Effects of Vinylphosphonate Internucleotide Linkages on the Cleavage Specificity of Exonuclease III and on the Activity of DNA Polymerase I†

Zara A. Doddridge, Richard D. Bertram, Christopher J. Hayes, and Panos Soultanas*

School of Chemistry, University of Nottingham, University Park, Nottingham NG7 2RD, U.K.

Received October 10, 2002; Revised Manuscript Received January 11, 2003

ABSTRACT: We have previously reported the synthesis of vinylphosphonate-linked thymidine dimers and their incorporation into synthetic oligonucleotides to create vinylphosphonate internucleotide linkages in the DNA. Such linkages have a profound effect on DNA backbone rotational flexibility, and we have shown that the PcrA helicase, which requires such flexibility, is inhibited when it encounters these linkages on the translocating strand. In this study, we have investigated the effects of these linkages on the dsDNA specific exonuclease III and on the ssDNA specific mung bean nuclease to establish whether our modification confers resistance to nucleases making it suitable for antisense therapy applications. We also investigated the effect on DNA polymerase I to establish whether we could in the future use this enzyme to incorporate these linkages in the DNA. Our results show that a single modification does not affect the activity of DNA polymerase I, but four vinylphosphonate linkages in tandem inhibit its activity. Furthermore, such linkages do not confer significant nuclease resistance to either exonuclease III or mung bean nuclease, but unexpectedly, they alter the cleavage specificity of exonuclease III.

The use of short oligonucleotides to disrupt the expression of disease-related genes forms the basis of antisense therapies and is an idea potentially applicable to many genetically based diseases (*1*). The theoretical goal is straightforward, but because it must be achieved in vivo, within a living cell it becomes harder in practice than in theory. One of the most important hurdles to overcome is the susceptibility of therapeutic oligonucleotides to a variety of nucleases. To make potentially therapeutic nucleic acids resistant to nucleases, different strategies have been employed. Chemical modification is one strategy, and various chemically modified nucleic acids, like for example phosphorothioates, have been synthesized (*2*). Another approach is to use oligonucleotide analogues, with peptide nucleic acids (PNAs)[1] being the best-known example. In PNAs, the entire nucleic acid backbone is replaced with a chain resembling a peptide, with the end result being a macromolecule that retains the correct alignment of the bases but is resistant to nucleases (*3*). Base analogues have also been explored as tools for DNA modifications with various applications (*4*).

Exploring alternative chemical modifications, we have previously reported the chemical synthesis of vinylphosphonate thymidine dimers and their incorporation into synthetic oligonucleotides (*5*). Such vinylphosphonate internucleotide linkages could be used in therapeutic oligonucleotides since they offer minimal steric hindrance and do not disturb the formation of normal duplex DNA. However, they have a profound effect on DNA backbone rotational freedom. This unique property also makes these modifications potentially useful in studying the enzymatic mechanism of nucleic acid metabolizing enzymes, particularly those requiring DNA backbone rotational flexibility. One such enzyme is the monomeric DNA helicase PcrA from *Bacillus stearothermophilus*, and we have previously shown that vinylphosphonate internucleotide linkages inhibit the activity of this enzyme, as long as they are positioned on the translocating strand, either at a single strand−double strand junction or within the DNA duplex (*6*). Although the molecular mechanism of this inhibitory effect is not known, it is likely to be due to the restriction of the DNA backbone rotational flexibility with a consequent inhibition of the "Mexican Wave" mechanism of translocation of this enzyme (*7*, *8*).

In this study, we examined the effects of these modifications on the activities of the commercially available nucleases, exonuclease III and mung bean nuclease, as well as DNA polymerase I. Exonuclease III is the major repair dsDNA specific nuclease in *Escherichia coli*, whereas mung bean nuclease is a ssDNA specific nuclease. The choice of these enzymes will allow us to determine the effects of these linkages as part of either ds- or ssDNA substrates for nucleases. We constructed synthetic substrates by incorporating vinylphosphonate internucleotide linkages at strategically placed positions to study their effects on the activities of these enzymes. Our results show that one modification does not inhibit the activity of DNA polymerase I, whereas a stretch of four modifications inhibits the polymerase activity. Therefore, DNA polymerase I could be used to incorporate

† This work was supported by Wellcome Trust Grant 064751/Z/01/Z to P.S. and a BBSRC committee studentship to R.D.B.

* To whom correspondence should be addressed. Telephone: 44-(0)115-9513525. Fax: 44-(0)115-9513564. E-mail: panos.soultanas@nottingham.ac.uk.

[1] Abbreviations: ExoIII, exonuclease III; DNA pol I, DNA polymerase I; PNA, peptide nucleic acid; PNK, polynucleotide kinase; CIP, calf intestinal phosphatase; dNTPs, deoxynucleotide triphosphates; dATP, deoxyadenosine triphosphate; dGTP, deoxyguanosine triphosphate; dCTP, deoxycytosine triphosphate; DNase I, deoxyribonuclease I; RNase I, ribonuclease I; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide electrophoresis; EDTA, ethylenediaminetetraacetic acid.

