chap. 6]. Thus, deformation mechanisms for the postglacial rebound in the upper mantle near the deglaciated region are similar to those involved in mantle convection. A large difference between the two processes is the strain magnitude. Strain involved in the postglacial rebound is small ($\sim 10^{-5}$), and transient rather than steady-state creep might occur [J. Weertman, *Philos. Trans. R. Astron. Soc. London Ser. A* **288**, 9 (1978)]. However, recent analysis of transient creep in olivine suggests that the transient period in olivine is much shorter than that of most metals, and so the transient creep proposed by Weertman may not be important [S. Karato, in (*3*), pp. 176–208].

53. The ice sheet was interpreted to have a parabolic profile. The ice thickness at the load center and the time of deglaciation were adjusted to give the best fit to the RSL data at the center of the load. A thickness of 3500 m was chosen in models A, B, C, D, and E and a thickness of 1600 m was chosen in model F. We chose a deglaciation time of 12,000 years before present except in model E, where 8,000 years before present was chosen. For each model, the first-order approximations to the sea level equations were solved, with the effects of eustatic sea level change, unloading of the ice, and loading of the ocean floor taken into account. As demonstrated by Wu and Peltier in (*51*), such an approximation is valid for RSL in the last 7000 years.
54. This result is consistent with that of W. R. Peltier [*J. Geophys. Res.* **89**, 11303 (1984)], who used the RSL data in the Laurentide region, but not with that of M. Nakada and K. Lambeck [*Geophys. J. Int.* **96**, 497 (1989)], who used the RSL data in and near Australia where a much thinner lithosphere (50 to 100 km) was inferred. This discrepancy may be due in part to the difference in the geothermal gradient between the two regions, because the recent high-resolution seismic tomography indicates a colder upper mantle in the Laurentide than in Australia [D. L. Anderson, T. Tanimoto, Y.-S. Zhang, *Science* **256**, 1645 (1992)].
55. This observation is consistent with some of the findings of Peltier, Wu, and Yuen in (*21*) but not with those of Nakada and Lambeck in (*23*). Part of this discrepancy may result from the regional difference in geothermal regimes (*54*).
56. J. Revenaugh and T. H. Jordan, *J. Geophys. Res.* **96**, 19781 (1991).
57. D. L. Kohlstedt and M. S. Weathers, *ibid.* **85**, 6269 (1980).
58. Y.-S. Zhang and T. Tanimoto, *Nature* **355**, 45 (1992).
59. T. Kawasaki, *Lithos* **20**, 263 (1987).
60. P. van Keken, D. A. Yuen, A. van den Berg, *Earth Planet. Sci. Lett.* **112**, 179 (1992).
61. An alternative way to get nearly isoviscous upper mantle with a large activation volume is to invoke a large decrease in activation volume with pressure. Borch and Green in (*33*) proposed that in the relation between strain rate and temperature and pressure

$$\dot{\epsilon} = A\sigma^n \exp[-gT_m(P)/T]$$

in which g is a constant and $T_m(P)$ is the melting temperature, this second variable should be identified as the solidus rather than the melting temperature of the constituting mineral or minerals. The solidus of peridotite changes with pressure with a large curvature [E. Takahashi, *J. Geophys. Res.* **91**, 3985 (1986)], and so the presence of nearly isoviscous upper mantle would be interpreted from this assumption. However, this assumption contradicts the experimental observations. If the solidus rather than the melting temperature determines rheology, then creep rate in olivine should increase by a factor of 10^4 to 10^5 when a small amount of pyroxenes is added, because the solidus will be reduced by 500 to 600 K. The observed increase in strain rate by the addition of orthopyroxene is only a factor of about 3 [Q. Bai, S. J. Mackwell, D. L. Kohlstedt, *J. Geophys. Res.* **96**, 2441 (1991)]. Therefore, the change in activation volume with pressure under the upper mantle conditions appears to be small, and so an activation volume as high as 27 cm^3 mol^{-1} leads to unacceptably high viscosities in the deep upper mantle (Fig. 2).
62. Sidereal age is based on the sidereal day—the time required for the Earth to rotate once about its axis; E. Bard, B. Hamelin, R. G. Fairbanks, A. Zindler, *Nature* **345**, 405 (1990).
63. We thank D. A. Yuen, W. R. Peltier, and D. L. Kohlstedt for reading the manuscript and for stimulating discussions. Supported by the National Science Foundation (EAR-9017811) (S.K.) and by the Natural Sciences and Engineering Research Council (Canada) (P.W.).

RESEARCH ARTICLE

Structure-Specific Endonucleolytic Cleavage of Nucleic Acids by Eubacterial DNA Polymerases

Victor Lyamichev,* Mary Ann D. Brow, James E. Dahlberg†

Previously known 5′ exonucleases of several eubacterial DNA polymerases have now been shown to be structure-specific endonucleases that cleave single-stranded DNA or RNA at the bifurcated end of a base-paired duplex. Cleavage was not coupled to synthesis, although primers accelerated the rate of cleavage considerably. The enzyme appeared to gain access to the cleavage site by moving from the free end of a 5′ extension to the bifurcation of the duplex, where cleavage took place. Single-stranded 5′ arms up to 200 nucleotides long were cleaved from such a duplex. Essentially any linear single-stranded nucleic acid can be targeted for specific cleavage by the 5′ nuclease of DNA polymerase through hybridization with an oligonucleotide that converts the desired cleavage site into a substrate.

