Stimulation of DNA Strand Slippage Synthesis by a Bulge Binding Synthetic Agent†

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ABSTRACT: It has been postulated that bulged structures may be intermediates in the DNA strand slippage synthesis associated with the expansion of nucleotide repeats in various neurodegenerative diseases and cancer. To probe the possible role of bulged structures in this process, we have synthesized a wedge-shaped spirocyclic molecule, DDI (double-decker intercalator), on the basis of our earlier work with the bulge-specific derivative prepared from the enediyne antitumor antibiotic neocarzinostatin chromophore. Using a series of primers/templates containing nucleotide repeats [$(AAT)_3/(ATT)_5$, $(ATT)_3/(AAT)_5$, $(CAG)_3/(CTG)_5$, $(CA)_4C/(GT)_7G$, $(GT)_4G/(CA)_7C$, T_9/A_{30}, T_{20}/A_{30}] with the Klenow fragment of *Escherichia coli* DNA polymerase I, we find that DDI markedly enhances the formation of long DNA products, whose synthesis would require strand slippage to occur. DDI-induced slippage synthesis is more pronounced as the incubation proceeds and at limiting enzyme levels. The gel band pattern of the synthesized DNA products reflects the particular nucleotide repeat unit and is not altered by DDI. The lack of any drug effect on primer extension on M13 DNA and heteropolymeric 62-mer templates, where strand slippage is much less likely to occur, suggests that stimulation of slippage synthesis by DDI is not due to a direct effect on the enzyme. By contrast, other DNA-binding agents, such as ethidium bromide, distamycin, and doxorubicin, inhibit the formation of slippage-induced DNA products, but this block can be overcome by DDI, presumably by its destabilizing duplex DNA-binding sites for these other agents. We propose that DDI binds to or induces the formation of a bulge or related structure, which promotes DNA strand slippage and its consequent expansion of nucleotide repeats during replication by DNA polymerase I and that this action provides insight into the development of agents that interfere with nucleotide expansions found in various disease states.

Bulged structures have been identified in nucleic acids and have been shown to be of functional biological significance (*1*, *2*). They have been proposed as intermediates in a number of biological processes, including RNA splicing, frame-shift mutagenesis, intercalator-induced mutagenesis, and binding motifs for regulatory proteins in viral replication (e.g., the TAR region of HIV), as targets for repair enzymes in imperfect homologous recombination, as essential elements in naturally occurring antisense RNAs, and as elements in ribosomal protein synthesis (*3*). Further, bulged structures have been implicated as intermediates in the slipped synthesis of DNA associated with the unstable expansion of triplet repeats in at least 12 human neurodegenerative diseases, such as Huntington's disease, Friederich's ataxia, and fragile X syndrome, as well as in nucleotide expansions found in certain human cancers (*4*−*8*). On the basis of entropic considerations, simple bulges (one or more unpaired bases) rather than hairpin structures appear to be favored in the slipped structure (*6*), although it remains unclear precisely what factors cause DNA sequences to slip during replication. Slippage was first observed in bacterial systems involving RNA polymerase (*9*) and DNA polymerase (*10*) with templates containing nucleotide repeats. Recently, using newer techniques of analysis there has been a renewed interest in the study of simple systems capable of nucleotide expansions as models for the diseased states.

We have recently described the synthesis of small molecules capable of preferential binding to DNA bulge sites, with the expectation that such molecules might be used to study the role of bulged structures in nucleic acid function (*11*, *12*). This result was the outcome of our earlier studies with the enediyne antitumor antibiotic neocarzinostatin (NCS-chrom) (*13*). It was shown that in the absence of the usual thiol activation NCS-chrom undergoes a general base catalyzed, intramolecular activation to generate a biradical species that selectively cleaves nucleic acids at bulge sites preferably of two to three unpaired bases (*14*−*16*). In the absence of a bulged DNA substrate a wedge-shaped spirocyclic molecule (Figure 1A) is generated that resembles the bulge cleaving species and binds to bulged DNA with great specificity (*17*, *18*). Binding of this compound to the bulge site in DNA was found by NMR analysis to involve major groove recognition by its amino sugar moiety and tight fitting

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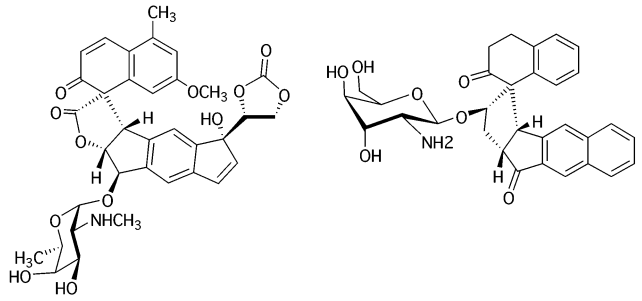