10.1021/bi026985+ CCC: $25.00
Published on Web 02/27/2003

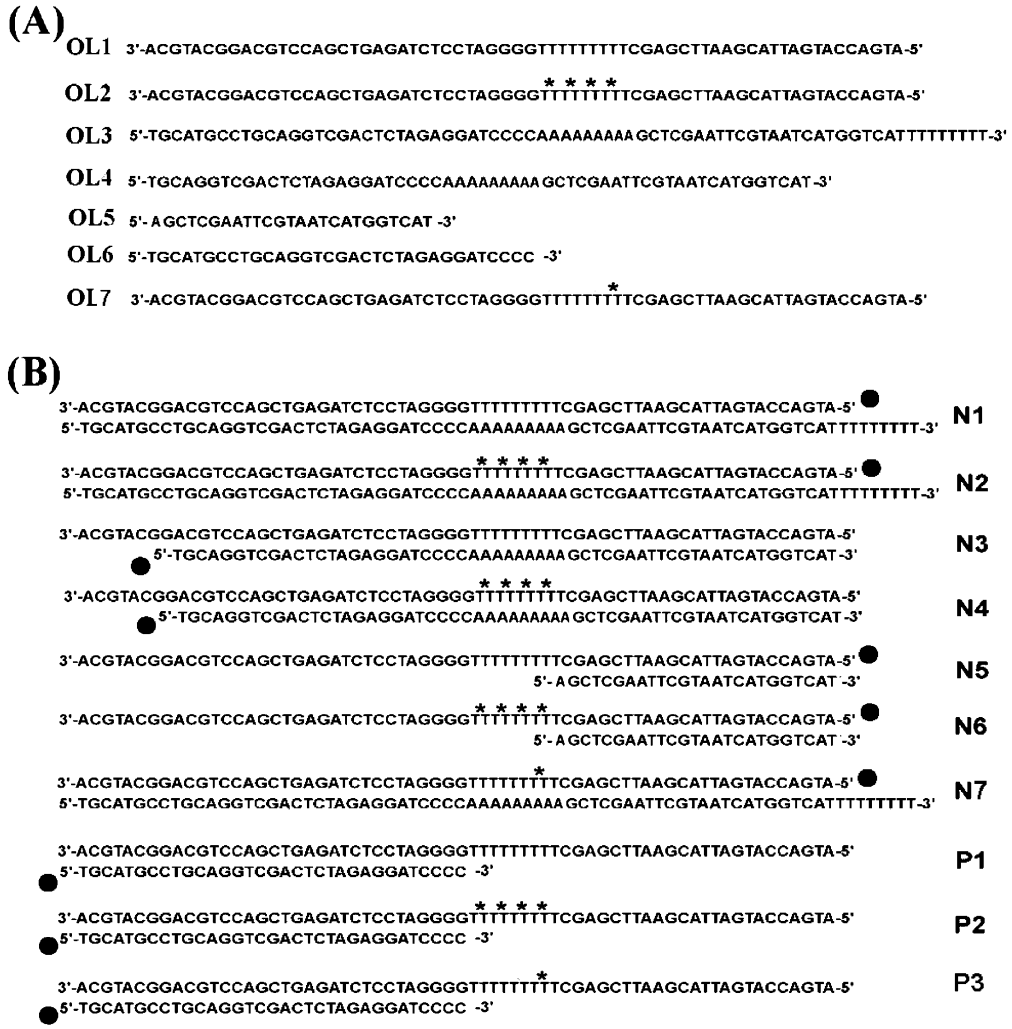

FIGURE 1: Synthetic oligonucleotides (A) and the DNA substrates (B) used in this study. The vinylphosphonate internucleotide linkages are denoted with asterisks, whereas the positions of the radioactive phosphate labels are denoted with bullets. Substrates N1−N4 and N7 were used for exonuclease III digestions and substrates N5 and N6 for mung bean nuclease digestions. Substrates P1−P3 were used for primer extension reactions catalyzed by Klenow and DNA polymerase I.

such linkages into the DNA directly. Furthermore, our modifications do not confer significant resistance to either exonuclease III or mung bean nuclease, suggesting that they will not confer any advantage on antisense therapy strategies, but unexpectedly, they induce an activity on exonuclease III that was previously undetected.

With the help of already available structural information, we discuss the molecular basis and implications of these effects and suggest that they are mediated by restriction of the rotational flexibility of the DNA backbone.

EXPERIMENTAL PROCEDURES

Construction of DNA Substrates for Exonuclease III Digestions. Vinylphosphonate synthetic oligonucleotides were prepared as described previously (*5*). Oligonucleotides OL1, OL2, and OL7 (7 pmol) were radioactively labeled at the 5′-end using [γ-^{32}P]ATP and T4 polynucleotide kinase (T4 PNK) and subsequently annealed to 50 pmol of oligonucleotide OL3 to construct substrates N1, N2, and N7, respectively, as shown in Figure 1. The labeled DNA substrates were purified away from unincorporated [γ-^{32}P]ATP by gel filtration through S200 Sephadex mini-spin columns (Amersham Pharmacia Biotech) according to the manufacturer's instructions. The labeled DNA substrates were dissolved in 100 μL of 5 mM Tris (pH 8.2) and 1 M NaCl to give a final concentration of 0.07 pmol/μL for stock solutions (assuming 100% recovery from the S200 mini-spin columns). The high ionic strength stabilizes duplex formation and prevents duplex destabilization during storage. Subsequent addition of the labeled substrates to the reaction mixtures resulted in a 1/20 dilution of the salt. Substrates N3 and N4 were prepared in a similar manner, but with OL4 labeled radioactively and annealed to either OL1 or OL2 to give N3 or N4, respectively. Oligonucleotides of known sizes (16, 22, 25, 32, and 38 bases long) were also labeled radioactively and used as size markers.

Construction of DNA Substrates for Mung Bean Nuclease Digestions. Substrates N5 and N6 were prepared as described above, with OL1 and OL2 labeled radioactively and subsequently annealed to OL5 which gave N5 and N6, respectively.

Construction of DNA Substrates for Primer Extension Reactions. Substrates P1−P3 were prepared by annealing radioactively labeled OL6 to OL1, OL2, and OL7, respectively.

Purity of Commercially Available Enzymes. The purity of the commercially available enzymes, exonuclease III, mung bean nuclease, DNA pol I, and Klenow, was assessed by SDS−PAGE (data not shown). Exonuclease III enzymes from Hybaid-AGS, New England Biolabs, and Amersham Pharmacia Biotech as well as Klenow and DNA polymerase I from New England Biolabs appeared to be very pure, as only single bands corresponding to approximately 28 kDa for exonuclease III, 68 kDa for Klenow, and 103 kDa for DNA polymerase I were visible after Coomassie blue staining. No protein bands could be detected for mung bean nuclease enzymes obtained from New England Biolabs and Amersham Pharmacia Biotech, presumably because the concentrations were very low and could not be detected by SDS−PAGE. Therefore, we cannot be certain of the purity of this enzyme.

In addition, to eliminate the possibility that very low concentrations of spurious contaminant proteins were responsible for the effects we were detecting (see below), we performed our exonuclease III digestions and primer extension experiments using commercially available enzymes from different suppliers (exonuclease III from Hybaid-AGS, Amersham Pharmacia Biotech, and New England Biolabs, DNA pol I and Klenow from New England Biolabs, ABgene, Sigma, and Amersham Pharmacia Biotech, and mung bean nuclease from New England Biolabs). In all cases, the results were the same (data not shown).