Important functions of several DNA polymerases (DNAPs) include the removal of nucleotides from the 5′ and 3′ ends of DNA chains (*1*). For example, the 5′ exonuclease activities located in the NH_2-terminal domains of DNAP from *Escherichia coli* (DNAP-Ec1) and *Thermus aquaticus* (DNAP-Taq) can participate in (i) removal of the RNA primers of lagging strand synthesis during replication and (ii) the removal of damaged nucleotides during repair (*1*, *2*). Although mononucleotides predominate among the digestion products, short oligonucleotides (≤12 nucleotides) can also be observed, implying that these so-called 5′ exonucleases can function endonucleolytically (*3*, *4*). Thus, we like to call these activities 5′ nucleases.

For removal of primers or damaged nucleotides, a 5′ nuclease must be able to cleave RNA and DNA strands regardless of their sequences. However, indiscriminate cleavage of the nucleic acids would be lethal. We propose that this problem is resolved by having the 5′ nuclease recognize its substrate by structure rather than sequence. An appropriate structure would be the junction where the two strands of a duplex separate into single-stranded arms; the arm with a free 5′ end would be recognized as the displaced strand that is to be cleaved from the remaining duplex (Fig. 1A). To avoid ambiguity, we refer to the top and bottom strands of the complex as the substrate and template strands, respectively. A primer that would be extended during nick translation is paired to the 3′ arm of the template strand. To test our model of recognition and cleavage by the 5′ nuclease, we used the substrate and template strands shown in Fig. 1A, covalently joined at the end of the duplex, for experimental convenience. We also used this model to develop a method for cleaving single-stranded nucleic acids efficiently at desired sites with extreme sequence specificity.

The 5′ nuclease of DNAP-Taq. Incubation of the structure shown in Fig. 1A with DNAP-Taq (*5*) and deoxynucleoside triphosphates (dNTPs) resulted in the re-

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lease of a few fragments whose mobilities indicated that they contained most or all of the unpaired 5′ arm (Fig. 1B). Direct sequence analysis confirmed that cleavage occurred near the end of the duplex, releasing the 5′ extension. The released fragments terminated with 3′ hydroxyl groups, as evidenced by their ability to be extended by terminal deoxynucleotidyl transferase (6). The fragments were not released by incubation with the Stoffel fragment (Stf), a derivative of DNAP-Taq lacking the 5′ nuclease domain (7).

Neither primer nor dNTPs were required for cleavage (Fig. 2A, lanes 1 through 6), consistent with the fact that the polymerization and 5′ nuclease functions of DNAP-Taq, like DNAP-Ec1, are in separate domains (2, 3). Nuclease activity required magnesium or manganese ions, and neither zinc nor calcium ions supported the cleavage reaction (lanes 7 to 10). The reaction occurred over a broad temperature range, from 25° to 85°C, with the rate of cleavage increasing at higher temperatures. The pH optimum was about pH 9 (measured at 23°C) although the reaction still occurred at pH 6.5 (6).

Roles of the primer. Although the primer was not required for cleavage, it did influence both the site and the rate of cleavage of the hairpin. The change in the site of cleavage (Fig. 2A) apparently resulted from a change in the substrate structure, and from alteration of the substrate-enzyme interaction. In the absence of primer, sequences such as those indicated in Fig. 1A by underlining could pair, forming extended duplexes (8); cleavage at the ends of these duplexes would release shorter fragments (Fig. 2A, lanes 5 and 6). Addition of excess primer (Fig. 2A, lanes 3 and 4) or incubation at elevated temperatures (Fig. 2B) disrupted these short extensions of the duplex and resulted in a longer 5′ arm and, hence, longer cleavage products.

The 3′ end of the primer also helped to fix the precise site of cleavage. In the absence of primer, cleavage occurred at the ends of the substrate duplexes (either the extended or shortened forms) between the first and second base pairs (23- and 25-nucleotide bands, Fig. 2C, lane 1). Likewise, cleavage also occurred at the end of the duplex when the primer extended up to the base of the duplex (Fig. 2C, lane 2). However, when a gap of four or six nucleotides existed between the 3′ end of the primer and the end of the substrate duplex, the cleavage site was shifted four to six nucleotides in the 5′ direction (Fig. 2C, lanes 3 and 4) (9). The primer-directed shifting of the site of cleavage suggests that precise orientation of the 5′ nuclease on the substrate is dominated by the interaction of the polymerization domain of DNAP-Taq with the primer.