(A) NCS-chrom derivative (B) DDI

FIGURE 1: Structures of the natural and synthetic spirocyclic compounds. (A) DNA bulge-specific compound derived from NCS-chrom upon base catalysis. (B) Synthetic compound mimicking (A) and having selectivity for binding to DNA bulge sites (*12*).

of the wedge-shaped molecule in the triangular prism pocket formed by the two looped-out DNA bulge bases and the neighboring base pairs (*19*). Of particular significance, this agent was actually found to induce the formation of the bulge binding pocket in the oligonucleotide under study from a relatively unstructured form, rather than to enter a preformed bulge site (*20*).

DDI (Figure 1B) was designed to mimic the wedge-shaped natural product in having a 35° right-handed twist imposed on the two ring systems comprising the wedge by the spirocyclic junction, along with a 60° transposition of the ring systems. This close resemblance was confirmed by the superimposability of the two aglycon structures, that of the natural product derived from NMR analysis of the bulge-bound compound and the crystal structure of the chemically synthesized DDI (*12*). The synthetic agent is more useful in biochemical studies than the natural product, which possesses a labile spirolactone ring (half-life about 5 h at pH 8.2, room temperature) and has a more stringent (and therefore more restrictive) sequence requirement surrounding the bulge for binding.

Since bulges and hairpins have been postulated as intermediates in DNA slippage synthesis involving templates with nucleotide repeats (*8*), we reasoned that DDI, by virtue of its ability to bind to preformed bulge sites or possibly to induce bulge formation in an otherwise unstructured region of the nascent DNA chain, might influence the expansion process. Our results show that DDI stimulates reiterative synthesis. Possible mechanisms are discussed.

MATERIALS AND METHODS

Materials. Oligodeoxyribonucleotides were purchased from commercial sources. M13mp18 (+) strand DNA and deoxynucleoside triphosphates were purchased from Amersham Pharmacia. The 24-mer primer (−47) for M13 DNA was from New England Bio Labs or from custom synthesis. Radioactive materials were from New England Nuclear. T4 polynucleotide kinase, the Klenow fragment of *Escherichia coli* DNA polymerase I, and the Klenow fragment lacking the 3′ to 5′ exonuclease activity were purchased from New England Biolabs. Ethidium bromide, distamycin, and doxorubicin were obtained from Sigma and were dissolved in H_2O. The primers were 5′-^{32}P-end-labeled using [γ-^{32}P]ATP and polynucleotide kinase, and the labeled oligomers were purified by electrophoresis on a 15% sequencing gel by standard procedures (*21*). The product was recovered from the gel eluate using a desalting Sephadex column (Pharmacia). Synthesis and characterization of DDI have been reported elsewhere (*12*). It was purified by HPLC using a reverse-phase C18 column and a methanol/H_2O gradient. The peak fractions containing the product were dried in a Speed Vac concentrator, and the pellet was dissolved in 50% dimethyl sulfoxide. The concentration of DDI was determined using the extinction coefficient ($\epsilon_{253} = 4.12 \times 10^4$ M^{-1} cm^{-1}).

Table 1: Oligonucleotides and DNA Used as Primer/Templates in Slippage Synthesis Studies[a]

$(AAT)_3/(ATT)_5$	T_9/A_{30}
$(ATT)_3/(AAT)_5$	T_{20}/A_{30}
$(CAG)_3/(CTG)_5$	24-mer primer (−47) for M13 DNA
$(CA)_4C/(GT)_7G$	M13mp18 (+) strand DNA
$(GT)_4G/(CA)_7C$	

[a] The subscripts following the parentheses denote the number of times the triplet, doublet, or monucleotide is repeated in the oligomer.

DNA Polymerase Assays. The primer/templates used in this study are listed in Table 1. A standard reaction (15−25 μL) contained 50 mM Tris-HCl, pH 7.5, 15 mM $MgCl_2$, 4 mM dithiothreitol, 4 μM each of the primer and template, and 2−4 mM each of the deoxynucleoside triphosphates and the Klenow fragment of DNA polymerase I. The DNA was severalfold in molar excess of the enzyme, with a DNA to enzyme ratio of 37−67. Unless otherwise indicated, the enzyme was at a level of 0.07−0.13 unit (0.05−0.095 pmol) per microliter of the reaction. Similar results were obtained with Klenow fragment proficient or deficient in its 3′ to 5′ exonuclease activity. A mixture of 5′-^{32}P-end-labeled primer and unlabeled template, generally in equimolar concentrations, was annealed by heating in Tris-HCl, pH 7.5, and $MgCl_2$ to 95 °C followed by slow cooling to room temperature. The concentrations of the components at the annealing stage were 30−50% higher than those in the final assay to accommodate the dilution resulting from the addition of the rest of the components in the subsequent stage. Following addition of dithiothreitol and deoxynucleoside triphosphates to the annealed mixture, it was distributed for assays. The compound to be tested was added as a solution in 50% dimethyl sulfoxide. Controls lacking the compound received an equal volume of 50% dimethyl sulfoxide, the final concentration of which was 2% in the assay. The reaction was started by the addition of the enzyme. The incubation was at room temperature (23 °C) or at 37 °C for the times indicated in the figure legends. The reaction was terminated by the addition of EDTA to a final concentration of 50 mM.