Exonuclease III Digestions. Time course digestions were carried out at 37 °C, in a buffer containing 66 mM Tris-HCl, 0.66 mM $MgCl_2$, and 3.5 nM DNA substrate. All reaction mixtures were preincubated at 37 °C for 5 min, and the reactions were initiated by the addition of exonuclease III (5 units). At appropriate time intervals, 20 μL aliquots were removed, and the reaction was terminated by addition of 7 μL of stop buffer [formamide loading buffer, 0.1% (w/v) bromophenol blue, and 0.1% (w/v) xylene cyanol] and 2 μL of 0.5 M EDTA to the aliquot, which was then temporarily stored on ice. Different size oligonucleotides were resolved by electrophoresis through a 12% polyacrylamide denaturing gel in 1× TBE electrophoresis buffer at 2000 V. Gels were dried and visualized using an FX molecular imager and associated software (Bio-Rad), according to the manufacturer's instructions.

Treatment with T4 polynucleotide kinase (T4 PNK, New England Biolabs) was carried out by pooling all the exonuclease III time course reaction mixtures together, ethanol precipitating the DNA fragments, washing the pellet with 70% cold ethanol to remove excess salt, and then treating with 1 unit/μL T4 PNK in 70 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, and 5 mM DTT at 37 °C for 3 h. The reaction was terminated by addition of stop buffer, and electrophoresis and visualization were carried out as described above.

In similar experiments, the pooled exonuclease III fragments were prepared as above and then treated with 20 units (New England Biolabs) of calf intestinal alkaline phosphatase (CIP) for 90 min at 37 °C. CIP was inactivated by heating at 94 °C for 15 min, followed by cooling on ice. The DNA fragments were extracted with a phenol/chloroform mixture, ethanol precipitated, washed in 70% ethanol, dried, and then relabeled radioactively using T4 PNK, as described above. Electrophoresis and visualization of the relabeled DNA fragments were carried out as described above.

Mung Bean Nuclease Digestions. Time course digestions were carried out at 30 °C in a buffer containing 50 mM sodium acetate, 30 mM NaCl, 1 mM $ZnSO_4$, and 3.5 nM DNA substrate. All reaction mixtures were preincubated at 30 °C for 5 min, and the reactions were initiated by the addition of mung bean nuclease (1 unit, New England Biolabs). At appropriate time intervals, 20 μL aliquots were removed, and the reaction was terminated as described above. Different size oligonucleotides were resolved by electrophoresis, and gels were dried and visualized, as described above.

Primer Extension by Klenow and DNA Polymerase I. Primer extension reactions were carried out in 50 mM $KHPO_4$ (pH 7.0), 5 mM $MgCl_2$, 1 mM DTT, 14 nM DNA substrate (P1, P2, or P3), and the appropriate mixture of dNTPs (312.5 μM each) at 37 °C for 30 min in a total reaction volume of 20 μL. The reactions were terminated by addition of 7 μL of stop buffer [formamide loading buffer, 0.1% (w/v) bromophenol blue, and 0.1% (w/v) xylene cyanol] and 2 μL of 0.5 M EDTA. The products were resolved by electrophoresis, and gels were dried and visualized, as described above.

RESULTS

Exonuclease III Digestion. The effect of the vinylphosphonate internucleotide linkages on the activity of exonuclease III was studied using DNA substrates N1 and N2. The 3′-$d(T)_8$ single-strand tail blocks the activity from one end of the duplex and directs the enzyme specifically on the modified strand. Time course reactions revealed that there is a delay as the enzyme encounters the stretch of the modified thymidines (Figure 2). The delay is apparent 37−28 nucleotides away from the radioactively labeled 5′-end of the modified oligonucleotide. Since the four vinylphosphonate linkages are situated between nucleotides 26 and 27, 28 and 29, 30 and 31, and 32 and 33, it is apparent that the enzyme is partially delayed approximately four nucleotides prior to the first linkage as it moves in the 3′−5′ direction. Prolonged incubation results in complete digestion of the modified substrate N2 (data not shown). The crystal structure of exonuclease III suggests that the DNA binding site is four to six nucleotides ahead of the active site (*9*). The enzyme binds to the duplex DNA ahead and cleaves four to six nucleotides behind as it moves along the 3′−5′ direction (see the Discussion). This mechanism is also consistent with the inability of exonuclease III to digest 3′-overhangs of four or more nucleotides. The apparent delay in the progression of the enzyme over the modified stretch of thymidines is evident only over the first three vinylphosphonate linkages, and it does seem to resume normal progression over the last linkage. As it reaches the last linkage, the duplex DNA ahead of it is normal (unmodified), and thus, there is no apparent effect on its progression. Interestingly, when the modification is placed in the "non-digested" strand in substrate N4, no effect on exonuclease III can be detected compared to control substrate N3 (data not shown).

An intriguing observation was the appearance of four "triplet bands" (triplets), apparently between nucleotides 28 and 29, 30 and 31, 32 and 33, and 34 and 35 (Figure 2A,B). Their appearance was time-dependent as they became visible

