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Two Different Reactions Involved in the Primer/Template-Independent Polymerization of dATP and dTTP by *Taq* DNA Polymerase

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***Taq* and Tth DNA polymerases catalyzed polymerization of dATP and dTTP into poly d(A-T) without requiring added primer/template (Hanaki *et al., Biochem. Biophys. Res. Commun.* 238, 113–118), while the Stoffel fragment of *Taq* DNA polymerase and Δ*Tth* DNA polymerase with respective deletions of ca. 290 and 250 N-terminal amino acids did not. The primer/template-independent polymerization appeared to proceed via two reactions, the slow process of formation of 16–19 nt long oligo d(A-T) without primer/template and the rapid process of elongation of the oligo d(A-T) by self-priming. As the former step was more sensitive to *N*-ethylmaleimide than the elongation reaction, probably the formation of the oligonucleotide preceded the elongation. But when the substrates were depleted, *Taq* DNA polymerase degraded the high molecular weigh d(A-T) polymer to the oligomers which were resistant to the further digestion by the 5′ → 3′ exonuclease activity of *Taq* DNA polymerase. Probably, the elongation and the degradation reactions proceeded simultaneously, the former process being faster than the latter in the presence of enough dATP and dTTP.** © 1998 Academic Press

Thermostable DNA polymerases from thermophile bacteria have made a revolution in molecular biology owing to their use in the polymerase chain reaction (PCR) (1, 2). Recently, we found Taq DNA polymerase had an activity which was not described so far, i.e., polymerization of dATP and dTTP to poly d(A-T) copolymer without requiring primer/template in the temperature range of 60–70°C, 5–10°C lower than the optimal temperature for PCR (3). A similar 'unprimed, template non-requiring' polymerase reaction was reported and studied extensively in 1960's for *Escherichia coli* DNA polymerase (4–9) and calf thymus DNA polymerase α (10). The further studies indicated that the reaction was at least partly due to contaminating nucleotide oligomers or other enzymes (10–14), but the nature of the reaction still remains obscure. As for the newly found primer/template-independent polymerization described by our group (3), the enzyme preparations were highly purified ones from various manufacturers, such as Perkin-Elmer, Gibco BRL, Toyobo, etc., and contamination of primer oligonucleotides in the enzyme preparation was excluded, at least, by failure of priming the terminal deoxynucleotidyl transferase (3). We recently found the reaction proceeded via two reactions, the slow process of polymerization of dATP and dTTP into low molecular weight oligo d(A-T) without requiring primer/template and the rapid process of polymerization into high molecular weight DNA using the low molecular weight copolymer as primer/template. The information is detailed in this report.

MATERIALS AND METHODS

DNA polymerases. The thermostable DNA polymerases examined were AmpliTaq DNA polymerase (Perkin-Elmer) (15), AmpliTaq DNA polymerase LD (Perkin-Elmer), AmpliTaq Gold (Perkin-Elmer), AmpliTaq DNA polymerase Stoffel Fragment (Perkin-Elmer) (15, 16), BcaBEST DNA polymerase (Takara) (17), ΔTth DNA polymerase (Toyobo) (18), Hot Tub DNA polymerase (Amersham), KOD DNA polymerase (Toyobo) (19), Pfu DNA polymerase (Stratagene) (20), Pwo DNA polymerase (Boehringer Mannheim) (21), Red Hot DNA polymerase (Advanced Biotechnologies), rTaq DNA polymerase (Toyobo), rTth DNA polymerase (Perkin-Elmer) (22), TaKaRa Taq (Takara) (23), Taq DNA polymerase (Gibco BRL), Tfl DNA polymerase (Promega) (24), Tth DNA polymerase (Toyobo), Ultma DNA poly-

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TABLE 1

Primer/Template-Independent Poly d(A-T) Synthetic Activities of Various DNA Polymerases

Commercial name	Manufacturer	Origin	Primer/template-independent poly d(A-T) synthesis	5′ → 3′ exonuclease activity	3′ → 5′ exonuclease activity	TdT like activity
AmpliTaq DNA Polymerase [15]	Perkin-Elmer	*Thermus aquaticus*	+	+	−	+
AmpliTaq DNA Polymerase, LD	Perkin-Elmer	*Thermus aquaticus*	+	+	−	+
AmpliTaq Gold	Perkin-Elmer	*Thermus aquaticus*	+	+	−	+
Taq DNA Polymerase	Gibco BRL	*Thermus aquaticus YT-1*	+	+	−	+
rTaq DNA Polymerase	Toyobo	*Thermus aquaticus YT-1*	+	+/−	−	+
TaKaRa Taq [23]	Takara	*Thermus aquaticus*	+	+	−	+
Hot Tub DNA Polymerase	Amersham	*Thermus flavus ubiquitos*	+	+	−	+
Tfl DNA Polymerase [24]	Promega	*Thermus flavus*	+	+	−	+
Tth DNA Polymerase	Toyobo	*Thermus thermophilus HB8*	+	+/−	−	+
rTth DNA Polymerase [22]	Perkin-Elmer	*Thermus thermophilus*	+	+	−	+
Red Hot DNA Polymerase	Advanced Biotechnologies	*Thermus icelandicus*	+	+	−	+
BcaBEST DNA Polymerase [17]	Takara	*Bacillus cardotenax YT-G*	−	−	+	n.i.
KOD DNA Polymerase [19]	Toyobo	*Pyrococcus sp. KOD1*	−	n.i.	+	+/−
Pfu DNA Polymerase [20]	Stratagene	*Pyrococcus furiosus*	−	+/−	+	+/−
Pwo DNA Polymerase [21]	Boehringer Mannheim	*Pyrococcus woesei*	−	−	+	−
Ultma DNA Polymerase [20, 25]	Perkin-Elmer	*Thermotoga maritima*	−	+/−	+	−
Vent DNA Polymerase [26]	New England Biolabs	*Thermococcus litoralis*	−	−	+	−
AmpliTaq, Stoffel Fragment [15, 16]	Perkin-Elmer	*Thermus aquaticus*	−	−	−	+/−
ΔTth DNA Polymerase [18]	Toyobo	*Thermus thermophilus HB8*	−	−	−	+
Vent(exo-) DNA Polymerase [27]	New England Biolabs	*Thermococcus litoralis*	−	−	−	+/−

n.i.: no information available.

merase (Perkin-Elmer) (20, 25), Vent DNA polymerase (New England Biolabs) (26), and Vent(exo-) DNA polymerase (New England Biolabs) (27). Except Tth, Pfu, Hot Tub, and Pwo DNA polymerases, all the enzymes were the recombinant DNA products from *E. coli*.