In primer extension assays using M13 single-stranded DNA as template the 24-mer primer and the template (16 nM each) were annealed as described for oligonucleotides. The reaction contained 4 mM dithiothreitol, 0.5 mM each of all four deoxynucleoside triphosphates, and the enzyme. In cases where [α-^{32}P]dTTP (0.3 μCi/μL) was included in the reaction, the concentration of unlabeled dTTP was reduced by half.

Product Analyses. Portions of the reaction mixtures were dried, and the pellets were dissolved in 80% formamide containing 1 mM EDTA and marker dyes for analysis on a 15% polyacrylamide sequencing gel. The gels were exposed to X-ray film, and the band intensities were quantitated on

a Phosphor Imager (Molecular Dynamics). Agarose gel (0.6−0.7%) electrophoresis of the products was done by standard procedures (*21*).

The polymerase reaction products were also quantitated by measuring the incorporation of acid-precipitable [^{32}P]-dTMP radioactivity from [α-^{32}P]dTTP. At termination of the incubation, 5−10 μL of the reaction mixture was mixed with 100 μL of a stop solution containing 200 μg/mL salmon sperm DNA and 20 mM EDTA followed by 3 mL of 10% trichloroacetic acid. After being chilled in ice for 15 min, DNA was collected by filtration on GF/C filters and washed with 10% trichloroacetic acid, followed by 95% ethanol. The radioactivity on the filters was determined by liquid scintillation counting.

RESULTS AND DISCUSSION

Effect of DDI on a Triplet Repeat Expansion. Expansion of a variety of simple sequence repeats using short complementary oligomers and DNA polymerases has been recently reported by several groups (*22*−*24*). Schlotterer and Tautz, for example, demonstrated the generation of long DNA products in reactions containing 9-mer/15-mer combinations of di- or trinucleotide motif repeats (*22*). The efficiency of reiterative synthesis depended on several factors including the length of the repetitive unit, its sequence, and the characteristics of the enzyme. In vitro studies on the expansion of triplet repeats such as CAG, CGG, and GAA, which are associated with human hereditary disease genes, have helped in understanding the possible mechanism of slippage and the molecular basis of the diseases (*8*, *25*−*27*).

The in vitro expansion assays provide a convenient method to test compounds as potential probes to study the mechanism of DNA slippage itself. We expected that agents that enhance bulge formation or its stability should facilitate slippage. We, therefore, tested the synthetic double-decker intercalator (DDI, Figure 1B) for its effect on the expansion of several triplet, doublet, and monucleotide repeats (Table 1) catalyzed by the Klenow fragment of DNA polymerase I. In Figure 2A is shown the extension of the AAT triplet repeat and the resolution of the products on a denaturing polyacrylamide gel. The reaction contained 5′-^{32}P-end-labeled 9-mer primer $(AAT)_3$ that had been annealed with unlabeled $(ATT)_5$, dATP, dTTP, and the Klenow fragment. In the control reaction the 9-mer was extended to a length ranging from 30 to about 62 nucleotides (lanes 1 and 3), the size of which is more than the combined length of the primer and the template and thus indicative that slippage occurred during synthesis. In the presence of DDI the slippage synthesis was enhanced to the extent that over 90% of the radioactivity is in DNA longer than 62 nucleotides (lanes 2 and 4).

Experiments similar to those in Figure 2A were performed for the expansion of the complementary ATT triplet using the 5′-^{32}P-end-labeled $(ATT)_3$ and $(AAT)_5$ template. The overall band patterns in the controls (Figure 2B, lanes 5 and 7) are similar to those obtained for AAT expansion. Again, DDI stimulated the synthesis to generate products much longer than those in the controls (lanes 6 and 8). It should also be noted that in the $(AAT)_3/(ATT)_5$ system double bands are observed (Figure 2A, lanes 1−4), whereas with $(ATT)_3/(AAT)_5$ the band spacing appears to reflect the triplet repeat unit (Figure 2B, lanes 5−8). DDI does not influence this pattern.