The effect of added primer on the rate of cleavage is evident in the extent of cleavage observed in lanes 1 and 2 of Fig. 2C (10). The influence of primer on cleavage rates

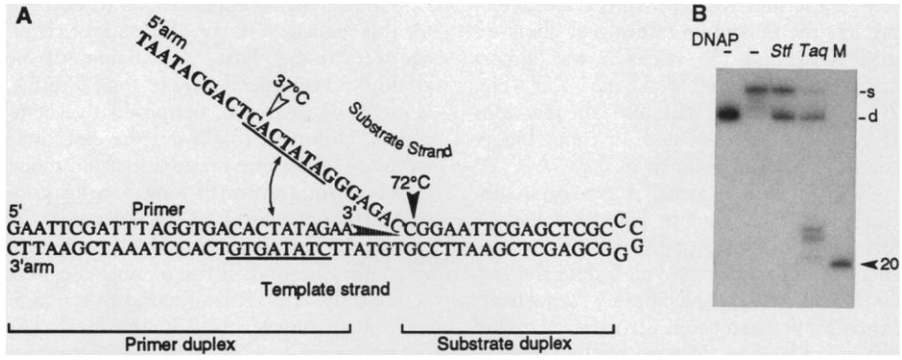

Fig. 1. Cleavage of a DNA hairpin by DNAP-Taq. (**A**) The proposed hairpin structure of a substrate for the 5′ nuclease of DNAP-Taq. One of three primers used with this substrate is shown, leaving a gap of four nucleotides between the 3′ arm and the bifurcation; the other primers leave no gaps or gaps of six nucleotides. The major cleavage sites at 37° and 72°C without primer are indicated by arrowheads; the filled one is also the cleavage site with a full-length primer. Complementary DNA sequences in 3′ and 5′ arms are underlined. This molecule was generated in a polymerase chain reaction (PCR) catalyzed by DNAP-Stf (22), a variant of DNAP-Taq lacking the 5′ nuclease; the molecule was labeled on the 5′ arm by the use of a ^{32}P-labeled primer. The nomenclature indicated is used throughout the text to describe various regions of the structure. (**B**) Endonucleolytic cleavage products of the 5′ nuclease of DNAP-Taq. The 5′ end-labeled hairpin was formed by snap-back of complementary sequences after denaturation of the PCR-generated duplex. The hairpin and associated primer shown in (A) were incubated with each dNTP at 50 μM for 5 minutes at 55°C alone or in the presence of either DNAP-Stf or DNAP-Taq. The products were resolved by electrophoresis (5). The mobilities of uncleaved substrates that were in the snap-back form (denoted as s) or that had been converted to duplex (d) by oligonucleotide extension are indicated. A 20-nucleotide size marker is also indicated.

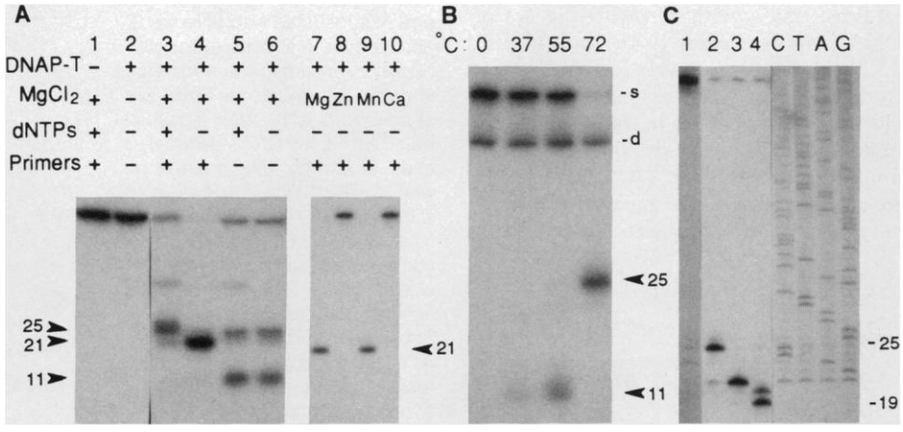

Fig. 2. Influence of reaction components and conditions on cleavage. (**A**) Determination of components required for cleavage (5). The 5′ end-labeled hairpin DNA shown in Fig. 1A was incubated for 30 minutes at 55°C, in 20 mM KCl, and in the presence of the indicated components. When included, dNTPs were present at 40 μM each. The primer left a gap of four nucleotides between its 3′ end and the first base pair of the hairpin duplex. The experiment in lane 7 is a repeat of lane 4, to serve as a marker; alternative cations were substituted at 1.5 mM. (A to C) Products were resolved by electrophoresis (5), and their lengths, in nucleotides, are indicated. (**B**) Effect of temperature on the site of cleavage in the absence of added primer. Reactions were incubated in the absence of KCl for 10 minutes at the indicated temperatures. The mobilities of the uncleaved substrate that either had (d) or had not (s) reannealed with its unlabeled complement (5) are indicated. (**C**) Effect of primer 3′ end locations on the sites of cleavage. The hairpin was incubated at 55°C for 10 minutes, with 50 mM KCl, in the absence (lane 1) or presence of primers that extended to various positions on the 3′ arm. The strong band in lane 2 was produced in the presence of a primer that extended to the end of the substrate duplex. In lanes 3 and 4, shorter primers were used, leaving gaps of four and six nucleotides, respectively, between the 3′ end of the primer and the end of the substrate duplex. The right side shows a dideoxynucleotide sequence ladder of the substrate, as a marker.