Standard reaction condition. The standard reaction mixture contained 5 U of Taq DNA polymerase (AmpliTaq DNA polymerase [N808-0171], Perkin-Elmer) and 200 μM each of dATP and dTTP (GeneAmp dNTPs [N808-0007], Perkin-Elmer) in 100 μl PCR buffer (1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl [pH 8.3]) (GeneAmp PCR Buffer [N808-0006], Perkin-Elmer). The mixture was incubated at 65°C for indicated period using GeneAmp PCR System 2400 (Perkin-Elmer). The reaction product was ethanol precipitated, dissolved in the distilled water and electrophoresed in the agarose gel (Agarose type I; Low EEO [A6013], Sigma). The gels were stained with ethidium bromide (1 mg/l) and photographed with FAS-II (Toyobo). The molecular size marker was the 1 kb DNA Ladder ([15615-016], Gibco BRL). For polymerases other than Taq DNA polymerase, reaction buffer which was optimized for each of the polymerases and supplied as a kit solution was used.

RESULT

Primer/template-independent dAdT polymerizing activity of various thermostable DNA polymerases. We previously reported that Taq and Tth DNA polymerases catalyzed primer/template-independent polymerization of dATP and dTTP into poly d(A-T) copolymer. We called the reaction dAdT polymerization (3). Table 1 summarizes the primer/template-independent dAdT polymerizing activities of various thermostable DNA polymerases so far tested. All the enzymes derived from the genus Thermus, except the artificially modified Stoffel fragment (15, 16) and ΔTth DNA polymerase (18), were positive for the reaction, while those derived from *Bacillus*, *Pyrococcus*, *Thermotoga* and *Thermococcus* were negative. *Thermus*, *Bacillus*, and *Thermotoga* belong to *Eubacteria*, while *Pyrococcus* and *Thermococcus* belong to *Archaea*.

The 5′ → 3′ exonuclease activity (28, 29), 3′ → 5′ exonuclease activity (30, 31, 32) and the terminal deoxynucleotidyl transferase (TdT)-like activity which adds one nucleotide (preferentially A) to the 3′ end of the double stranded DNA (33, 34) are shown in Table 1 together with the dAdT polymerizing activity. Among the natural products, i.e., except the Stoffel fragment (15, 16), ΔTth (18) and Vent(exo-) (27) DNA polymerases, those having the dAdT polymerizing activity had the TdT-like activity and the 5′ → 3′ exonuclease activity but not the 3′ → 5′ exonuclease activity, while those without the dAdT polymerizing activity had the 3′ → 5′ exonuclease activity but no or only weak TdT-like activity and 5′ → 3′ exonuclease activity. The removal of ca. 290 amino acid-long N-terminal sequence from Taq (15, 16) (Stoffel fragment) and *ca.* 250 amino acid sequence from Tth (35) (ΔTth DNA polymerase) to abolish the 5′ → 3′ exonuclease activity resulted in the abolishment of dAdT polymerizing activity, suggesting that the N-terminal region was responsible not only for the 5′ → 3′ exonuclease activity but also for the dAdT polymerizing activity.

Two reactions-involved in dAdT polymerization. Fig. 1A shows the time course of the dAdT polymeriza-