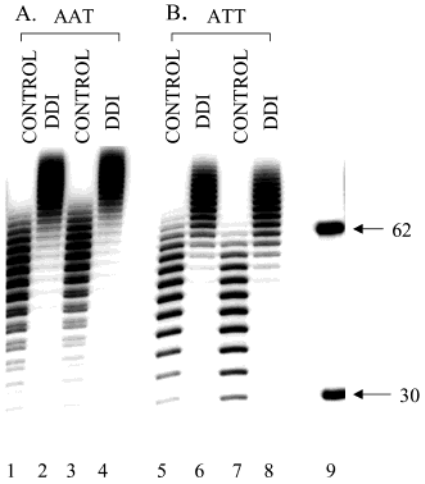

FIGURE 2: Expansion of the trinucleotide repeat AAT or ATT and effect of DDI. (A) A standard reaction (23 °C, 7 h) containing 5′-^{32}P-end-labeled $(AAT)_3$ and unlabeled template $(ATT)_5$ was catalyzed by the Klenow fragment. (B) The reaction conditions were the same as in (A) except for the use of 5′-^{32}P-end-labeled $(ATT)_3$ and unlabeled $(AAT)_5$. In both (A) and (B) the DDI concentration was 98 μM. The products were resolved on a 15% sequencing gel. The arrows in this and the succeeding figures point to the size markers of 30 and 62 nucleotides in length.

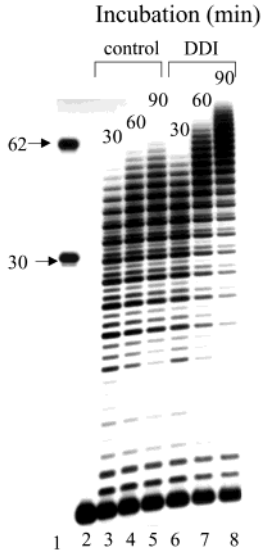

FIGURE 3: Time course of expansion of the trinucleotide repeat AAT and stimulation of synthesis by DDI (98 μM). Standard reactions similar to those in Figure 2A were carried out at 37 °C. At the times indicated samples were withdrawn for gel analysis. Lane 2 has the 9-mer primer.

In experiments similar to those described above but using $(CAG)_3/(CTG)_5$, quantitation of the gel bands showed that in the presence of DDI the products longer than 30 nucleotides more than doubled in comparison with those in the control.

Time Course of AAT Expansion. A time course of extension of the AAT triplet repeat was done (Figure 3) in assays similar to those in Figure 2, except that the incubation was at 37 °C. In the control there is a time-dependent increase in products in the region of 30−62 nucleotides (lanes 3−5). In the presence of DDI the radioactivity in the bands corresponding to the long fragments steadily increased with time concomitant with a decrease in products shorter than 30 nucleotides in length (lanes 6−8). This shows that DDI

Table 2: Dose Response for DDI in Slippage Synthesis[a]

DDI (μM)	fragments longer than 62-mer (%)
0	1.3
24	13
48	48.7
71	64.0
96	82.5

[a] Data come from experiments similar to those described in Figure 2 using ^{32}P-labeled $(AAT)_3$ and $(ATT)_5$ except for the substitution of Klenow fragment with its exonuclease-deficient counterpart. After gel analysis of the products the band intensitites were quantitated on a Phosphor Imager.

has stimulated slippage synthesis, and it is most pronounced at 90 min by which time, unlike in the control, a significant amount of the radioactivity is in DNA longer than the 62-mer (lane 8). Again, the double band pattern is apparent on the gel, but a third very faint band can also be observed where the double bands are more intense. Interestingly, there is a hierarchy of intensities of the three bands, each apparently separated by one nucleotide, which is faithfully repeated every three nucleotides throughout the lane. The size of the slippage synthesis products depends on several factors including reaction temperature, duration of the incubation, and the amount of enzyme. At 37 °C, but not at 23 °C, a fraction of the primer remained without extension, most likely as a result of its dissociation from the template. Low temperature may also favor tighter binding of the test compound as determined by the stability properties of the DNA structure to which it binds.

DDI Dose Effect on Slippage Synthesis. The concentration of DDI was varied in an AAT triplet expansion assay similar to that in Figure 2, except for the use of the Klenow fragment lacking 3′ to 5′ exonuclease activity. After resolution of the products on the gel, the total radioactivity in each lane and that in the region of DNA longer than 62 nucleotides (a length, arbitrarily chosen) were quantitated (Table 2). The data show a DDI dose-dependent increase in the length of the products, and at the highest level used (96 μM) over 80% of the radioactivity is present in DNA longer than the 62-mer, whereas in the control only 1.3% is this size. Further, the finding that DDI stimulates slippage synthesis catalyzed by the Klenow fragment, proficient or deficient in 3′ to 5′ exonuclease activity, rules out the possibility that the DDI enhancement of slippage synthesis is related to the exonuclease activity of the enzyme.