was accentuated when both reactions were run in 50 mM KCl. In the presence of primer, the rate of cleavage was optimal at about 50 mM (Fig. 3A), but it approached zero at 150 mM KCl (6). In the absence of primer, the maximum rate was at about 20 mM KCl, and the reaction was almost completely inhibited at 50 mM KCl (Fig. 2C, lane 1, and Fig. 3B); also, the reactions (Fig. 3B) were incubated 15 times longer than the reactions shown in Fig. 3A.

The 5′ and 3′ arms. A potential substrate similar to that in Fig. 1A could be formed in a region of DNA containing a single-stranded gap provided that the gap contained self-complementary sequences. Since primer extension across a structured gap could lead to cleavage of the template strand, such a structure must be distinguished from a displaced arm in a bona fide cleavage substrate. A significant difference between in vivo complexes and the substrate that we used is the length of the 5′ and 3′ arms. Nick translation occurs without cleavage on templates with long 3′ arms, and therefore it seemed likely that the distinguishing feature would be the free end of the 5′ arm. As would be expected with this model, substrates that lack a free 5′ end such as circular M13 DNA, but not linear M13 DNA, are resistant to cleavage even in the presence of added primers and pilot oligonucleotides (see below) (6).

The importance of a free 5′ end on cleavage was investigated with the use of DNA hairpins similar to the one in Fig. 1A, but with progressively longer 5′ arms. In the presence of primer and 50 mM KCl, cleavage of a 5′ extension that is 27 nucleotides long was essentially complete within 2 minutes at 55°C (Fig. 4A, lanes 1 to 4). In contrast, cleavage of molecules with 5′ arms of 84 and 188 nucleotides was only about 90 and 40 percent complete after 20 minutes (lanes 5 to 8 and 9 to 12, respectively) in the presence of a molar excess (2.5 times greater) of enzyme.

Three possible explanations to account for this reduction in the efficiency of cleavage were tested. First, if available enzyme could bind nonspecifically to long 5′ arms, it might be tied up in nonproductive complexes. However, this is not the case since exposure of the enzyme to a fourfold molar excess of substrate with long 5′ arms prior to addition of substrate with short arms did not inhibit the cleavage of the shorter arms (Fig. 4B). Second, if the 5′ nuclease were active only when it was bound to a free 5′ end, then the reduction in the local concentration of such ends near the cleavage site, because of longer arms, might reduce the efficiency of the cleavage. This explanation is unlikely, since increasing the concentration of 5′ ends by the addition of a fortyfold molar excess of nonspecific oligonucleotides did not increase the efficiency of cleavage of long 5′ arms (6).

A third model postulates that the appropriate structure for cleavage is achieved only when the 5′ arm is threaded through the enzyme; in contrast to the clamp associated with *E. coli* DNAP-III (*11*), such a mechanism in DNAP-Taq would keep inappropriate DNA strands away from the 5′ nuclease site rather than hold DNA on the enzyme. Longer 5′ arms would be likely to contain structures that could bind the enzyme and impede movement of the strand through the enzyme. Consistent with this model, cleavage was inhibited by an oligonucleotide complementary to the 31 nucleotides at the 5′ end of the 84-nucleotide arm, but not by a nonspecific oligonucleotide (Fig. 4C). In addition, a 268-nucleotide 5′ arm was cleaved efficiently when the temperature was raised to 72°C and the KCl was omitted from the reaction (6), both being measures that would reduce adventitious structures on long arms. Because gapped templates, such as might exist during gap repair, replication, or transcription, do not have free 5′ ends that could be threaded to the cleavage domain of the enzyme, they would not be cleaved.

Cleavage does not appear to be inhibited by long 3′ arms, at least up to 2 kilobases (6). At the other extreme, very short 3′ arms can support cleavage in a primer-independent reaction. A complex containing a template strand with no 3′ arm did not elicit cleavage of the DNA substrate (Fig. 4D, lane 2), but a complex with the same template extended by a single mismatched dideoxynucleotide did support

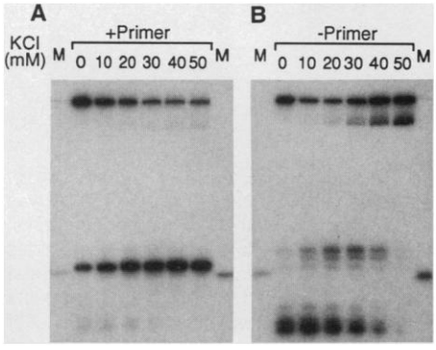

Fig. 3. Effect of KCl on cleavage in the presence and absence of primer. Reactions (*5*) were incubated at 55°C for 2 minutes or for 30 minutes in the presence (**A**) or absence (**B**) of a primer, respectively. The primer left a four-nucleotide gap. Undigested substrate is near the top of the gel; M is a 5′ end-labeled 19-nucleotide oligonucleotide. Substrates and cleavage products were resolved by gel electrophoresis (*5*).