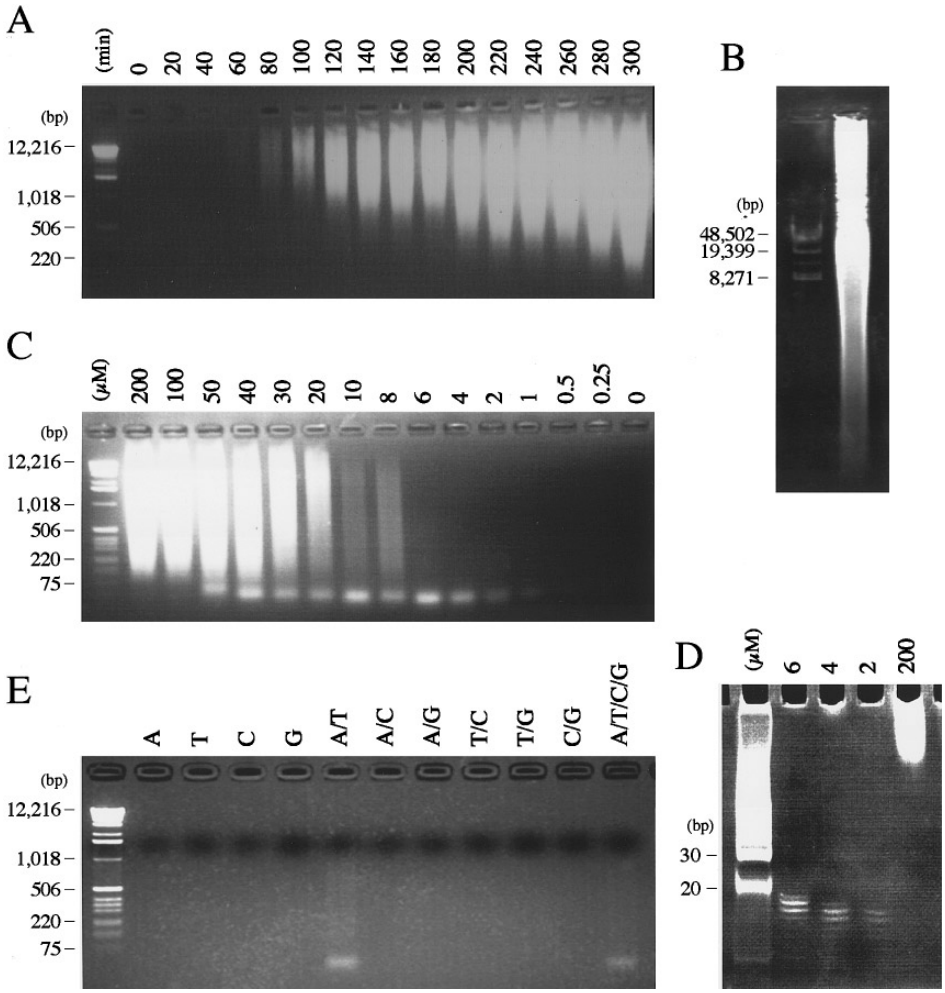

FIG. 1. Stoichiometric analysis of dAdT polymerization. (A) Tubes containing 5 U Taq DNA polymerase and 200 μM each of dATP and dTTP in 100 μl PCR buffer were incubated at 65°C, and one of the tubes was taken at 20 min interval for analysis of the product. (B) The reaction mixture consisting of 5 U Taq DNA polymerase and 200 μM each of dATP and dTTP in 100 μl PCR buffer was incubated for 5 hr at 65°C. The product was ethanol-precipitated and electrophoresed in 0.3% agarose gel (SeaKem Gold Agarose [50152], FMC BioProducts) (right lane) together with the high molecular weight DNA markers ([15618-010], Gibco BRL) (left lane). (C) Effect of substrate concentrations on the formation of polymers. The reaction mixture consisted of 5 U Taq DNA polymerase and various concentrations of 1:1 mixture of dATP and dTTP in 100 μl PCR buffer. After 3 hr incubation, the reaction mixture was directly electrophoresed in 2% agarose gel. (D) Size estimation of the oligonucleotides in the polyacrylamide gel electrophoresis. The reaction mixture consisted of 2, 4, 6 or 200 μM dNTPs and 2.5 U Taq DNA polymerase in 50 μl PCR buffer. The reaction was conducted at 65°C for 3 hr. The reaction mixture was directly applied to the 20% polyacrylamide gel. The size marker on the left most lane was 10 bp Ladder ([15631-013], Gibco BRL) for double stranded DNA. (E) Requirement of dATP and dTTP for the synthesis of 'precursor oligonucleotides'. The reaction mixture consisted of 2.5 U Taq DNA polymerase and various combinations of 4 μM dNTPs in 50 μl PCR buffer. The reaction was conducted at 65°C for 90 min. The reaction mixture was directly electrophoresed in 2% agarose gel.

tion by Taq DNA polymerase under the standard condition, where the concentrations of dATP and dTTP were 200 μM. The product became detectable at 80 min. Fig. 1B shows the co-electrophoresis of the product obtained after incubation for 5 hr and the reference high molecular weight DNAs. The molecular weight of the majority of the products was found to exceed 50 kb. Fig. 1C shows the dose response. The reaction mixture contained 5 U Taq DNA polymerase and varying amount of 1:1 mixture of dATP and dTTP in 100 μl PCR buffer. The reaction period was 3 hr. The high molecular weight DNA was produced in a dose responsive manner. In the optimum condition (200 μM each of dATP and dTTP and 5 U/100 μl Taq DNA polymerase), near 90% of dATP and dTTP were polymerized into the poly d(A-T) (3). At concentrations lower than 50 μM each of dATP and dTTP, in addition to the high molecular weight diffuse bands, a discrete low molecular weight band appeared, and at concentrations of 1-6 μM, only the low molecular weight band was detected. In order to estimate the molecular weight of the band more precisely, the products obtained at substrate concentra-

tions 2–6 μM were electrophoresed in 20% polyacrylamide gel together with the low molecular weight marker (Fig. 1D). A DNA ladder appeared in the region of 16–19 bp double stranded DNA. Therefore, in this range of the substrate concentration the 16-19 nt oligomers were produced and probably annealed into the double stranded DNA. When the various combinations of dATP, dTTP, dCTP and dGTP were tried as substrates for the synthesis of the oligomers, the oligomers were synthesized only in the presence of dATP and dTTP (Fig. 1E).

As the discrete low molecular weight oligonucleotide band was not detected at substrate concentrations higher than 100 μM, it was speculated that the elongation of the oligonucleotides came to halt at low substrate concentrations and, at higher substrate concentrations, the oligonucleotides represented by the low molecular weight band were quickly elongated to the high molecular weight DNA. In order to test this possibility, the initial reaction was conducted with the mixture containing 2.5 U Taq DNA polymerase and 4 μM each of dATP and dTTP in 50 μl PCR buffer for 1.5 hr, which allowed production of the low molecular weight band, and then the concentration of dATP and dTTP was increased to the final concentration of 200 μM by adding 50 μl of PCR buffer containing 400 μM each of dATP and dTTP and 2.5 U Taq DNA polymerase. As shown in Fig. 2A, the low molecular weight band which is barely detectable in the figure shifted to the positions around 250 nucleotides in 2 min and further to higher position in a few minutes while its density being increased. This suggested that the low molecular weight DNA could serve as a precursor of the high molecular weight DNA. The DNA represented by the discrete low molecular band will be called as 'precursor oligonucleotide' hereafter.

Molecular nature of the 'precursor oligonucleotide'. As the 'precursor oligonucleotide' was rapidly converted to the high molecular weight DNA by supply of sufficient amount of dATP and dTTP, the 80 min incubation before the appearance of high molecular weight DNA (Fig. 1A) was considered as the time required for formation of the 'precursor oligonucleotides'. As its production required only dATP and dTTP and not dCTP nor dGTP (Fig. 1E), the 'precursor oligonucleotide' must have been either double stranded oligo dA·oligo dT, single stranded oligo d(A-T), or double stranded oligo d(A-T).

As it was difficult to characterize the 'precursor oligonucleotide' directly, we examined which of dA_{18}, dT_{18}, mixture of dA_{18} and dT_{18}, $d(A\text{-}T)_8$, $d(T\text{-}A)_9$, and mixture of $d(A\text{-}T)_8$ and $d(T\text{-}A)_9$ could be used as the precursor of the high molecular weight DNA. The oligonucleotides were boiled for 10 min, and then cooled rapidly on ice to avoid annealing (Fig. 2B, RC) or slowly in the room temperature to facilitate annealing (Fig. 2B, SC); renaturation of mixture of dA_{18} and dT_{18} to form the double stranded DNA in the latter condition was confirmed by the positive ethidium bromide staining (Fig. 2C, lane c/SC; the rapid cooling too resulted in a renaturation, though to a lesser extent, as shown in lane c/RC). The oligonucleotides thus prepared were added to the standard reaction mixture containing 2.5 U Taq DNA polymerase and 200 μM each of dATP and dTTP in 50 μl PCR buffer. The reaction was stopped after 30 min, and the products were electrophoresed. It was found that $d(A\text{-}T)_8$, $d(T\text{-}A)_9$ and mixture of $d(A\text{-}T)_8$ and $d(T\text{-}A)_9$ were utilized as precursors. The slow cooling which favored the annealing of the complementary strands did not confer the dA_{18}-dT_{18} mixture the capacity of becoming the precursor (Fig. 2B, SC, lane c). It was therefore suggested that the 'precursor oligonucleotide' was oligo d(A-T).