Effect of DDI on the Expansion of a Doublet Motif. The efficiency of slippage synthesis depends on the sequence and the length of the repeated motif (*22*, *23*), whereas DDI, by design, is expected to bind to DNA bulges relatively independently of their sequence (*12*). In Figure 4 is shown the expansion of the doublet CA in reactions containing the primer $(CA)_4C$ and the template $(GT)_7G$, supplemented with all four deoxynucleoside triphosphates and the Klenow fragment of DNA polymerase I. In the control reaction (lanes 2−4) there is a time-dependent increase in products longer than 30 nucleotides. As in the case of triplet expansion DDI enhanced the synthesis to give rise to products significantly longer than those in the respective controls (lanes 5−7). The bands are in a doublet pattern with alternating strong and weak bands; this pattern was not altered by DDI. The

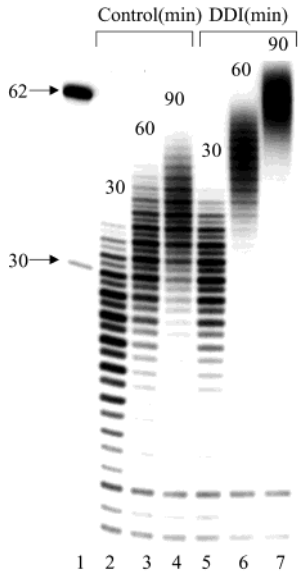

FIGURE 4: Time course of expansion of the dinucleotide repeat CA and effect of DDI (98 μM). A standard reaction (37 °C) containing 5′-^{32}P-end-labeled $(CA)_4C$ and $(GT)_7G$ and all four deoxynucleoside triphosphates was catalyzed by the Klenow fragment. At the times indicated samples were withdrawn for gel analysis.

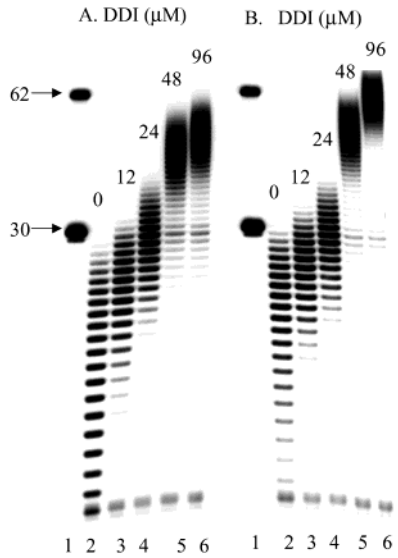

FIGURE 5: Effect of DDI concentration on a mononucleotide repeat expansion. Standard reactions containing the 5′-^{32}P-end-labeled T_9 and A_{30} template, dATP, dTTP, and the Klenow fragment were carried out in the absence or presence of varying amounts of DDI. Incubation was at 23 °C for 6 h in (A) and for 10.5 h in (B). The number on top of each lane indicates the concentration of DDI (0−98 μM).

expansion of the complementary doublet GT was also stimulated by DDI in experiments using the primer $(GT)_4G$ annealed to the template $(CA)_7C$ (data not shown).

Effect of DDI on a Mononucleotide Repeat Expansion. Expansion of the T residue was studied using T_9 as the primer with an A_{30} template in a reaction containing dATP, dTTP, and the Klenow fragment under conditions where slippage will be minimal (limiting enzyme and 23 °C). In a 6 h incubation (Figure 5A) control synthesis produced a series of discrete bands, each differing in lengh by one nucleotide and all shorter than 30 nucleotides (lane 2). Addition of increasing amounts of DDI caused a progressive increase in

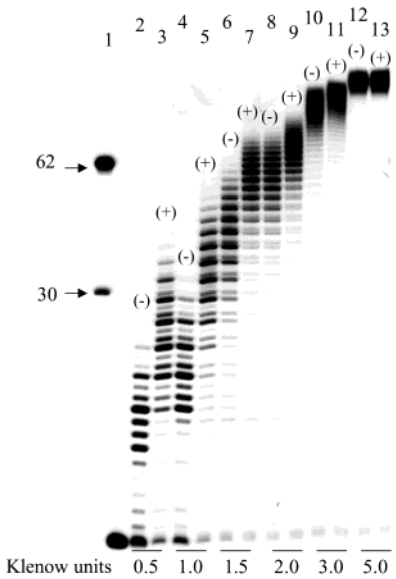

FIGURE 6: Effect of varying enzyme levels on DDI stimulation of a triplet repeat expansion. In assays similar to that in Figure 2A, using ^{32}P-labeled $(AAT)_3$ and $(ATT)_5$, the amount of Klenow fragment was varied from 0.5 to 5 units (per 15 μL assay). Incubation was at 23 °C for 6 h. In each set (−) refers to controls lacking DDI and (+) indicates the presence of DDI. Lane 2 has the 9-mer primer.