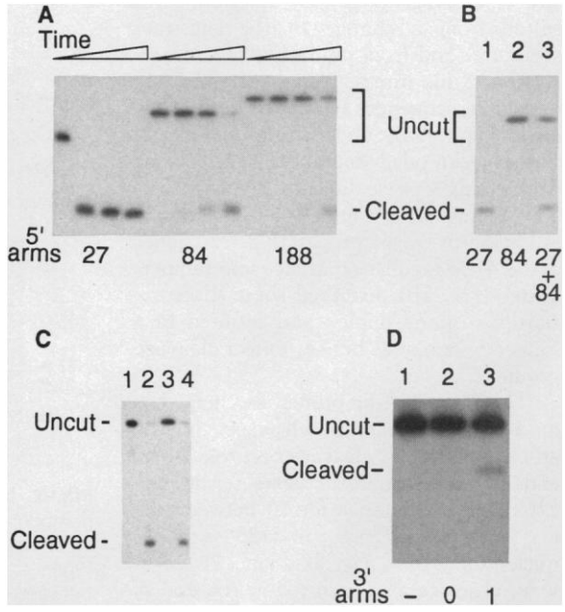

Fig. 4. Effect of arm length on the efficiency of cleavage. (**A**) Cleavage of substrates with different 5′ arm lengths. A substrate was produced by inserting the sequence shown in Fig. 1A into the Sma I site of plasmid pUC19. After digestion with Bam HI, the DNA was labeled at the 3′ end by filling in the ends with [α-^{32}P]dNTPs, and was recut with Kpn I, Bst NI, or Bgl I to create 5′ arms of 27, 84, or 188 nucleotides, respectively. Cleavage (*5*) was at 55°C for 0, 2, 6, and 20 minutes (left to right). (A to D) Substrates and cleavage products were resolved by electrophoresis (*5*). (**B**) Lack of interference by long 5′ arms. Two substrates, with 5′ extensions of 27 and 84 nucleotides, were incubated separately (lanes 1 and 2), or the shorter substrate was added to a reaction containing the longer substrate just prior to the initiation of cleavage (*5*) by addition of Mg^{2+} (lane 3). Incubations were at 55°C for 1 minute, and the DNAP-Taq was reduced to 0.05 unit in a 10-μl reaction mixture. (**C**) Inhibition of cleavage by structure in the 5′ arm. The substrate hairpin with a 5′ arm of 84 nucleotides was incubated for 20 minutes at 55°C with DNAP-Taq without any additional oligonucleotide (lane 2) or with an oligonucleotide that was complementary to 31 nucleotides at the 5′ end of the 84-nucleotide arm (lane 3), or a nonspecific oligonucleotide (lane 4). Material incubated without enzyme or oligonucleotide (lane 1) is shown as a marker. (**D**) Cleavage directed by a 3′ arm of one nucleotide. A non-hairpin PCR fragment (*22*) of 70 base pairs was heat-denatured and then incubated with DNAP-Taq in 20 mM KCl at 55°C for 30 minutes either alone (lane 1) or in the presence of an oligonucleotide, shown as the DNA pilot in Fig. 7. This latter molecule was either fully paired to the substrate (lane 2) or was extended by a single mismatched dideoxyadenosine (lane 3).

cleavage of the substrate, albeit inefficiently (lane 3).

The 5′ nuclease activities of other DNAPs. The hairpin substrate shown in Fig. 1A was used to monitor 5′ nucleases of other DNAPs under conditions reported to be optimal for DNA synthesis by each enzyme (*12*). DNAPs from the eubacteria *Thermus thermophilus* (Tth) and *Thermus flavus* (Tfl) cleaved the substrate at the same places as did DNAP-Taq, both in the presence and absence of primer (Fig. 5A). As is the case with DNAP-Taq, the sites of cleavage by these enzymes were influenced by the location of the 3′ end of the primer. No 5′ nuclease activity was detected for the DNAPs from the archaebacteria *Pyrococcus furiosus* (Pfu) and *Thermococcus litoralis* (Tli) (*13*).

