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# Distamycin Paradoxically Stimulates the Copying of Oligo(dA)·Poly(dT) by DNA Polymerases†

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ABSTRACT: Distamycin A, a polypeptide antibiotic, binds to dA·dT-rich regions in the minor groove of B-DNA. By virtue of its nonintercalating binding, distamycin acts as a potent inhibitor of the synthesis of DNA both in vivo and in vitro. Here we report that distamycin paradoxically stimulates *Escherichia coli* DNA polymerase I (pol I), its large (Klenow) fragment, and bacteriophage T4 DNA polymerase to copy oligo(dA)·poly(dT) in vitro. It is found that distamycin increases the maximum velocity ($V_{max}$) of the extension of the oligo(dA) primer by pol I without affecting the Michaelis constant ($K_m$) of the primer. Gel electrophoresis of the extended primer indicates that the antibiotic specifically increases the rate of addition of the first three dAMP residues. Lastly, in the presence of both distamycin and the oligo(dT)-binding protein factor D, which increases the processivity of pol I, a synergistic stimulation of polymerization is attained. Taken together, these results suggest that distamycin stimulates synthesis by increasing the rate of initiation of oligo(dA) extension. The stimulatory effect of distamycin is inversely related to the stability of the primer–template complex. Thus, maximum stimulation is exerted at elevated temperatures and with shorter oligo(dA) primers. That distamycin increases the thermal stability of [$^{32}$P](dA)$_9$·poly(dT) is directly demonstrated by electrophoretic separation of the hybrid from dissociated [$^{32}$P](dA)$_9$ primer. It is proposed that by binding to the short primer–template duplex, distamycin stabilizes the oligo(dA)·poly(dT) complex and, therefore, increases the rate of productive initiations of synthesis at the primer terminus.

Distamycin A, a nonintercalating oligopeptide antibiotic, binds to clusters of dA·dT base pairs within the minor groove of double-stranded B-form DNA [for reviews, see Zimmer (1975) and Zimmer and Wahnert (1986)]. Footprinting analyses with methidiumpropyl-EDTA·Fe(II) (Van Dyke et al., 1982; Van Dyke & Dervan, 1982) and with nuclease (Portugal & Waring, 1987) indicate that a minimum size of four dA·dT base pairs is required for the binding of distamycin to DNA. Upon binding of distamycin or its close relative netropsin to DNA, the inherently curved antibiotic molecules force open the minor groove and bend back the helix axis by 8° (Kopka et al., 1985; Ekambareswara et al., 1988). Further, an increased nuclease susceptibility of regions in the vicinity of the antibiotic binding site suggests that in addition to its direct effect on the structure of the bound base cluster, distamycin introduces structural variations in flanking base stretches (Fox & Waring, 1984).

Distamycin and netropsin are potent inhibitors of the growth of bacteria and viruses as well as of plant and animal cells, both exhibit antimitotic and antitumor activity, and both are cytotoxic [reviewed by Zimmer and Wahnert (1986)]. These effects of the antibiotics are mostly due to the inhibition of RNA and DNA synthesis consequent to their binding to the DNA template. In addition to blocking the in vitro activity of various RNA polymerases (Zimmer et al., 1971; Puschendorf et al., 1974; Muller et al., 1974; Jaros-Kaminska, 1981), distamycin was shown to inhibit the in vitro activity

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of every tested DNA polymerase (Muller et al., 1974; Wahnert et al., 1975; Wilkins, 1982; Grehn et al., 1983). Evidence indicates that it is the DNA template which serves as a target for the inhibitory action of distamycin and that replication of (A + T)-rich regions in DNA is preferentially blocked (Puschendorf & Grunicke, 1969; Muller et al., 1974).