The minimum size of oligo d(A-T) for becoming the precursor was determined (Fig. 2D). The high molecular weight DNA was synthesized with oligo $d(A\text{-}T)_6$, but not with oligo $dA(T\text{-}A)_4$. The minimum size was therefore considered to be in the range of 10-12 bases with alternating A and T. Various DNA polymerases lacking in the dAdT polymerization activity could polymerize dATP and dTTP using the 'precursor oligonucleotide' as primer/template (Fig. 2E); *E. coli* DNA polymerase I (lane d), T_4 DNA polymerase (lane e) and Klenow fragment (lane f) catalyzed the reaction at 37°C and the Stoffel fragment (lane c) and ΔTth DNA polymerase (lane b) did so at 65°C. The reactions catalyzed by *E. coli* DNA polymerase I and T4 DNA polymerase were weak, however.

Different sensitivities of the 'precursor oligonucleotide' synthesis and the elongation to N-ethylmaleimide. N-ethylmaleimide (NEM) is known as an inhibitor of eukaryotic polymerase α (36), rabbit bone marrow-derived polymerase δ (37), mitochondrial polymerase γ (38), plant chloroplast DNA polymerase (38), and dNDP-transferase (14). Pyridoxal 5′-phosphate (PLP) is an inhibitor of *E. coli* DNA polymerase I large fragment and the inhibition was noncompetitive with respect to dNTP (39). Adenosine 5′-triphosphate (ATP) reportedly inhibits the ordinary TdT (40) selectively but not eukaryotic polymerase α, β or γ (41, 42). Sensitivities of the dAdT polymerization to these compounds were measured. Effect on the formation of the 'precursor oligonucleotide' was measured by 90 min polymerization reaction in the presence of various concentrations of the inhibitors using the mixture of 4 μM each of dATP and dTTP and 2.5 U Taq DNA polymerase in 50 μl PCR buffer. Effect on the elongation reaction was measured as follows; 5 μl of the 'precursor oligonucleotide' product obtained in the absence of the inhibitors were added to 50 μl of reaction mixture containing 2.5 U Taq DNA polymerase, 200 μM each of dATP and dTTP and various concentrations of the inhibitors, and