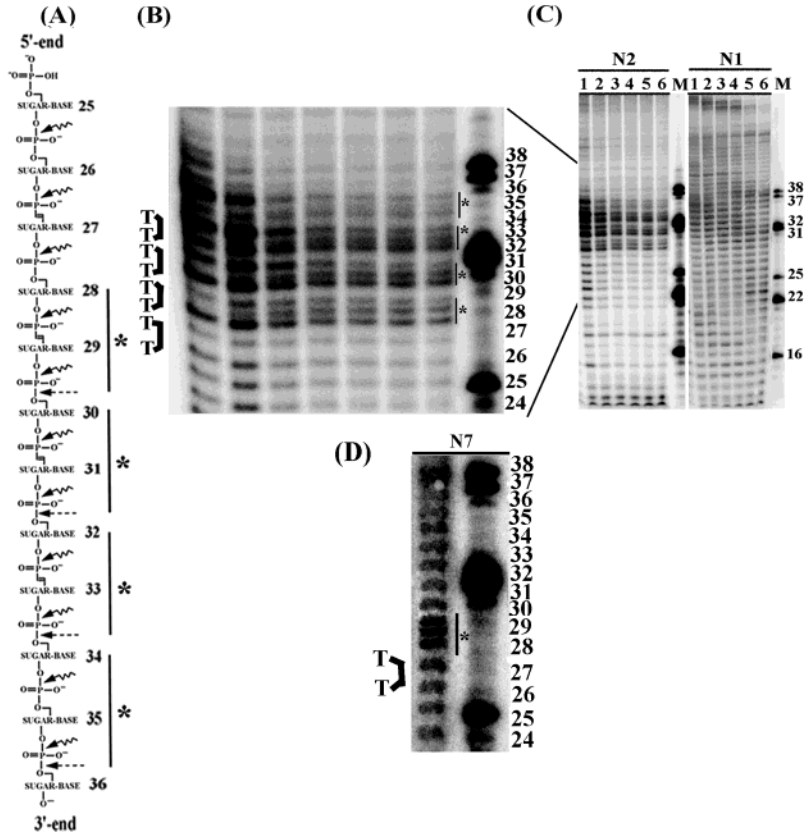

FIGURE 2: Time course reactions of exonuclease III digestions using substrates N1 and N2. The precise positions of the cleavages are shown by wiggly arrows, whereas the vinylphosphonate-induced cleavages are shown by interrupted arrows in panel A. The corresponding triplets are denoted with asterisks in both panels A and B. The magnified region (nucleotides 24−38) containing the triplets is shown in panel B. The complete gels showing the exonuclease III time course digestions using substrates N1 and N2 are shown in panel C. Labels 1−6 indicate termination of the reactions 15, 30, 45, 60, 90, and 120 s after addition of the exonuclease III, respectively. Lanes marked M contained size markers. Panel D shows similar reactions with substrate N7. All the samples from the time points were pooled and run in one lane alongside size markers. One triplet was apparent.

from approximately 30 s onward in the digestion time course reactions. The obvious interpretation of these data is that the middle bands of these triplets represent DNA fragments with 3′-phosphate groups (Figure 2A). The electrophoretic mobility of DNA fragments through the gel depends on the charge/mass ratio. The higher this ratio is, the faster the mobility will be. The middle fragment of the triplet will be identical to the fragment immediately above it but with an additional phosphate group at its 3′-end. Its negative charge will be greater, whereas its mass will be changed minimally, thus making it migrate faster. Such a suggestion implies that the enzyme has acquired the ability to cleave the P−O5′ bond in addition to cleaving the P−O3′ bond. The positioning of these triplets allowed us to pinpoint accurately the four P−O5′ cleavages. They took place between nucleotides 29 and 30, 31 and 32, 33 and 34, and 35 and 36 (Figure 2A). The first two are normal linkages interspersed between the vinylphosphonate linkages, whereas the last two are normal linkages just prior to the stretch of vinylphosphonates as the enzyme moves in the 3′−5′ direction. It is worth noting that a P−O5′ cleavage is not possible in the vinylphosphonate linkages.

The introduction of a single modification between nucleotides 26 and 27 in substrate N7 (Figure 1) resulted in a single triplet between nucleotides 28 and 29 (Figure 2D). The additional P−O5′ cleavage takes place between nucleotides 29 and 30. This pattern of cleavage is consistent with that obtained with substrate N2 and implies a footprint for alternative cleavage. When the vinylphosphonate linkage is three positions (between nucleotides five and six from the last one) ahead of the last internucleotide link to be cleaved, it induces an alternative cleavage by the enzyme.

We set out to prove the presence of a 3′-phosphate group at these alternative cleavages directly by employing T4 PNK, which also catalyzes the removal of 3′-phosphoryl groups from 3′-phosphoryl polynucleotides (*10*). We repeated the same time course experiment with substrate N2 and pooled all the samples together, before treating them with T4 PNK. This enzyme has a 3′-phosphatase activity and should have removed 3′-phosphate groups with the apparent disappearance of the triplet bands. However, even with prolonged incubations, we observed that the triplet bands did not disappear upon T4 PNK treatment (Figure 3A). Our immediate concern was that the 3′-phosphatase activity of T4 PNK may also be inhibited by the presence of the vinylphosphonate modification very close to the 3′-end. We adopted a different approach, employing calf intestinal alkaline phosphatase (CIP) that is also known to remove 3′-phosphoryl groups from DNA (*11*). We repeated the same experiment as described above for the PNK, but this time we treated the DNA fragments from the exonuclease III-treated substrate, N2, with CIP to remove all 5′- and 3′-phosphate groups from the DNA, followed by radiolabeling of the DNA at the 5′-end by T4 PNK treatment. This time we observed that the triplets disappeared, indicating that CIP was able to remove the 3′-phosphates from the DNA

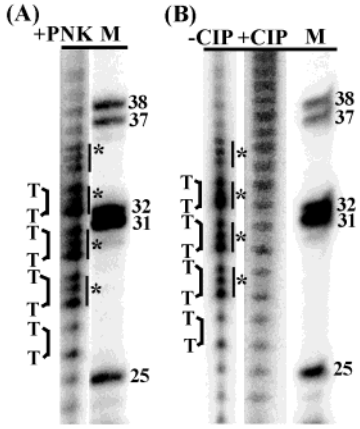

FIGURE 3: Treatment of exonuclease III-derived DNA fragments with T4 PNK (A) and CIP (B). DNA substrate N2 was used to generate fragments by exonuclease III treatment, as described in the legend of Figure 2. All the fragments were pooled together and then treated with either T4 PNK (A) or CIP (B). The CIP-treated DNA fragments were then radiolabeled again with T4 PNK before electrophoresis through a denaturing sequencing gel. The regions of the gels that contain the triplets (labeled with asterisks) are magnified for clarity. The positions of the vinylphosphonate internucleotide linkages are also shown. The lanes labeled M contained oligonucleotide size markers. T4 PNK-treated samples still contain the triplets, whereas in CIP-treated samples, the triplets have disappeared.