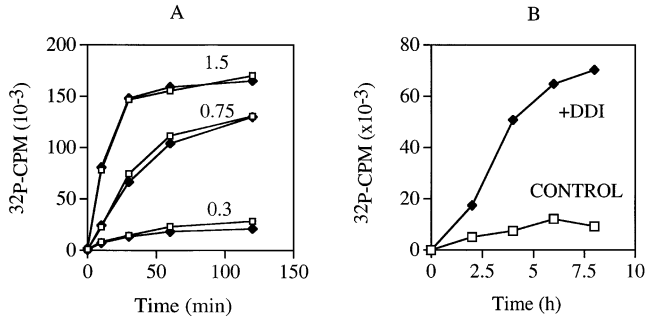

FIGURE 7: Effect of DDI on primer extension using M13 DNA or A_{30} as templates. (A) A time course of extension of a 24-mer primer on M13 single-stranded DNA at three levels of the Klenow fragment (units as shown in the figure) was carried out at 37 °C in the absence and presence of DDI (112 μM) as described in Materials and Methods. [^{32}P]dTMP incorporation from [α-^{32}P]dTTP into acid-precipitable products was determined at the indicated time intervals. In each set of curves the lines with open squares indicate ^{32}P incorporation in the control and the lines with closed squares are for that in the presence of DDI. (B) A time course of expansion of T_{20} on the A_{30} template by the Klenow fragment was carried out at 37 °C in the absence and presence of DDI (112 μM). [^{32}P]dTMP incorporation from [α-^{32}P]dTTP into acid-precipitable products was determined at the indicated time intervals. The lines with open squares indicate ^{32}P incorporation in the control, and the lines with closed squares are for that in the presence of DDI.

the length of the products at the expense of the short oligomers (lanes 3−6). A similar band pattern was obtained in the 10.5 h incubation (Figure 5B), except that the products are somewhat longer than those in the 6 h reaction. Significant stimulation of product length is found at the lowest concentration (12 μM) of DDI. Again, these data resemble those in Table 2 with the AAT triplet in which exonuclease-deficient Klenow enzyme was used.

Effect of Varying Enzyme Levels on DDI-Induced Stimulation of Slippage Synthesis. In determining the optimal conditions for DDI-induced enhancement of DNA product length, it was found that the stimulation of slippage synthesis was maximal with a limiting amount of enzyme (data not shown). To explore the relationship between DDI stimulation and the enzyme concentration in more detail, we varied the amount of enzyme in an AAT expansion assay similar to that in Figure 2. Each pair of lanes in Figure 6 represents reactions in the absence (−) or presence (+) of DDI at a particular enzyme concentration. It can be seen that the DDI-dependent enhancement of synthesis is most pronounced at 0.5−1.5 units of enzyme (lanes 2−9). As the enzyme level is increased, slippage synthesis in the control itself becomes very efficient and the DDI stimulation is relatively decreased (lanes 10 and 11). Again, the repeated three-band pattern found in Figure 3 is seen. The resolution of the products with 5 units of enzyme is not good enough to assess the length difference, if any, between the control and the DDI-containing sample (lanes 12 and 13).

Effect of DDI on DNA Synthesis Using M13 Single-Stranded DNA and a Heteropolymeric 62-mer as Templates. The finding that the DDI-dependent increase in slippage synthesis is maximal with limiting amounts of enzyme raises the possibility that the DDI interacts directly with the enzyme so as to enhance its efficiency in some way. To address this question, we used an assay involving the elongation of a 24-mer primer on single-stranded M13 DNA. In this system, which is devoid of slippage, chain elongation by an enzyme of low processivity will result in the accumulation of intermediates instead of full-length molecules of about 7000 nucleotides. A time course of DNA synthesis on the M13 template, as measured by the incorporation of ^{32}P radioactivity from [α-^{32}P]dTTP into acid-precipitable products at three levels of Klenow fragment, is shown in Figure 7A. An increase in enzyme efficiency is expected to enhance the rate of synthesis and hence the incorporation of ^{32}P radioactivity. In the control reactions the rate and extent of synthesis increased with increasing amounts (0.3−1.5 units) of enzyme. In the presence of DDI ^{32}P incorporation was virtually the same as that in the respective controls at all three enzyme levels. If DDI stimulated the enzyme, one would expect an enhanced synthesis, especially at the low enzyme levels (0.3 and 0.75 unit). The products obtained from chain extension of 5′-^{32}P-end-labeled 24-mer primer on M13 DNA were also analyzed on an agarose gel. Autoradiography of the gel showed that the stalled intermediates in the control were about 3000−4000 nucleotides in length, and the presence of DDI did not significantly alter the profile (data not shown). Since DDI showed no stimulation of chain elongation with the M13 template, it is highly unlikely that it is directly affecting the efficiency of the nucleotide addition process itself. The same conclusion was reached from an experiment using an assay involving the extension of a 5′-^{32}P-end-labeled 9-mer primer on a linear 62-mer template of heterogeneous composition and devoid of sequence repeats, in which the only product was a full-length 62-mer in the presence and absence of DDI (data not shown). Nevertheless, these studies do not eliminate the possibility that the drug directly affects the ability of the polymerase to facilitate the strand sliding process, independent of its interacting with DNA.