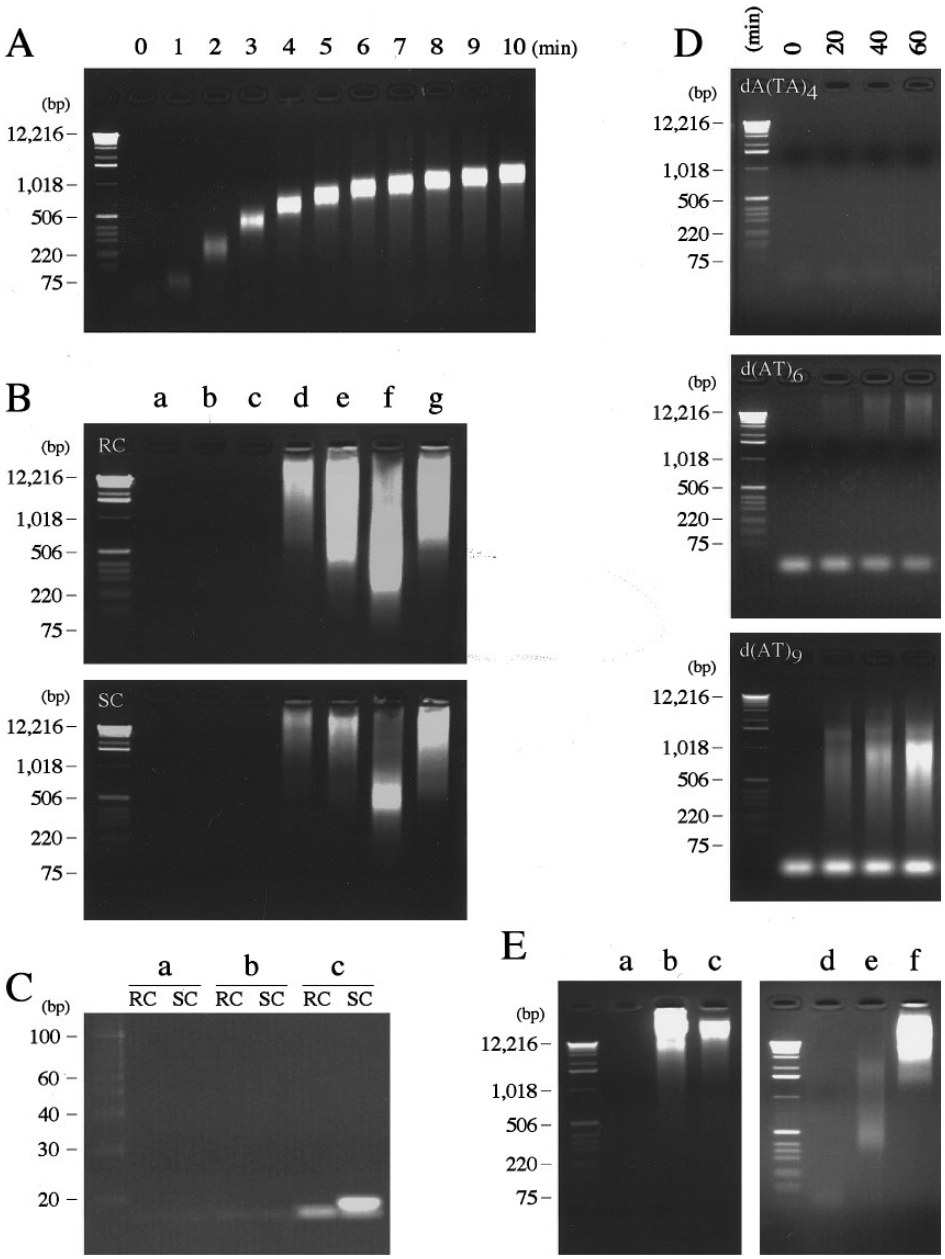

FIG. 2. Two reactions involved in the dAdT polymerization. (A) Conversion of the oligonucleotide into high molecular weight DNA by elongation. The initial reaction was conducted with 2.5 U Taq DNA polymerase ([N808-0171], Perkin-Elmer) and 4 μM each of dATP and dTTP in 50 μl PCR buffer in several tubes at 65°C for 90 min. Fifty μl of PCR buffer containing 2.5 U Taq DNA polymerase and 400 μM each of dATP and dTTP (warmed at 65°C beforehand) was then added to the above reaction mixture, and incubated. At the period indicated one of the tubes were cooled quickly on ice. The reaction mixture was directly electrophoresed in 2% agarose gel. (B,C) Capacity of oligonucleotides as primer/template for the elongation reaction. The reaction mixture consisted of 200 μM each of dATP and dTTP (GeneAmp dNTPs, [808-0007], Perkin-Elmer), 10 pmole oligonucleotides (BEX, Co., Tokyo) and 2.5 U AmpliTaq DNA polymerase in 50 μl PCR buffer. The oligonucleotides were boiled for 10 min and cooled rapidly on ice (RC) or slowly in the room temperature (SC). The reaction was conducted at 65°C for 30 min. The reaction mixture was electrophoresed in 2% agarose gel. The oligonucleotides were dA_{18} (lane a), dT_{18} (lane b), mixture of dA_{18} and dT_{18} (lane c), $d(A\text{-}T)_8$ (lane d), $d(T\text{-}A)_9$ (lane e), and mixture of $d(A\text{-}T)_8$ and $d(T\text{-}A)_9$ (lane f). The oligonucleotide obtained by incubating 4 μM each of dATP and dTTP, 2.5 U Taq DNA polymerase in 50 μl PCR buffer for 90 min at 65°C (6 μl) was also used as a primer/template (lane g). The reaction mixture was directly electrophoresed in 2% agarose gel. Figs in (C) show the gel electrophoresis 15–25% polyacrylamide gradient gel (Daiichi Pure Chemicals, Tokyo) of dA_{18} (a/RC, SC), dT_{18} (b/RC, SC) and their mixture (c/RC, SC) before the reaction. dA_{18} (200 pmole), dT_{18} (200 pmole) or 1:1 mixture of dA_{18} (100 pmole) and dT_{18} (100 pmole) in a volume of 100 μl was boiled and cooled rapidly on ice (RC) or slowly in the room temperature (SC). 10 μl aliquot was applied to each lane. The single strand DNAs were not stained by ethidium bromide (a and b). The oligo dA and oligo dT partially annealed even in the rapid cooling condition (c/RC). (D) Effect of the size of d(A-T) oligomer on the capacity as primer/template. The oligomers, $dA(T\text{-}A)_4$, $d(A\text{-}T)_6$, and $d(A\text{-}T)_9$ were used as the primer/template. The reaction was conducted with the mixture consisting of 5 U Taq DNA polymerase, 200 μM each of dATP and

incubated for 45 min for the elongation reaction. As the effect of inhibitors could be affected by the substrate concentration, the effect on the dAdT polymerization as a whole was also examined, i.e., the reaction was continued for 2 hr in the 50 μl reaction mixture containing 2.5 U Taq DNA polymerase, 200 μM each of dATP and dTTP and various concentrations of the inhibitors. ATP and PLP inhibited the 'precursor oligonucleotide' formation, the elongation and the standard dAdT polymerization reactions at similar doses, i.e., around 1,000 mM for both ATP and PLP (data not shown). NEM, however, inhibited the 'precursor oligonucleotide' formation at far lower doses than the elongation reaction; the 'precursor oligonucleotide' synthesis and the standard dAdT polymerization were inhibited at 0.002 μM (Fig. 3A, upper left panel), while the elongation was not inhibited even at 2,500 μM (Fig. 3A, bottom right panel). The dAdT polymerization as a whole at the high substrate condition was inhibited at 150-300 μM, i.e., the whole dAdT polymerization process was at least 16-fold more sensitive to NEM than the elongation reaction (Fig. 3A, right upper panel). The more than 10^6-fold higher sensitivity of the 'precursor oligonucleotide' synthesis relative to the elongation reaction suggested the two reactions were enzymatically different. The far higher sensitivity of the 'precursor oligonucleotide' synthesis and the whole dAdT polymerization relative to the elongation reaction suggested that the synthesis of the 'precursor oligonucleotide' was rate limiting and preceded the elongation reaction.

We checked effect of dideoxy NTPs (ddNTPs). The elongation reaction was inhibited by ddATP and ddTTP at concentrations of 100 and 200 μM, respectively, while ddCTP and ddGTP failed to do so at concentrations of 1,000 μM (Fig. 3B, lower panel). On the formation of the precursor oligonucleotide, ddATP and ddTTP were found inhibitory at concentrations of 200 μM. Dideoxy CTP and ddGTP were also inhibitory, though higher concentrations were required i.e., 600 and 800 μM, respectively (Fig. 3B, upper panel). As we obtained no evidence indicating the incorporation of C or G into the precursor oligonucleotides, the inhibition of the 'precursor oligonucleotide' synthesis by ddCTP and ddGTP was quite puzzling. It is possible that the ddCTP or ddGTP reacted with the nucleotide-interacting domain of the Taq DNA polymerase, and interfered with its interaction with dATP and dTTP.

