm/ 0.1 A_{260}. Separations were performed using Biogel P-150. The peak at fraction 4 represents undegraded polymer and the later peak is 5'-NMP. Digestions were 18h, 37° and performed as described in Methods. The amounts of substrate and enzyme were as follows:

A. 0.02 A_{260} polymer (30 nmoles nucleotide), 4.5 U Klenow.
B. 0.002 A_{260} polymer, 4.5 U Klenow. Incubation of poly[d(A-T)] in the absence of polymerase did not cause any measurable degradation.
C. 0.002 A_{260} polymer, 5 U T_4 DNA polymerase.

RESULTS

The unexpected finding that m⁴dT-containing poly[d(A-T)] completely inhibited 3' → 5' exonuclease digestion is illustrated in Figure 1. Neither the Klenow fragment of Pol I, nor T_4 DNA polymerase, even at extremely high ratios of enzyme/polymer (50-fold that used for synthesis), released any low molecular weight material from polymers with 5-10% m⁴dT. In contrast, exonuclease digestion of poly[d(A-T)] was almost complete in 18 h, with both polymerases. As shown in Figures 1B and 1C, less than 15% of the [³H]dA remained in the polymer peak. When the ratio of enzyme/polymer was reduced 10-fold, 70% of the label remained in the polymer (Figure 1A).

Other experiments showed that the extent of digestion was a function of enzyme concentration (data not shown) and time (Fig. 2). This Figure also presents data on a parallel digestion of the alternating polymer in which dAMP is replaced by dAMPαS. The phosphorothionate linkage has been reported to be resistant to the nuclease activity of Pol I, and the products of digestion are

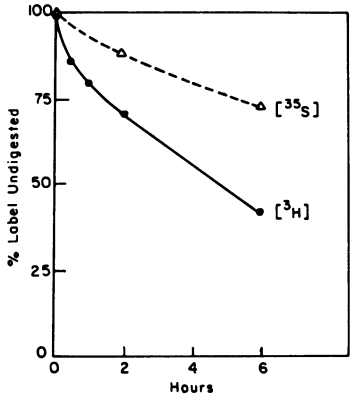

Figure 2. Rates of Klenow-associated exonuclease digestion of poly [d(^{3}H)A-T] and poly[d(^{35}S-αS)A-T]. 0.002 A_{260} polymer and 5 units Loeb prepared Klenow were used. The remaining label in the polymer, at various time points, was determined using a filter assay.

tetramers and dimers which have the structures $(pdTp_{S}dA)_2$ and $pdTp_{S}dA$ (11). Using the filter assay, which does not retain such oligomers, or the Biogel P-150 column chromatography system shown in Figure 1, both the [^{3}H] and [^{35}S] polymers are susceptible to 3' → 5' exonuclease action, although the phosphorothionate linkages are somewhat inhibitory, as noted by Burgers and Eckstein (10).

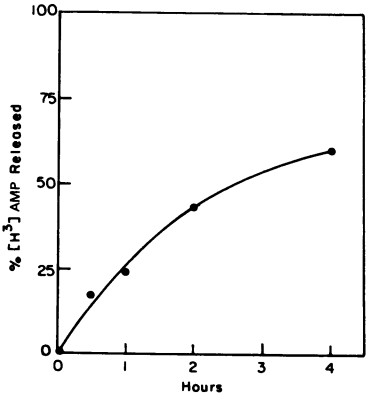

Figure 3. Rate of release of [^{3}H]5'-AMP from poly[d(A-T)]. The same conditions were used as in Figure 2. The 5'-nucleotide released was quantitated using TLC.

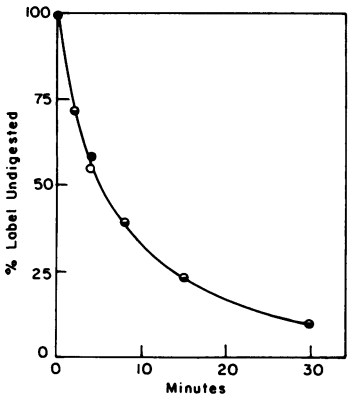

Figure 4. Rates of release of 3'-terminal[^{3}H]dAMP (●—●) or [^{3}H]dTMP (o---o). 0.12 A_{260} polymer and 5 units Loeb prepared Klenow were used. Quantitation was by a filter assay.