DNAP-Ec1 also cleaved the bifurcated duplex endonucleolytically (Fig. 5B), with the precise site of cleavage fixed by the location of the 3′ end of the primer (*14*). At 37°C, in the absence of added primer (lane 3), the major product released by DNAP-Ec1 was the same 11-nucleotide fragment released by DNAP-Taq from the extended duplex (Fig. 2). Addition of a primer that left a gap of four nucleotides (lane 4) greatly increased the extent of cleavage, but the products were primarily di- and trinucleotides; these would be generated if the sequence A-A-T-A-C near the free end of the 5′ arm were paired with the 3′ arm, adjacent to the bifurcation. A primer extending right up to the bifurcation directed cleavage between the first two base pairs in the hairpin duplex (lane 5). The diagrams to the right of the photograph depict ways that the complex could fold to

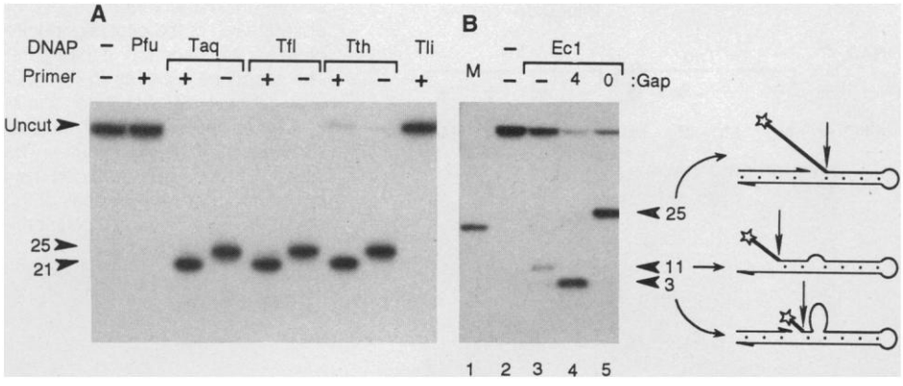

Fig. 5. Survey of several DNAPs for their ability to cleave the hairpin substrate in the presence (+) or absence (−) of added primer. (**A**) Endonucleases of DNAPs of several thermophilic bacteria (*12*). The 5′ end-labeled substrate and the primer shown in Fig. 1A were incubated at 55°C for 10 minutes with the enzymes indicated in the presence of primer (+) or at 72°C for 30 minutes in the absence of primer (−). The products were resolved by electrophoresis (*5*), and the lengths of the products, in nucleotides, are indicated. (**B**) Endonucleolytic cleavage by the 5′ nuclease of DNAP-Ec1. DNAP-Ec1 was incubated for 5 minutes at 37°C with the substrate in the absence of primer (lane 3) or with primers that left gaps of four or no nucleotides (lanes 4 and 5). No enzyme was added to the incubation in lane 2. A 20-nucleotide marker oligonucleotide is shown (lane 1); the mobilities of the fragments indicated on the right were determined by coelectrophoresis of known markers (Fig. 2) on the same gel (not shown) and by homochromatography. The diagram on the right shows possible base-paired structures that might form between the 5′ and 3′ arms of the hairpin, and which could give rise to the observed products.