'Precursor oligonucleotide' formation mediated by degradation from high molecular weight DNA. The experiments shown in Fig. 2 proved that the oligonucleotides formed in the low substrate condition could be used as primer/template for the elongation reaction, and suggested, together with the NEM experiment (Fig. 3A) that the 'precursor oligonucleotides' were formed first without primer/template and then they were elongated to form the high molecular weight DNA. However, since Taq DNA polymerase has the $5' \rightarrow 3'$ exonuclease activity, it was also possible that the formation of the 'precursor oligonucleotides' was contributed by a degradation from the high molecular weight DNA. In addition, if the 'precursor oligonucleotides' were formed only through the halt of polymerization due to an exhaustion of the substrates, it is curious why the size of the oligonucleotides were so uniform. Therefore, we examined if Taq DNA polymerase could mediate degradation of the high molecular weight DNA into the oligonucleotides when the substrates were depleted. As shown in the top panel of Fig. 4, Taq DNA polymerase degraded the high molecular weight DNA to produce the oligonucleotides of the discrete size though very slowly. In contrast, Stoffel fragment devoid of the $5' \rightarrow 3'$ exonuclease activity did not (Fig. 4, the second panel). *E. coli* polymerase I and Vent DNA polymerase, both having the $3' \rightarrow 5'$ exonuclease activity, degraded the high molecular weight DNA completely; no oligonucleotides of the discrete size were formed (Fig. 4, the third and bottom panels). The experiments suggested that the formation of the 'precursor oligonucleotides' was mediated by polymerization and subsequent trimming of the poly d(A-T) by the $5' \rightarrow 3'$ exonuclease activity of the Taq DNA polymerase. It was also shown that the oligonucleotides thus produced were not further degraded by the $5' \rightarrow 3'$ exonuclease activity of Taq DNA polymerase. *E. coli* polymerase I

dTTP and either one of the d(A-T) oligomers (purified by the reversed phase cartridge, Sawady Tech, Co., Tokyo) at a concentration of 200 pmole in 100 μl PCR buffer. The reaction mixture was distributed into tubes at 15 μl aliquot. At the period indicated, one of the tubes was cooled quickly on ice to stop the reaction. The product was analyzed by the electrophoresis in 2% agarose gel. (E) The elongation reaction by various DNA polymerases which are lacking in the primer/template-independent dAdT polymerization activity. The reaction period was 2 hr for all the polymerases. The temperature was 65°C for ΔTth DNA polymerase and the Stoffel fragment, and 37°C for *E. coli* DNA polymerase I, T4 DNA polymerase, and Klenow fragment. The 'precursor oligonucleotide' was prepared by incubating the reaction mixture consisting of 5 U Taq DNA polymerase and 4 μM each of dATP and dTTP in 100 μl PCR buffer at 65°C for 90 min. The elongation reaction mixture consisted of one of the DNA polymerases at a concentration of 2.5 U, 200 μM each of dATP and dTTP in 55 μl mixture of 5 μl of the precursor oligonucleotides and 50 μl of the buffer optimized for each DNA polymerase (provided as a part of the reaction kit by the supplier). a: no added DNA polymerases. The precursor oligonucleotide band is undetectable on account of the small quantity. As the high molecular weight DNA was undetectable here, it is clear that Taq DNA polymerase present in 5 μl precursor oligonucleotide preparation did not contribute to the polymerization reactions by other enzymes (lanes b-f). b: ΔTth DNA polymerase ([TTH-201], Toyobo). c: AmpliTaq DNA polymerase, Stoffel fragment ([N808-0007], Perkin-Elmer). d: *E. coli* DNA polymerase I ([2130A], Takara). e: T4 DNA polymerase, ([TPL-101], Toyobo). f: Klenow fragment of *E. coli* DNA polymerase I ([2140A], Takara).