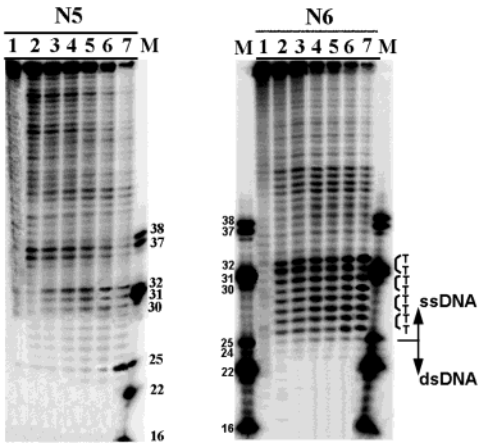

FIGURE 4: Time course reactions of mung bean nuclease digestions using DNA substrates N5 and N6, as indicated. Lanes 1−7 represent reactions terminated 0, 2, 4, 8, 10, 12, and 14 min after addition of the nuclease, respectively. Size markers are shown in lanes marked M. The positions of the vinylphosphonate linkages and the ssDNA−dsDNA boundary are marked for clarity.

fragments (Figure 3B). A control sample that was not treated with CIP clearly exhibited the triplets as before.

The same results were obtained with exonuclease III enzymes from three different suppliers (Hybaid-AGS, New England Biolabs, and Amersham Pharmacia Biotech), and we were interested in finding out whether this effect is observed exclusively with exonuclease III or whether it is also manifested in the activities of other nucleases. We examined the effect of these modifications on the activity of mung bean nuclease.

Mung Bean Nuclease Digestion. Mung bean nuclease is a commercially available single-strand specific DNA and RNA endonuclease which is used to remove single-strand extensions from the ends of DNA molecules, leaving blunt ligatable ends (*11*). However, it can also cleave double-strand DNA at high concentrations and after prolonged digestions. Utilizing its single-strand specificity, we constructed the partially single-stranded DNA substrates N5 (unmodified) and N6 (modified) (Figure 1B). Time course reactions of mung bean nuclease digestions revealed that the modifications conferred partial resistance to cleavage within the modified region (Figure 4). The pattern of cleaved products revealed that the specificity of cleavage of this enzyme was not altered. Cleavage occurred at the P−O3′ bonds as normal.

Having determined the effects of vinylphosphonate internucleotide linkages on the exonuclease III and mung bean nucleases, we proceeded to determine whether these linkages serve as normal DNA substrates for DNA polymerase.

Primer Extension Studies. We employed a simple primer extension assay to determine the efficiency and accuracy of DNA polymerase I activity on substrates P1 and P2 (Figure 1B). The polymerase should extend the radioactively labeled primer (OL6) oligonucleotide from the 3′-end. We controlled the extension reaction by limiting the availability of dNTPs in the reaction mixture. In the presence of only dATP, extension should be observed over the nine thymidines immediately after the 3′-end, and in the presence of both dATP and dGTP, the polymerization should be extended by one further nucleotide; in the presence of dATP, dGTP, and dCTP, one additional nucleotide will be added, whereas in the presence of all four dNTPs, the enzyme should be able to complete the polymerization of the DNA substrate. In the presence of the unmodified P1 substrate, both the whole DNA polymerase I and the Klenow fragment were able to extend the primer efficiently (Figure 5A,B). However, both enzymes were inhibited by the vinylphosphonate internucleotide linkages, as manifested by the poor extension of the primer in the modified substrate P2 (Figure 5A,B). There was marked stalling of the enzymes at position 5, and this stalling was particularly pronounced for the Klenow enzyme. Some extension beyond the nucleotide at position 5 was observed, albeit very poor indeed.

Interestingly, in similar primer extension experiments, there was no observed inhibition of either Klenow or DNA polymerase I activities when only one vinylphosphonate internucleotide linkage was introduced into the DNA, as was the case with substrate P3 (Figure 5C). The same results were obtained with enzymes from two different suppliers (Boehringer Mannheim and Amersham Pharmacia Biotech).

DISCUSSION

Effect on Exonuclease III. Exonuclease III is a 3′−5′ exonuclease (*12*). It catalyzes the stepwise removal of mononucleotides from the 3′-end of the duplex DNA. It is not a processive enzyme, and thus, a limited number of nucleotides are removed during each binding event, resulting in progressive deletions of the DNA. The preferred substrates for this reaction are blunt and 3′-recessed termini (*13*). Single-stranded DNA and 3′-overhangs, four bases or longer, on duplex DNA as well as thioester-linked nucleotides are resistant to exonuclease III cleavage (*14*). The enzyme has also been reported to have RNase H, 3′-phosphomonoesterase, 3′-repair diesterase, and apurinic/apyrimidinic DNA repair endonuclease activities (*12*, *15*).

The high-resolution crystal structure of exonuclease III complexed with Mn^{2+} and dCMP revealed similarities to both DNase I and RNase H (ref *9* and Figure 6A). The

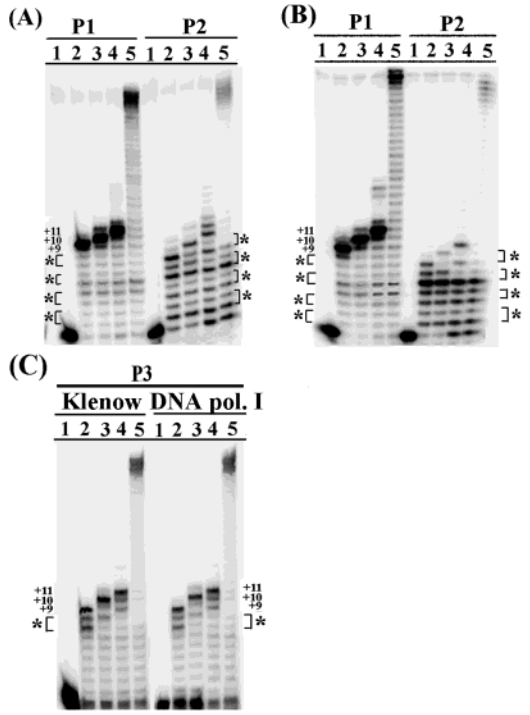

FIGURE 5: Primer extension reactions by DNA polymerase I (A) and Klenow (B). All reactions were carried out using DNA substrates P1 (unmodified) and P2 (modified) in the presence of dATP (lane 2), dATP and dGTP (lane 3), dATP, dGTP, and dCTP (lane 4), and dATP, dGTP, dCTP, and dTTP (lane 5). Lane 1 shows the radioactively labeled substrate, whereas the positions of the vinylphosphonate internucleotide linkages are marked with asterisks. Panel C shows similar reactions carried out with Klenow and DNA polymerase I but this time using DNA substrate P3 (one modification).