The processive nature of exonuclease action is confirmed by separate experiments which independently measured the amount of undegraded polymer and the amount of 5'-dAMP released (Fig. 3). For example, after 4 h digestion about 45% of poly[d(A-T)] remains bound to a DEAE filter (Fig. 2) while about 60% of the radiolabel is chromatographically identical to 5'-dAMP. Further identification of 5'-dAMP as the sole product of both sources of the Klenow fragment, Pol I and T_4 DNA polymerase was obtained by TLC of the two peaks found after Biogel P-150 separation. The polymer peak radioactivity was entirely at the origin, while the radioactivity in the later peak all migrated as 5'-dAMP. Occasionally, some deoxyadenosine formed, particularly with long digestion times. This is attributed to low levels of contaminating phosphatases.

The technique of 3'-end labeling was found useful in examining the kinetics of release of 5'-dAMP and 5'-dTMP (Fig. 4). The $t_{\frac{1}{2}}$ of release of both nucleotides was identical and in repeated determinations averaged 5 min, under the conditions used. The [^{3}H]dA-label from DNA (made in the absence of dTTP) was also removed rapidly with a $t_{\frac{1}{2}}$ of 10 min. These figures cannot be directly compared with the $t_{\frac{1}{2}}$ for extensively elongated poly[d(A-T)] which averaged 4.5 h for about 200 nucleotides (or 1.4 min/nucleotide), since the latter experiments used a 60-fold greater enzyme/polymer ratio.

DISCUSSION

The major and surprising finding of the resistance of m^4dT-containing poly[d(A-T)] to the 3'-5' exonuclease activity of two DNA polymerases, in the absence of dNTPs, was even more unexpected in view of the low m^4dT content. The probability of m^4dT being the terminal nucleotide is small since the preferred dTTP (9) is also present. On the other hand, we cannot exclude that most chains do carry either terminal or proximal m^4dT. Regardless of which model we choose, the polymer was highly resistant to exonuclease digestion.

We could easily detect the nuclease action of the Klenow fragment of Pol I on poly[d(T_{S}A)] containing the resistant thiophosphoryl linkage (10,11) since the products of degradation are tetramers and dimers which are not retained in the filter assay (Fig. 2) and are also separated from polymer using column chromatography. The rate of hydrolysis of the [^{35}S] polymers was lower than for the [^{3}H] polymers, as noted by Burgers and Eckstein (10). Normal poly[d(A-T)] was degraded in the conventional manner and release of labeled 5'-dAMP or 5'-dTMP followed expected kinetics (14).

Enzyme resistance conferred by modified bases has been reported, but usually the enzymes are endonucleases such as snake venom phosphodiesterase (SV-DE), micrococcal nuclease and spleen phosphodiesterase (15-20), although in some instances the authors attribute this, in part, to phosphotriesters which are not degraded by these enzymes (21).

This laboratory found that even the phosphodiester linkage of dinucleoside diphosphates (e.g., pGpN where N represents m^3C, m^3U, e^2U, e^4U, e^2C) were resistant to SV-DE (22). More recently we reported that O^4-ethyl dT and O^4-isopropyl dT, incorporated into poly[d(A-T)], were not appreciably digested by a variety of nucleases (3). The same problem in susceptibility to enzymes forming 3' nucleotides was noted by Preston et al. (23) who, however, were using the sequence ...GpT*pA (T* representing any of three O^4-alkyl dTs) whereas Singer et al. were studying the sequence ...ApT*pA. It would appear, in the absence of additional data, that the difficulty in endonuclease recognition may be the 3' neighbor. Further experiments are in progress to test this hypothesis.

Regarding the editing function of prokaryotic DNA polymerases, in the presence of dNTPs, the removal of mismatched or poorly matched bases may be restricted to certain 3'-OH terminal mismatches during replication. In the absence of replication, normal basepairs are excised stepwise. When such a polymer contains m^4dT, which prevents excision, we postulate that the enzyme

occupies a domain containing one or more m^4dT residues which inhibit the polymerase from assuming the proper configuration for the exonuclease reaction. On the basis of recent crystallographic data on the Klenow fragment complexed with dTMP (24), this postulate is reasonable. It is generally accepted that the polymerizing and editing functions are separate (25) and we also find evidence for this. When O^4-alkyl dTTPs are 90% substituted for dTTP in poly[d(A-T)] primed synthesis and such synthesis ceases prematurely but excision of nucleotides is not occurring, synthesis can be reinitiated by addition of dTTP (3).

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