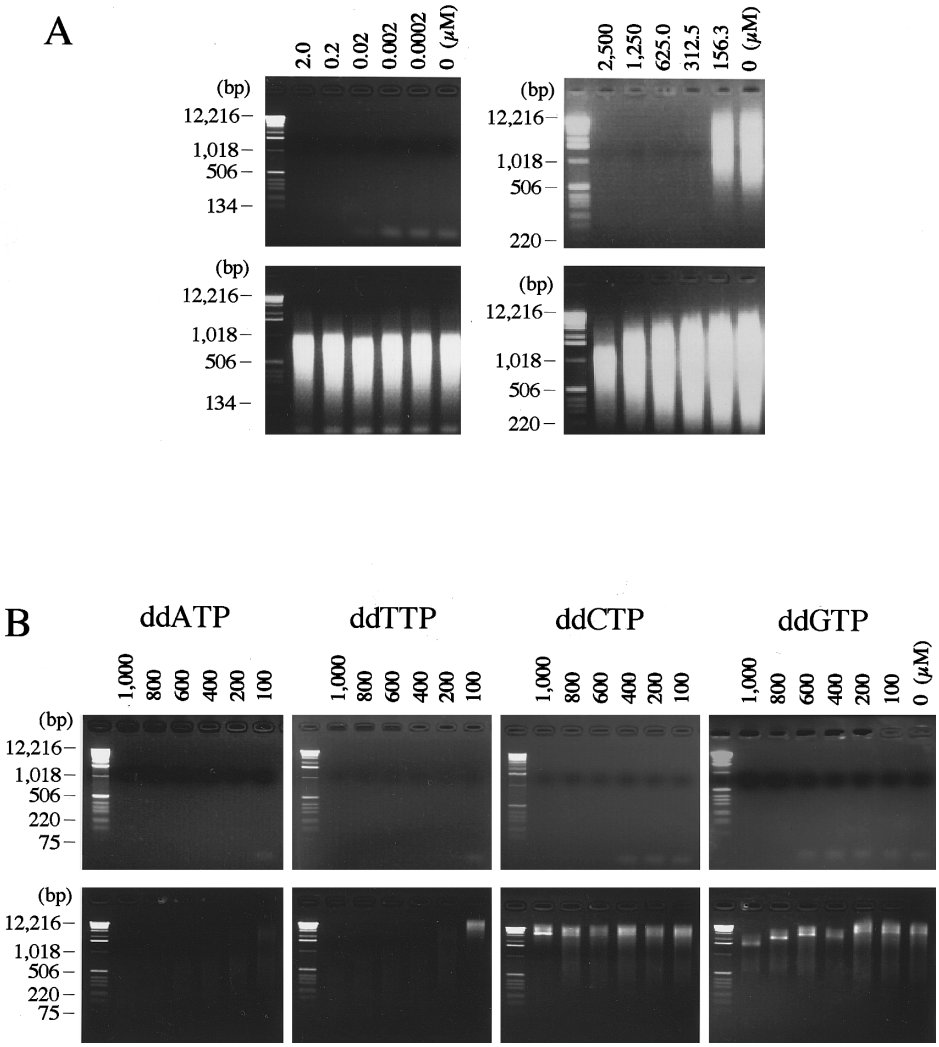

FIG. 3. Effect of inhibitors on the precursor nucleotide formation and the elongation reaction. (A) Effect of NEM, on the synthesis of precursor oligonucleotides (left upper panel), the whole process of dAdT polymerization (right upper panel) and their elongation (lower panels). The reaction mixture for testing the effect of inhibitors on the oligonucleotide synthesis contained 2.5 U Taq DNA polymerase, 4 μM each of dATP and dTTP, and various concentration of NEM (Wako Pure Chemicals, Osaka) in 50 μl PCR buffer. The reaction was continued for 90 min at 65°C. For testing the effect on the whole process of dAdT polymerization, the condition was the same as for testing the effect on the oligonucleotide formation except that the concentration of dATP and dTTP was 200 μM. For testing the effect on the elongation, 5 μl of the precursor product obtained without inhibitors and NEM at various concentrations were added to 50 μl PCR buffer containing 200 μM each of dATP and dTTP and 2.5 U Taq DNA polymerase, and the mixture was incubated for 45 min. The reaction products were electrophoresed in 2% agarose gel. (B) Effect of ddNTPs on the synthesis of precursor oligonucleotides (upper panel) and their elongation (lower panel). The reaction conditions were the same as those in the experiments shown in Fig. 3A except that either one of the ddNTPs (ddNTPs set [1008382], Boehringer Mannheim) was added at indicated concentrations.

and Vent DNA polymerase were unable to produce the oligonucleotides probably on account of the complete digestion by the 3′ → 5′ exonuclease activity.

DISCUSSION

We found thermostable DNA polymerases derived from the genus Thermus, *T. aquaticus, T. flavus, T. icelandicus* and *T. thermophilus,* had a primer/template-independent polymerization of dATP and dTTP into high molecular weight DNA (dAdT polymerization). The thermostable DNA polymerases derived from *Bacillus cardotenax,* three species belonging to genus Pyrococcus, and *Thermotoga maritima* did not show that activity (Table 1). Removal of around 250 and 290 N-terminal amino acids from Tth DNA polymerase (3) and from Taq DNA polymerase (this report) respectively resulted in loss of not only the 5′ → 3′ exonuclease activity but also the dAdT polymerizing activity. Namely, the N-terminus of the Thermus-derived DNA polymerases carried a sequence essential for the reaction. When the amino acid sequences of Taq (43), Tth (44) and BcaBEST (45) DNA

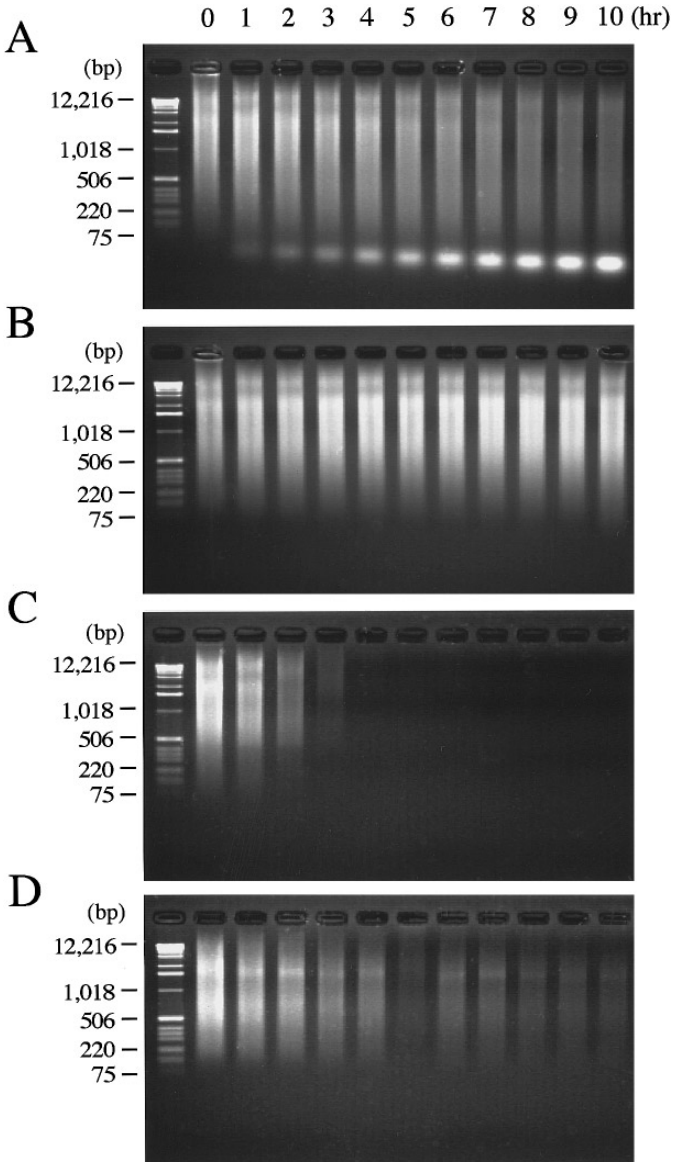

FIG. 4. Degradation of the high molecular weight DNA by the exonuclease activities of DNA polymerases. High molecular weight poly d(A-T) was produced by reaction for 3 hr at 65°C with the mixture consisting of 25 U of Taq DNA polymerase, 200 μM each of dATP and dTTP in 1,000 μl PCR buffer. DNA was purified three times by ethanol precipitation, and 8.5 μg aliquots were incubated for indicated periods with 5 U of Taq DNA polymerase (top), 5 U of Stoffel fragment (second), 5 U of *E. coli* DNA polymerase I (third), or 5 U of Vent DNA polymerase (bottom) in 50 μl PCR buffer without substrates.

polymerases, all derived from the thermophile eubacteria, were compared by using GENETYX-MAC Ver. 8 (Software Development Co., Ltd., Tokyo), Taq and Tth were highly homologous (71%/514 amino acids) while the homology between Taq and BcaBEST was very low (19.4%/509 amino acids) except at the C-terminal region where weak homology (about 30%/120 amino acids) was found.