structure also revealed the likely mechanism for the hydrolytic cleavage of the P−O3′ bond. The active site His259 acts as a general base and abstracts a proton from the incoming water molecule. Asp229 forms a H-bond with His259 and stabilizes the developing positive charge. The resulting hydroxide acts as a nucleophile and attacks the phosphate group, through a pentacovalent transition state (Figure 6B). Other active site residues and a metal ion also participate in the reaction mainly via interactions with the phosphate group, helping to position it in the correct orientation for cleavage, stabilize the transition state, or polarize the P−O3′ bond. Asp151 is believed to play the role of the Brönsted acid to protonate the O3′ leaving group. The same mechanism may also apply to all the other known nucleolytic activities of exonuclease III. Modeling studies suggest that the enzyme binds the DNA duplex ahead of the active site, via interactions of a protruding α-helix with the major groove and a $\beta-\beta$ loop with the minor groove of the duplex (Figure 6A). The whole of the DNA-interacting area spans four to six base pairs and explains quite nicely the insensitivity of 3′-overhangs of four or more bases to exonucleolytic cleavage. These interactions ahead of the active site are likely to involve some conformational changes of the enzyme, the DNA, or both, to enable the DNA to fit into the active site and also determine the specificity of cleavage.

Our data show that exonuclease III is somewhat delayed when it encounters a stretch of four vinylphosphonate internucleotide linkages alternating with normal linkages. It

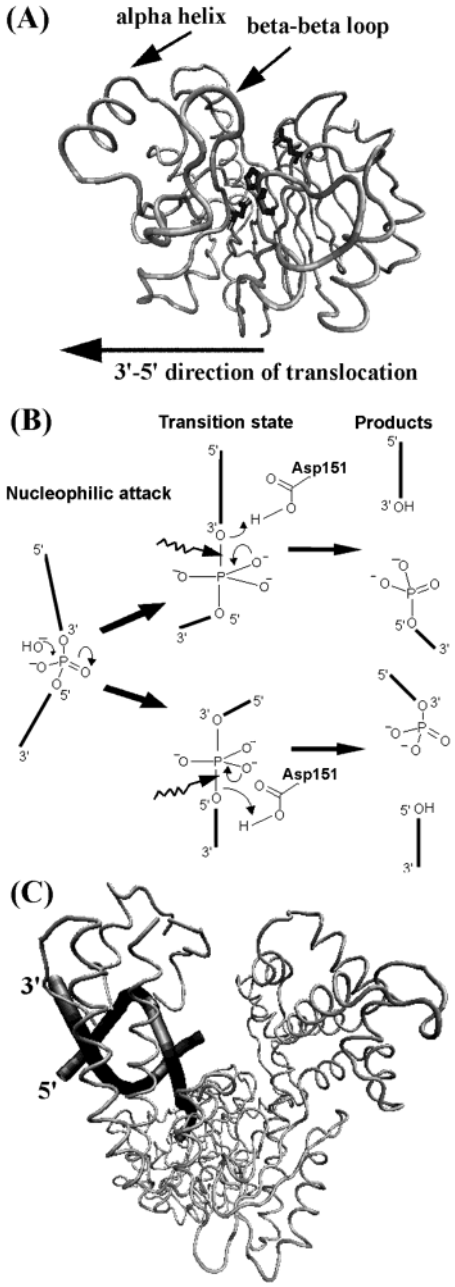

FIGURE 6: Crystal structure of exonuclease III (A) (PDB entry1AKO). Three active site residues (E34, D229, and H259) are shown in bold, and the $\beta-\beta$ loop and α-helix both suggested to be involved in interactions with the duplex DNA are shown with arrows. The 3′−5′ direction of translocation of the enzyme along the duplex DNA is also indicated by an arrow. A schematic diagram showing the formation of the trigonal bipyramid pentacoordinate transition state in the active site of exonuclease III is given in panel B. A conserved Asp151 has been suggested to protonate the O3′ leaving group, but repositioning of the phosphate group in the active site may affect the geometry and favor protonation of the O5′ group instead or even confer promiscuity in the cleavage reaction by not preferring the P−O3′ cleavage over the P−O5′ cleavage. Other active site residues also participate in the reaction, but they are omitted for the sake of clarity. The crystal structure of the Klenow fragment of DNA polymerase I bound to a duplex DNA with a three-base 3′-extension is given in panel C (PDB entry 1KLN). The polymerase active site is situated at the bottom of the deep cleft in the center of the molecule. The 5′- and 3′-ends of the duplex are labeled for clarity. Distortion of the duplex is clearly visible, and although this is an editing complex, it has been suggested that bending of the duplex will be required for the DNA to fit into the polymerase active site (*18*).

cleaves the DNA backbone at the normal P−O3′ sites, but when it encounters the vinylphosphonate linkages, it also cleaves at P−O5′ sites in the intervening normal linkages between the vinylphosphonate-linked dimers (Figure 2A). Somehow, it acquires a 5′-phosphomonoesterase activity. Such an activity for exonuclease III has not been reported before. This alteration in the cleavage specificity seems to be specific for exonuclease III as the same modifications do not affect the cleavage specificity of the ssDNA specific mung bean nuclease. However, this enzyme is also delayed when it encounters a stretch of four vinylphosphonate internucleotide linkages alternating with normal linkages (Figure 4). This effect is only apparent when the modifications are placed on the "digesting" strand, as the enzyme digests the DNA normally when the modifications are in the "nondigested" strand. Although this is an important observation, its significance is not clear at present. One speculative suggestion is that the interactions of the enzyme with the digesting strand may determine the specificity of the cleavage in the active site. Such a notion would also suggest that important DNA−enzyme interactions with the digesting strand have been disrupted in the modified substrate. Our data with substrate N7 (single modification) also suggest a "footprint" for the enzyme, as the vinylphosphonate internucleotide linkage between nucleotides 4 and 5 from the 3′-end of the digesting strand seems to induce P−O5′ cleavage of the terminal nucleotide. Again, another speculative suggestion is that the modified linkage at this particular position ahead of the translocating nuclease determines the specificity of cleavage in the active site.