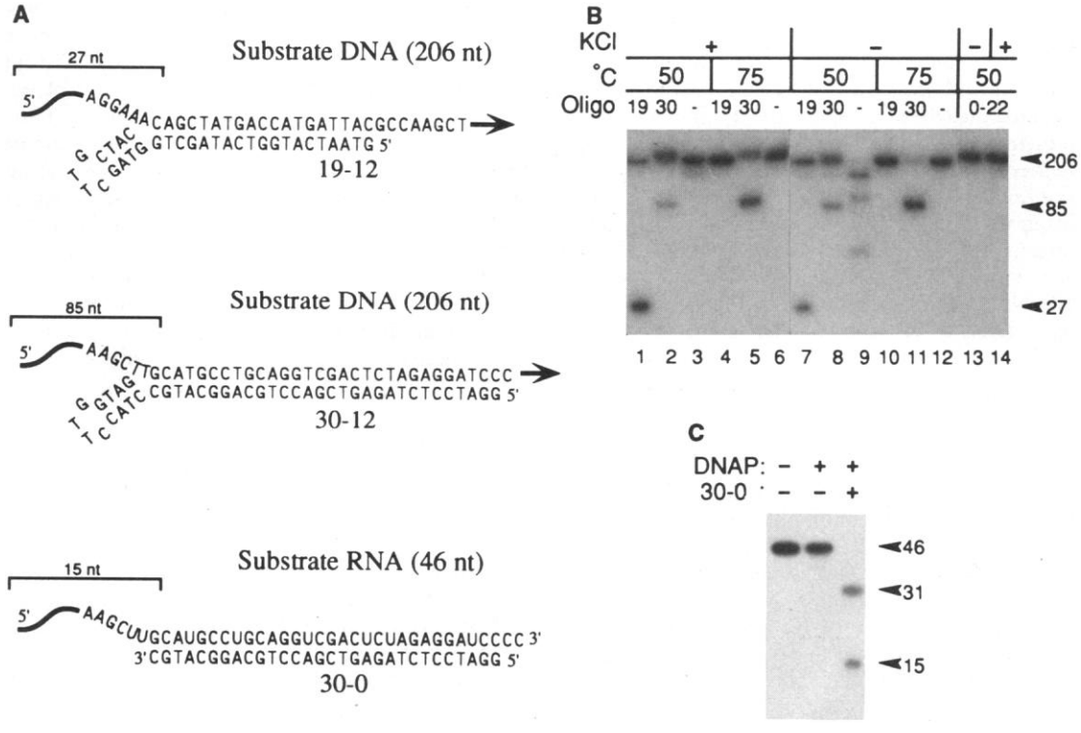

Fig. 6. Specific cleavage of nucleic acids targeted by pilot oligonucleotides. (**A**) Structures of pilot oligonucleotides base-paired to their complements in the substrate DNA or RNA. Two different sequences were used, which would pair to two different sites in the targeted nucleic acids, making complexes with different stabilities and distinguishable digestion products. The 206-nucleotide DNA substrate was 5′ end-labeled during PCR amplification of the polylinker region of plasmid pGEM3Z (*22*) with the use of the "−47 forward" and "−48 reverse" sequencing primers. (B and C) Products were resolved by electrophoresis (*5*), and their lengths, in nucleotides, are indicated. (**B**) Cleavage (*5*) of single-stranded DNA directed by pilot oligonucleotides 19–12 or 30–12 or an unrelated oligonucleotide 0–22. Incubations were for 10 minutes at 50° or 75°C, in the presence or absence of 50 mM KCl, as indicated. The 20-μl reaction mixtures contained 10 fmol of single end-labeled substrate DNA, 1 unit of DNAP-Taq (estimated to be 50 fmol) and 5 pmol of pilot oligonucleotide. The DNAs were heated to 95°C for 1 minute to denature the double-stranded substrate DNA, enzyme that had been heated was added, and the reactions were cooled to their final incubation temperatures as indicated. (**C**) Cleavage of RNA by DNAP-Taq in the presence of 30–0. The RNA substrate, made by T7 RNA polymerase in the presence of [α-^{32}P]UTP, corresponded to a truncated version of the DNA substrate used in (B). Reaction conditions resembled those used in (B), with 50 mM KCl, and incubation was for 40 minutes at 55°C.

give rise to the observed products. It is unclear whether DNAP-Taq would behave similarly at 37°C.

Since the amino acid sequences of the 5′ nuclease domains of DNAP-Ec1 and DNAP-Taq are about 34 percent similar, it is not surprising that they cleave the substrate DNA by similar mechanisms. It is unknown if other DNAPs and their associated 5′ exonucleases behave like the 5′ nucleases of eubacteria. The 5′ nuclease domain of DNAP-Taq shares about 19 percent homology with the 5′ exonuclease encoded by gene 6 of bacteriophage T7 (*15*). This nuclease, which is not covalently attached to a DNAP polymerization domain, is also able to cleave DNA endonucleolytically both in the presence and absence of primer, at a site similar or identical to the site that is cut by the 5′ nucleases described above (6). We have not yet tested eukaryotic DNAPs or associated exonucleases (*16*) for 5′ nuclease activity, but it has been observed that α-type DNAPs are homologous to the enzymes of the archaebacteria (*13*), which showed no 5′ nuclease activity (Fig. 5A).

Transcleavage of DNA and RNA. To learn whether a 5′ nuclease could be directed to cleave efficiently at any specific sequence of our choosing, we hybridized a partially complementary oligonucleotide (called a pilot) to sequences at the desired point of cleavage; the noncomplementary part of the pilot oligonucleotide provided a structure analogous to the 3′ arm of the template, whereas the 5′ region of the targeted nucleic acid became the 5′ arm. A primer was provided by designing the 3′ region of the pilot so that it would fold on itself, creating a short hairpin with a stabilizing tetra-loop (*17*). Two potential pilot oligonucleotides are shown in the structures at the top of Fig. 6A. Oligonucleotides 19–12 and 30–12 have 19 or 30 nucleotides, respectively, that are complementary to different sequences in the target DNA and should melt off their complements at about 50° and 75°C; both pilots have 12 nucleotides at their 3′ ends, which act as 3′ arms with base-paired primers attached.

Cleavage of the substrate DNA occurred in the presence of the pilot oligonucleotide 19–12 at 50° (Fig. 6B, lanes 1 and 7) but not at 75°C (lanes 4 and 10); in the presence of oligonucleotide 30–12, cleavage was observed at both temperatures. Cleavage did not occur at 80°C (6) or in the absence of added oligonucleotides (lanes 3, 6, or 12) except at 50°C where adventitious structures in the substrate allowed primer-independent cleavage in the absence of KCl (Fig. 6B, lane 9). A nonspecific oligonucleotide with no complementarity to the DNA target (but with the potential to form a hairpin with a stem of nine base pairs and a tetraloop at its 3′ end), called 0–22, did not direct cleavage at 50°C, either in the absence or presence of 50 mM KCl (lanes 13 and 14). Thus, the specificity of the cleavage reactions can be controlled by the extent of complementarity to the target and by the conditions of incubation.