The dAdT polymerization appeared to proceed via two reactions. At low substrate concentrations (1-6 μM each of dATP and dTTP), only the low molecular weight band appeared, and addition of dATP and dTTP to a final concentration of 200 μM quickly shifted the band to the more dense and diffuse high molecular weight bands. The formation of the low molecular weight DNA was a slow process requiring about 80 min and did not require added primer/template. The upper shift of the band after addition of 200 μM each of dATP and dTTP was a rapid process of the elongation of the oligomers into the high molecular weight DNA. As the 15–20 mer 'precursors' were shifted to around 250 nt polymers in 2 minutes, the elongation speed was around 125 nt/min. As the elongation speed for PCR at 72–80°C was 2,000–4,000 nt/min (the suppliers' information), the elongation of d(A-T) copolymer by Taq DNA polymerase at 65°C was much slower.

The 'precursor oligonucleotide' was formed only when the both dATP and dTTP were supplied to the Taq DNA polymerase reaction mixture (Fig. 1E). Therefore, the 'precursor oligonucleotide' must have been either double stranded oligo dA·oligo dT or single or double stranded oligo d(A-T). As only oligo d(A-T) was shifted to the high molecular weight DNA when incubated for 30 min with Taq DNA polymerase and 200 μM each of dATP and dTTP, the 'precursor oligonucleotide' must be oligo d(A-T). The minimum size required for the oligo d(A-T) to become the precursor was estimated to be 10–12 bases.

The elongation of the 'precursor oligonucleotide' probably through self-priming was mediated not only by the intact Thermus-derived DNA polymerases but also by the truncated derivatives, ΔTth DNA polymerase and the Stoffel fragment. *E. coli* DNA polymerase I, Klenow fragment of *E. coli* DNA polymerase I and T_4 DNA polymerase too could catalyze the elongation reaction at 37°C.

NEM known as an inhibitor of eukaryotic polymerase α (36), rabbit bone marrow-derived polymerase δ (37), mitochondrial polymerase γ (38), plant chloroplast DNA polymerase (38), and dNDP-transferase (14), inhibited the 'precursor oligonucleotide' synthesis at a very low dose but not the elongation even at 10^6-fold higher dose. As the dAdT polymerization reaction as a whole also was far more sensitive than the elongation, it was deduced that the 'precursor oligonucleotide' synthesis was a rate limiting process, i.e., the oligonucleotides were first produced and then elongated to the high molecular weight DNA. Taq DNA polymerase, however, degraded the high molecular weight DNA into the oligonucleotides of the sizes of the 'precursor' when the dATP and dTTP were depleted. Therefore, the formation of the 'precursor oligonucleotides' was found a complex process, i.e., [1] polymerization of dATP and dTTP into poly d(A-T) initiated without added primer/template and [2] trimming of the poly

d(A-T) into the 'precursor oligonucleotides' which could not be further degraded by the exonuclease activity. When enough amounts of substrates were available, the oligonucleotides were elongated to form the high molecular weight DNA, the elongation being faster than the 5′ → 3′ digestion.

A similar primer/template-independent polymerization of poly d(A-T) was reported for *E. coli* DNA polymerase I and studied extensively in 1960's (4–9). As reported (3), we were unable to reproduce the phenomenon with the highly purified enzyme preparation obtained commercially (Toyobo). Nevertheless, our observation with Thermus-derived DNA polymerase and that with *E. coli* DNA polymerase I share similar characters. In the both, elimination of the 5′ → 3′ exonuclease activity by deleting N-terminal region resulted in a loss of initiating the primer/template independent DNA synthesis, but the deleted enzymes, Stoffel and Klenow fragments, could mediate the elongation reaction. The initiation reaction was slow and the elongation reaction was quick in the both. The minimum size of the oligonucleotides to initiate the elongation reaction was small sized in the both. One of the major differences between the *E. coli* DNA polymerase I and Taq DNA polymerase was that the 3′ → 5′ exonuclease activity was present in the former but not in the latter. Therefore, it was possible that, though the both enzymes had the activity of primer/template-independent synthesis of poly d(A-T), its detection was easier for Taq DNA polymerase on account of the absence of the 3′ → 5′ exonuclease activity which could degrade synthesized poly d(A-T) extensively (Fig. 4). However, elimination of the 3′ → 5′ exonuclease from Vent DNA polymerase failed to confer this enzyme with the activity of the primer/template-independent poly d(A-T) synthesis (Table 1), i.e., the absence of the 3′ → 5′ exonuclease in Taq DNA polymerase alone could not account for its dAdT polymerization activity. One difference between Vent and Taq DNA polymerases was the 5′ → 3′ exonuclease which was absent in the former and present in the latter. The DNA polymerases positive for the dAdT polymerization all had the 5′ → 3′ exonuclease activity. This exonuclease activity degraded the high molecular DNA to the oligonucleotides of about 20 nt but not further. It is possible that the formation of the stable oligo d(A-T) was crucial for the primer/template independent dAdT polymerization. Probably, during the dAdT polymerization, both the degradation by the 5′ → 3′ exonuclease and the chain elongation proceeded in parallel; if enough substrates were available, the elongation proceeded more rapidly than the degradation, while if the substrates were limited, the degradation predominated.

Non-requirement of primer/template in the apparent *de novo* synthesis of poly d(A-T) by *E. coli* DNA polymerase I has been a matter of the long debate. Though they raised the possibilities of the real *de novo* synthesis and the synthesis primed by contaminating trace amount of oligonucleotides, Kornberg and Baker preferred the latter hypothesis based upon the argument that the affinity between the enzyme and the oligonucleotides was very high and the enzyme molecules in tight association with the oligo d(A-T) could initiate the poly d(A-T) synthesis (11). In the various thermostable DNA polymerases tested so far, only the Thermus-derived DNA polymerases but not the DNA polymerases of other origins had the activity. It was remarkable that the activity of poly d(A-T) synthesis in Taq DNA polymerases was really high (3) and that high activity was detected in all the Thermus-derived enzymes obtained from the eleven different manufacturers (Table 1), i.e., the association of the enzymes with oligonucleotides, if ever present, will not be fortuitous. As we reported previously, the priming activity for terminal deoxynucleotidyl transferase attributable to oligonucleotides could not be detected in the Taq DNA polymerase preparation (3), i.e., the oligonucleotides, if ever contaminating, are not in a free state. Therefore, there remain two possibilities. First, the dAdT polymerization was a real primer/template-independent DNA synthesis. Second, the dAdT polymerization was dependent upon primer/template oligonucleotides in a close association with the Taq enzymes and the association was strong enough for copurification; such a situation has been actually reported for telomerase (46).

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