In this paper, we report that in contrast to the inhibition by distamycin of the in vitro synthesis of poly(dA)·poly(dT) and natural DNA templates, this antibiotic paradoxically stimulates the copying of oligo(dA)·poly(dT) by *Escherichia coli* DNA polymerase I and by bacteriophage T4 DNA polymerase. Presented evidence suggests that distamycin exerts this unexpected stimulatory activity by stabilizing the association between the short oligo(dA) primer stem and the poly(dT) template.

## Materials and Methods

*Materials and Enzymes.* Tritium-labeled deoxynucleoside 5′-triphosphates ([$^3$H]dNTPs), deoxyadenosine [$\alpha$-$^{32}$P]triphosphate ([$^{32}$P]dATP), and adenosine 5′-[$\gamma$-$^{32}$P]triphosphate ([$^{32}$P]ATP) were products of Amersham, United Kingdom. Unlabeled dNTPs, poly(dA)·poly(dT), poly[d(G-C)], poly(dT), poly(dA), $(dG)_8$, $(dT)_8$, and oligo(dA) of different chain lengths were supplied by Sigma Chemical Co., St Louis, MO. Annealing of the oligomers to their respective complementary polydeoxynucleotides was conducted by heating the hybrids at 70 °C for 30 min, followed by heating at 37 °C for an additional 30 min and incubation at room temperature for 30 min. Circular single-stranded DNA from bacteriophage M13mp2 was a gift from Dr. B. Preston, University of Washington. A universal 17-mer primer to M13 DNA, *E. coli* DNA polymerase I, bacteriophage T4 DNA polymerase, and bacteriophage T4 polynucleotide kinase were the products of United States Biochemical Corp., Cleveland, OH. Calf intestine alkaline phosphatase was supplied by Pharmacia, Uppsala, Sweden. Highly purified large (Klenow) fragment of pol I and immunoaffinity column purified polymerase $\alpha$-primase from calf thymus were the generous gifts of Drs. L. A. Loeb and F. Perrino, University of Washington. Factor D (phosphocellulose-purified fraction) was isolated from rabbit liver as described in the past (Sharf et al., 1988). Distamycin A, which was a product of Sigma, was kindly contributed by Dr. Y. Shlomai, Hebrew University, Jerusalem.

*Assay Conditions for the Activity of DNA Polymerases.* Activities of DNA polymerases were determined by the extent of incorporation of $^3$H-labeled dNMPs into acid-insoluble DNA. Polymerization was conducted in 25-$\mu$L reaction mixtures whose specific compositions for the different polymerases were as described recently (Fry et al., 1987a). Duration and temperature of the incubation were varied for specific experiments as described under Results.

*Electrophoretic Analysis of the Products of Primer Extension Reactions.* Unlabeled $(dA)_9$ primer was annealed to poly(dT) at a primer to template ratio of 1:5 and extended at 37 °C for 1 min by pol I in reaction mixtures for the assay of polymerase activity that contained [$\alpha$-$^{32}$P]dATP (950 cpm/pmol) and in some cases distamycin, factor D, or both. In other experiments, the polymerase extended 5′-$^{32}$P-labeled $(dA)_9$ with unlabeled dAMP. For this procedure, unlabeled $d(pA)_9$ was digested by alkaline phosphatase to remove the 5′-phosphate group. The dephosphorylation reaction was terminated by the addition of ethylenediaminetetraacetic acid (EDTA) to a final concentration of 40 mM, and the phosphatase was inactivated by heating at 65 °C for 20 min. Residual protein was extracted by chloroform–isoamyl alcohol (1:24 v/v), and salts were subsequently removed by a Sephadex G-50 spun column (Maniatis et al., 1982). The dephosphorylated and desiccated oligomer was resuspended in a reaction mixture for the activity of T4 polynucleotide kinase and was end-labeled with [$\gamma$-$^{32}$P]ATP (Maniatis et al., 1982). The 5′-end-labeled oligomer was diluted with unlabeled $(dA)_9$ and annealed to poly(dT) at a primer to template ratio of 1:5 (w/w). Extension of the annealed primer by pol I was conducted at 37 °C for 2 min by pol I as described above. Primer extension was terminated by the addition of EDTA to a final concentration of 16 mM, and the mixtures were desalted by filtration through Sephadex G-50 spun columns (Maniatis et al., 1982). The collected samples were desiccated and dissolved in 2–4 $\mu$L of deionized formamide containing 2 mM EDTA, 0.1% bromophenol blue, and 0.01% xylene cyanol. After denaturation by boiling, the products of the extension reactions were separated by DNA sequencing electrophoresis on gels of 12% polyacrylamide in 8 M urea as described in a previous work (Fry et al., 1987b). The resolved labeled DNA chains were visualized by autoradiography.