In the absence of a structure of exonuclease III complexed to duplex DNA, we can only speculate about the molecular details involved in the induction of the 5′-phosphomonoesterase activity. However, it is clear that the modified duplex ahead of the active site alters the specificity of the cleavage in the active site. Major alterations of the duplex conformation in the modified region can be ruled out, as previous NMR analysis showed that the modified duplex is virtually identical to the normal unmodified duplex (*6*). The suggestion by Tainer and co-workers (*9*) that a DNA conformational change may be needed for the DNA to fit both onto the protruding α-helix and into the active site is very interesting, and such a conformational change is likely to require rotational freedom of the DNA backbone. It is feasible that the DNA−backbone rotational restrictions, encountered by the enzyme on the modified substrate, have prevented the correct positioning of the P−O3′ bond in the active site. Such mispositioning may have profound consequences on the pentacovalent transition state, and now instead of the Asp151 protonating exclusively the O3′ leaving group with a resulting inversion of configuration, it can also protonate the O5′ leaving group with a resulting P−O5′ cleavage (Figure 6B). The final result is a mixture of oligonucleotide products with either 3′- or 5′-phosphate groups. This may also explain the partial inhibition of the nuclease activity. Although at this stage we cannot rule out directly the disruption of potentially important protein−DNA contacts, an inhibitory mechanism based upon the restriction of DNA backbone rotational freedom is novel and distinctly different from that proposed for modifications involving 2′-*O*-alkyl substituents at the 2′-position of the deoxyribose sugar. The degree of resistance to nuclease degradation is directly related to the length of the alkyl chain (*2*, *16*). For one such modification, the 2′-*O*-aminopropyl substituent, it has also been suggested that it interferes with one of the two metal ion binding sites of the 3′−5′ exonuclease domain of the Klenow DNA polymerase (*17*).

This promiscuity of the cleavage reaction in the active site may be advantageous for a DNA repair nuclease like exonuclease III. It is desirable for a repair enzyme to be able to recognize a variety of alterations in the DNA duplex but at the same time to be able to cope with spatial alterations in the active site, thus avoiding inhibition of the cleavage reaction. Such promiscuity will enable exonuclease III to recognize and deal with a variety of defects in the DNA duplex. It remains to be determined whether other DNA duplex modifications also alter the cleavage specificity of exonuclease III and other repair nucleases.

Effect on DNA Polymerase I. The molecular details that underpin the inhibitory effect of vinylphosphonate internucleotide linkages on DNA polymerase activity also seem to involve restriction of DNA backbone flexibility. Structural information suggests that the DNA duplex approaches the polymerase active site from the side of the 3′−5′ exonuclease domain and that significant bending of the duplex must be accommodated to allow for correct positioning of the DNA in the polymerase active site (refs *18* and *19* and Figure 6C). However, initial structural evidence for the human DNA polymerase β suggested that the DNA adopts an orientation opposite of that observed for the Klenow and other DNA polymerases (*20*, *21*). This controversy about the correct orientation of the duplex DNA now appears to have been solved by a new structural alignment scheme that suggests a common DNA binding mode applicable to all polymerases (*22*). It remains to be determined whether our vinylphosphonate internucleotide linkages will also have an inhibitory effect on other DNA polymerases. It is clear, however, that in DNA polymerases complexed with primed DNA, the duplex appears to deviate from the normal B-DNA structure. In the HIV-1 reverse transcriptase DNA complex, five or six base pairs proximal to the primer terminus have an A-DNA-like geometry and the DNA is bent significantly by 40−45° (*23*), whereas in the DNA polymerase β DNA complex, two or three bases assume such a conformation (*20*, *21*). In the latter case, the crystal structure of the complex with a gapped DNA substrate revealed a kink in the active site at the 5′-phosphodiester link of the template residue which base pairs with the incoming dNTP (*21*). This kink is formed by rotating about the P−O5′ and P−O3′ bonds by approximately 180° each from their normal B-DNA torsion angles. Such rotations will clearly be problematic in our modified DNA substrates. The B- to A-DNA conversion is functionally significant as it was shown in the case of the *B. stearothermophilus* DNA polymerase I, which retained its catalytic activity within the crystal with the B- to A-DNA switch at the polymerase active site (*24*). Although we lack detailed structural information, it is likely that our modifications somehow restrict these DNA conformational changes in the active site of DNA polymerase I. A single modification ahead of the polymerase is not sufficient to inhibit its progression, presumably because the rotational restrictions imposed by a single vinylphosphonate internucleotide linkage do not impose a significant energetic barrier to the polymerase enzyme.

The importance of DNA backbone flexibility has not been examined in detail compared to other mechanistic features of the DNA polymerization reaction such as hydrogen bonding, base stacking, steric effects, active site tightness, and substrate fit (*25*, *26*). The significance of DNA flexibility on protein−DNA interactions is becoming clear with the use of methylphosphonate modifications that neutralize the phosphate backbone and significantly increase local flexibility (*27*, *28*). Such an increase in flexibility has been suggested to contribute to the energetics of protein−DNA binding by allowing the DNA a greater degree of bending and a wider range of structural conformations without additional energetic penalty (*29*). Vinylphosphonate internucleotide linkages offer us an additional useful tool in studying the importance of restricting DNA backbone flexibility. They reduce DNA "plasticity" without offering steric hindrance. It remains to be established whether other DNA metabolizing enzymes are also affected by this unusual modification.

ACKNOWLEDGMENT

We express our gratitude to John Keyte for assistance in the synthesis of the oligonucleotides used in this study.