In contrast to the results obtained with the M13 and the 62-mer heteropolymer templates, slippage synthesis as measured by [^{32}P]dTMP incorporation in a reaction containing a T_{20} primer, an A_{30} template, and [α-^{32}P]dTTP was

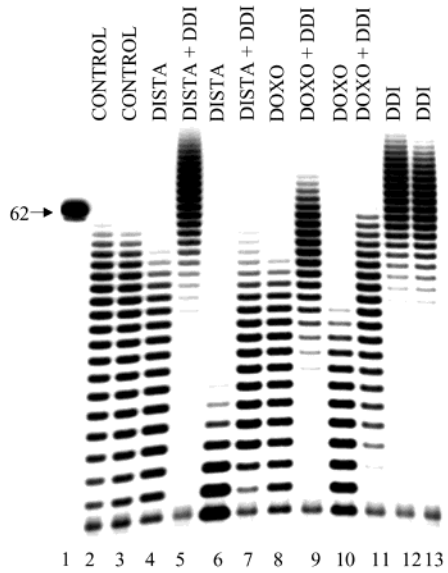

FIGURE 8: Comparison of the effect of different DNA-binding agents and DDI on DNA slippage synthesis. Assays for T_9 expansion on the A_{30} template (23 °C, 6 h) were carried out as described in Figure 4 with and without the addition of the indicated compounds. DISTA refers to distamycin, which is present at 4 μM in lanes 4 and 5 and at 8 μM in lanes 6 and 7. Doxo indicates doxorubicin, which in lanes 8 and 9 is at 20 μM and in lanes 10 and 11 at 40 μM. DDI concentration was 112 μM.

stimulated by DDI, as shown in a time course of the synthesis (Figure 7B). The stimulation was 4−6-fold in different assays. Taken together, the results in Figure 7 suggest that the stimulation of slippage synthesis by DDI is not due to its enhancement of the polymerase activity itself but rather is a consequence of its binding to some transient DNA structures such as bulges or hairpins.

Effect of Other DNA-Binding Agents on Slippage Synthesis. We compared three DNA-binding compounds with diverse structural features (*28*) with DDI for their effect on slippage synthesis. Ethidium bromide, a well-known simple DNA intercalator, inhibited AAT triplet expansion on gels (data not shown). The effect of two other DNA binders on T_9 expansion is shown in Figure 8. Distamycin, a polyamide antibiotic that binds in the minor groove of duplex DNA preferentially at AT-rich regions (*29*−*31*), inhibited slippage synthesis (lanes 4 and 6). When distamycin and DDI were present from the start of the reaction, DDI caused a reversal of the inhibiton so as to restore the synthesis almost to the control level (lane 7) or stimulated it to the extent obtained by itself (lane 5). Similarly, doxorubicin, an anthracycline glycoside that intercalates between DNA base pairs (*28*), inhibited T expansion (lanes 8 and 10). When both DDI and doxorubicin were present, the inhibition was again reversed or stimulated depending upon the concentration of the latter (lanes 9 and 11). DDI alone, as expected, stimulated the synthesis (lanes 12 and 13).