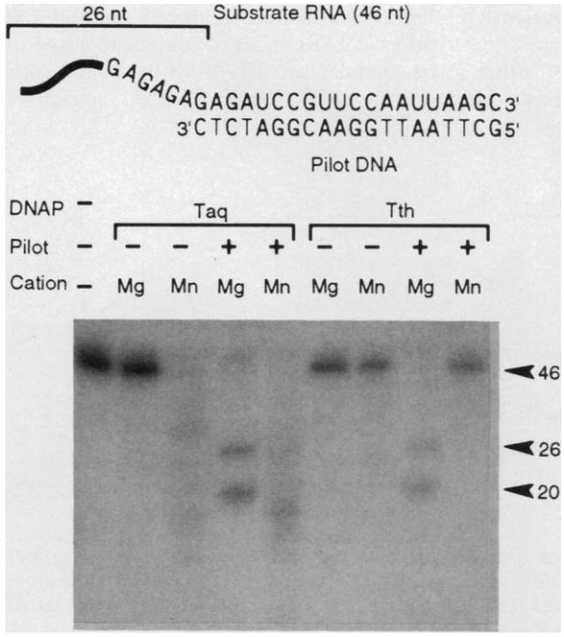

Fig. 7. Differential sensitivity of DNAPs to divalent cations during RNA cleavage. A 46-nucleotide-long RNA substrate, labeled during synthesis with [α-^{32}P]UTP, was incubated with either DNAP-Taq or DNAP-Tth in the presence (+) or absence (−) of the pilot oligonucleotide shown at the top of the figure. The reactions were as in Fig. 6C, with either 1 mM $MgCl_2$ or 1 mM $MnCl_2$, as indicated, and incubation was at 55°C for 20 minutes. The site of cleavage was deduced from the electrophoretic mobilities of the products in denaturing polyacrylamide gel electrophoresis (*5*).

An RNA version of the targeted sequence (Fig. 6A, bottom) was cleaved in a comparable place, in a reaction that was dependent on the presence of a pilot oligonucleotide resembling 30–12. In the case of RNA cleavage, a 3′ arm on the pilot oligonucleotide was not required (Fig. 6C). In contrast to previously described ribonuclease (RNase) H, which would be expected to cut the RNA in several places along the 30–base pair RNA-DNA duplex, the 5′ nuclease of DNAP-Taq is a structure-specific RNase H that cleaves at a single site opposite the 3′ end of the pilot DNA. It is surprising that an oligonucleotide lacking a 3′ arm can act as a pilot in directing cleavage of an RNA target since such oligonucleotides are unable to direct cleavage of DNA targets (Fig. 4C).

Both DNAP-Taq or DNAP-Tth cleaved RNA when incubated with an appropriate pilot oligonucleotide (Fig. 7, top) in the presence of Mg^{2+}. However, DNAP-Tth was unable to cleave the RNA in the presence of Mn^{2+}. The specificity of the 5′ nuclease of DNAP-Tth for Mg^{2+} may contribute to the ability of this enzyme to function as a reverse transcriptase in the presence of Mn^{2+} (*18*).

Some applications. An understanding of the 5′ nuclease activity has several practical consequences. Most exciting is the creation of cleavage reagents that can cut single-stranded polynucleotides in a highly specific manner at chosen sequences (*19*). Because cleavage depends on the ability of the 5′ nuclease to recognize substrates not by their sequence but by their structure, essentially any sequence can be targeted for cleavage by annealing it to an appropriate pilot oligonucleotide. By carrying out cleavage under very stringent hybridization conditions, such as at 75°C, a high degree of specificity of the site of cleavage is achieved (Fig. 6B), and the effects of long 5′ arms can be minimized. Sequences in one or both complementary strands of denatured DNA can be targeted for cleavage by the use of appropriate pilot oligonucleotides. Furthermore, use of conditions that favor cleavage may facilitate optimization of allele-specific PCR (*20*), so that primers with unpaired 3′ ends would act as pilot oligonucleotides to direct selective cleavage of unwanted templates.

If the 3′ extension of a pilot oligonucleotide forms a stable hairpin sequence (*17*), the pilot carries its own primer and can function under primer-dependent conditions (for example, in the presence of 50 mM KCl) (Fig. 6); this feature both greatly increases cleavage efficiency, and reduces the number of unwanted cleavages at regions of secondary structure in the target nucleic acid. Whether single-stranded RNA or DNA binding proteins can facilitate cleavage of long 5′ arms through disruption of secondary structures remains to be determined.

The 5′ nucleases of thermostable DNAPs may also be useful in probing for sequences or sequence variants in nucleic acid preparations. For example, the presence of a particular DNA might be detected

by using that molecule as the template strand for cleavage of an added substrate probe, analogous to the method of Holland *et al.* (*4*). In this case, however, cleavage of the probe need not be coupled to synthesis, so it can be applied to unamplified DNA fragments or in post-PCR analysis. Additional demands could be made of the system, such as requiring that the template strand also bind a primer adjacent to the probe, thereby adding another level of stringency. Other less direct sequence probing applications of the 5′ nuclease could involve the production of nucleic acid fragments of optimal size for analyses such as single-strand conformation polymorphism (*21*).

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8. Presumably the short extension of the duplex was stabilized by being in the same molecule, near to the hairpin.
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