REFERENCES

1. Crooke, S. T., and Crooke, S. T., Eds. (1998) *Handbook of Experimental Pharmacology: Antisense Research and Applications*, Vol. 131, pp 1−50, Springer, Heidelberg, Germany.
2. Freier, S. M., and Altmann, K. H. (1997) The ups and downs of nucleic acid duplex stability: structure−stability studies on chemically-modified DNA:RNA duplexes, *Nucleic Acids Res. 25*, 4429−4443.
3. Raney, K. D., Hamilton, S., and Corey, D. R. (1998) in *Peptide Nucleic Acids* (Nielsen, P. E., and Egholm, M., Eds.) Horizon Scientific Press, Wymondham, U.K.
4. Loakes, D. (2001) Survey and summary: the applications of universal DNA base analogues, *Nucleic Acids Res. 29*, 2437−2447.
5. Abbas, S., Bertram, R. D., and Hayes, C. J. (2001) Commercially available 5′-DMT phosphoramidites as reagents for the synthesis of vinylphosphonate-linked oligonucleic acids, *Org. Lett. 3*, 3365−3367.
6. Bertram, R. D., Hayes, C. J., and Soultanas, P. (2002) Vinylphosphonate internucleotide linkages inhibit the activity of PcrA DNA helicase, *Biochemistry 41*, 7725−7731.
7. Velankar, S. S., Soultanas, P., Dillingham, M. S., Subramanya, H. S., and Wigley, D. B. (1999) Crystal structures of complexes of PcrA DNA helicase with a DNA substrate indicate an inchworm mechanism, *Cell 97*, 75.
8. Soultanas, P., and Wigley, D. B. (2001) Unwinding the "Gordian knot" of helicase action, *Trends Biochem. Sci. 26*, 47−54.
9. Mol, C. D., Kuo, C.-F., Thayer, M. M., Cunningham, R. P., and Tainer, J. A. (1995) Structure and function of the multifunctional DNA-repair enzyme exonuclease III, *Nature 374*, 381−386.
10. Wang, L. K., Lima, C. D., and Shuman, S. (2002) Structure and mechanism of T4 polynucleotide kinase: An RNA repair enzyme, *EMBO J. 21*, 3873−3880.
11. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Plainview, NY.
12. Rogers, G. S., and Weiss, B. (1980) Exonuclease III of *Escherichia coli* K-12, an AP endonuclease, *Methods Enzymol. 65*, 201−211.
13. Henikoff, S. (1984) Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing, *Gene 28*, 351−359.
14. Putney, S., Benkovic, S., and Schimmel, P. (1981) A DNA fragment with an α-phosphorothioate nucleotide at one end is asymmetrically blocked from digestion by exonuclease III and can be replicated *in vivo*, *Proc. Natl. Acad. Sci. U.S.A. 78*, 7350−7354.
15. Demple, B., and Harrison, L. A. (1994) Repair of oxidative damage to DNA: enzymology and biology, *Annu. Rev. Biochem. 63*, 915−948.
16. Cummins, L. L., Owens, S. R., Risen, L. M., Lesnik, E. A., Freier, S. M., McGee, D., Guinosso, C. J., and Cook, P. D. (1995) Characterization of fully 2′-modified oligoribonucleotide hetero- and homoduplex hybridization and nuclease sensitivity, *Nucleic Acids Res. 23*, 2019−2024.
17. Teplova, M., Wallace, S. T., Tereshko, V., Minasov, G., Symons, A. M., Cook, P. D., Manoharan, M., and Egli, M. (1999) Structural origins of the exonuclease resistance of a zwitterionic, *Proc. Natl. Acad. Sci. U.S.A. 96*, 14240−14245.
18. Beese, L. S., Derbyshire, V., and Steitz, T. A. (1993) Structure of DNA polymerase I Klenow fragment bound to duplex DNA, *Science 260*, 352−355.
19. Yadav, P. N., Modak, M. J., and Yadav J. S. (1994) Identification and analysis of a template-primer (ds-DNA) binding cleft in *E. coli* DNA polymerase I: an electrostatic potential contour pattern of the modelled structure, *J. Mol. Recognit. 3*, 207−209.
20. Pelletier, H., Sawaya, M. R., Kumar, A., Wilson, S. H., and Kraut, J. (1994) Structures of ternary complexes of rat DNA polymerase beta, a DNA template-primer, and ddCTP, *Science 264*, 1891−1903.
21. Sawaya, M. R., Prasad, R., Wilson, S. H., Kraut, J., and Pelletier, H. (1997) Crystal structures of human DNA polymerase β complexed with gapped and nicked DNA: evidence for an induced fit mechanism, *Biochemistry 36*, 11205−11215.
22. Singh, K., and Modak, M. J. (1998) A unified DNA- and dNTP-binding mode for DNA polymerases, *Trends Biochem. Sci. 23*, 277−281.
23. Jacobo-Molina, A., Ding, J., Nanni, R. G., Clark, A. D., Lu, X., Tantillo, C., Williams, R. L., Kamer, G., Ferris, A. L., Clark, P., Hizi, A., Hughes, S. H., and Arnold, E. (1993) Crystal structure of human immunodeficiency virus type 1 reverse transcriptase complexed with double-stranded DNA at 3.0 Å resolution shows bent DNA, *Proc. Natl. Acad. Sci. U.S.A. 90*, 6320−6324.
24. Kiefer, J. R., Mao, C., Braman, J. C., and Beese, L. S. (1998) Visualizing DNA replication in a catalytically active *Bacillus* DNA polymerase crystal, *Nature 391*, 304−307.
25. Kool, E. T. (2001) Hydrogen bonding, base stacking, and steric effects in DNA replication, *Annu. Rev. Biophs. Biomol. Struct. 30*, 1−22.
26. Kool, E. T. (2002) Active site tightness and substrate fit in DNA replication, *Annu. Rev. Biochem. 71*, 191−219.
27. Strauss-Soukup, J. K., Vaghefi, M. M., Hogrefe, R. I., and Maher, L. J., III (1997) Effects of neutralization pattern and stereochemistry on DNA bending by methylphosphonate substitutions, *Biochemistry 36*, 8692−8698.
28. Strauss-Soukup, J. K., Rodrigues, P. D., and Maher, L. J., III (1998) Effect of base composition on DNA bending by phosphate neutralization, *Biophys. Chem. 72*, 297−306.
29. Okonogi, T. M., Alley, S. C., Harwood, E. A., Hopkins, P. B., and Robinson, B. H. (2002) Phosphate backbone neutralization increases duplex DNA flexibility: A model for protein binding, *Proc. Natl. Acad. Sci. U.S.A. 99*, 4156−4160.

BI026985+