CONCLUSION

As shown in these and earlier studies (*22*−*24*), given the size of the DNA products made by the DNA polymerase I based system using repeat nucleotide templates, slippage must be involved during synthesis. Further, slippage occurs on both strands of the duplex. This synthesis is markedly enhanced by an agent, DDI, known to bind preferentially to bulges of two to three unpaired bases in DNA, presumably by facilitating the slippage process. Once simple extension of the primer to the original template length is completed, slippage synthesis due to DDI becomes more pronounced as the incubation proceeds. Expansion of the primer $(ATT)_3$ is associated with a band spacing reflecting the triplet unit length, whereas the pattern found with $(AAT)_3$ is more complex, with three bands for each unit repeat with apparent single nucleotide spacing and a consistent hierarchy of intensities. Similarly, repeat doublets of alternating strong and weak bands are found in the CA nucleotide expansion system. Related observations by Schlotterer and Tautz have led to a model of synthesis in which unit nucleotide slippage, as reflected by the repeat unit length of the synthesis products, is rate determining, and the polymerase dissociates from the complex before each new slippage event occurs (*22*). They proposed that the presence of more than a single band is due to polymerase release from the DNA before the last one or two nucleotides are added to fill in the 5′ overhang of the template strand. The consistency of the relative band intensities further suggests that the latter is not a random event. In fact, it may be sequence related and indicate that more than a single triplet repeat is being replicated, e.g., TAT or TTA rather than ATT, which may have a propensity for generating an unstable polymerase−DNA complex before the terminal one or two nucleotides are added. In any case, DDI appears to stimulate product formation, with the expected repeat unit length distribution.

Although there is no direct evidence that DDI binds to a preexisting bulged structure or induces its formation under DNA replication conditions, it seems reasonable to conjecture that the agent might stabilize such a structure or some other unusual DNA structure, resulting in the stimulation of slippage and the resultant expansion of the oligonucleotide repeats. Our finding that the DDI-induced stimulation of reiterative synthesis is best observed at limiting polymerase concentrations suggests that once the enzyme−DNA complex is reestablished, the DDI-binding structure is less accessible. Accordingly, high levels of polymerase would be expected to compete with DDI action. Of course, it is also possible that the polymerase is more directly involved in the slippage process itself.

It may appear that the concentration of DDI required for the observed effect is rather high (somewhat less than 50 μM for half effect in Table 2 and somewhere below 12 μM for beginning stimulation in Figure 5), when compared with the previously observed K_ds in the range of 0.5−5 μM for binding to bulge-containing oligonucleotides (*12*). The latter values, however, were determined under optimal binding conditions at a much lower temperature (5 °C) in order to ensure the stability of the nucleic acid structure. Further, the K_d values rise significantly (about 5-fold) when binding was studied in bulged oligonucleotides lacking a hairpin structure, which was employed to maintain the stability of the bulged structure (*12*). It should also be noted (Figure 1) that while the aglycon structures of the natural product and of DDI are very similar, the amino sugar moieties differ both in structure and in linkage to their respective chromophoric moieties, and these may be the major reasons why the natural product binds to its optimal bulge substrate at least 10-fold better than DDI (*12*). The linkage difference, in particular, likely accounts for our preliminary NMR finding that DDI binds to the bulge

site via the minor groove, unlike the NCS-chrom derivative, which binds through the major groove (G.-S. Hwang, Z. Xi, and I. H. Goldberg, unpublished data). Accordingly, derivatives of the small molecule with the amino sugar attached to the same position on the chromophore as in the natural product are being synthesized.

It appears that, in conjunction with binding to a structure that is involved in DNA slippage, DDI destabilizes the binding of other DNA-interacting agents, such as ethidium bromide, distamycin, and doxorubicin. DDI has a weak affinity for duplex regions in DNA (*12*). It is possible that the duplex regions involved in the binding of these other agents are disrupted by DDI-induced bulge formation and consequent Watson−Crick hydrogen bond breakage associated with strand slippage, leading to their dissociation from the DNA. The results with distamycin are of particular interest, since it has been found that a related agent, DAPI (4′,6-diamidino-2-phenylindole), which preferentially binds to the minor groove of sequences rich in A and T, causes ATT repeats to adopt stable hairpin structures (*32*). Such structures have been proposed as intermediates in slippage synthesis. Our results, however, indicate that distamycin, presumably by its binding to duplex regions at the replication fork, inhibits T repeat expansion and that this effect is overcome by DDI.

Our first goal in designing small molecules that interact with the system for enzymatic expansion of nucleotide repeats has been met, although the precise DNA structure that DDI binds to in this system remains to be identified. Enhancement of expansion by a drug-induced slippage mechanism was the expected result. The next step, however, will be to prepare agents that interact with the same DNA structure as does DDI but interfere with the expansion process, such as that occurring in neurodegenerative diseases and cancer. We expect that by adding functional groups, such as alkylating or cleaving moieties, to a molecule such as DDI we shall have prepared an agent that will covalently interact with the slipped structure and interrupt the expansion process. Further, since expansion of triplets, such as CAG in Huntington's disease, results in expansion of a polyglutamine tract in the "mutant" protein (*33*), interference with triplet expansion or the generation of ineffectual DNA triplet templates for RNA transcription should prevent toxic protein formation.

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