*Electrophoretic Analysis of the Stability of [$^{32}$P]$(dA)_9$·Poly(dT).* The effect of distamycin on the stability of $(dA)_9$·poly(dT) was directly examined by electrophoretic separation of annealed from dissociated oligomer after incubation of the hybrid at 37 °C with or without distamycin. End-labeling of $(dA)_9$ at its 5′-terminus with $^{32}$P was conducted according to Maniatis et al. (1982) as described above, and the oligomer was annealed to poly(dT) at a primer to template ratio of 1:6 (w/w). Free [$^{32}$P]$(dA)_9$ or [$^{32}$P]$(dA)_9$·poly(dT) was incubated at 37 °C for 20 min with or without 3.0 $\mu$g/mL distamycin. The mixtures were loaded onto 4.0% polyacrylamide gels (16.0 × 20.0 cm × 0.8 mm) in TE buffer (10 mM Tris-HCl, pH 8.0, and 1.0 mM EDTA) and electrophoresed at 25 °C for 90 min at 150 V in TE buffer. Dissociated and annealed [$^{32}$P]$(dA)_9$ was detected by autoradiography of the dried gels.

Table I: Effect of Distamycin on Rates of Copying of Different Primer-Templates by *E. coli* Pol I[a]

| primer-template | substrates | pol act. (units) +dist | pol act. (units) −dist | stimulation (x-fold) |
|---|---|---|---|---|
| poly[d(G-C)] | [$^3$H]dGTP + dCTP | 0.033 | 0.031 | 0.94 |
| $(dG)_8$·poly(dC) | [$^3$H]dGTP | 0.620 | 0.572 | 0.92 |
| poly(dA)·poly(dT) | [$^3$H]dATP | 0.010 | 0.001 | 0.10 |
| M13-primed DNA | [$^3$H]dATP + dNTPs | 0.002 | 0.001 | 0.50 |
| $(dA)_8$·poly(dT) | [$^3$H]dATP | 0.003 | 0.050 | 16.70 |

[a] The indicated primer-templates (40 $\mu$g each/mL reaction mixture; primer to template ratio 1:5, w/w) were copied for 30 min at 37 °C by pol I (2.0 units/mL) under standard conditions and with or without 3.0 $\mu$g/mL distamycin. Specific activity of the labeled substrates ranged between 60 and 250 cpm/pmol of dXMP. One unit of polymerase activity is the activity that polymerizes 1 nmol of dXMP per 30 min at 37 °C.

## Results

*Distamycin Specifically Stimulates the Copying of Oligo(dA)·Poly(dT).* In the course of testing the effect of various agents on the in vitro replication of (A + T)-rich DNA templates, we noted that distamycin, a known inhibitor of DNA synthesis, unexpectedly stimulates the copying of oligo(dA)·poly(dT) by pol I. Results summarized in Table I indicate that distamycin did not affect the rates of copying of poly[d(G-C)] and oligo(dG)·poly(dC) to which it does not bind. By contrast, the antibiotic greatly suppressed the copying of templates to which it does bind: poly(dA)·poly(dT) and bacteriophage M13 primed circular single-stranded DNA (Table I). These results are in line with previous reports indicating that distamycin has no effect on the polymerase

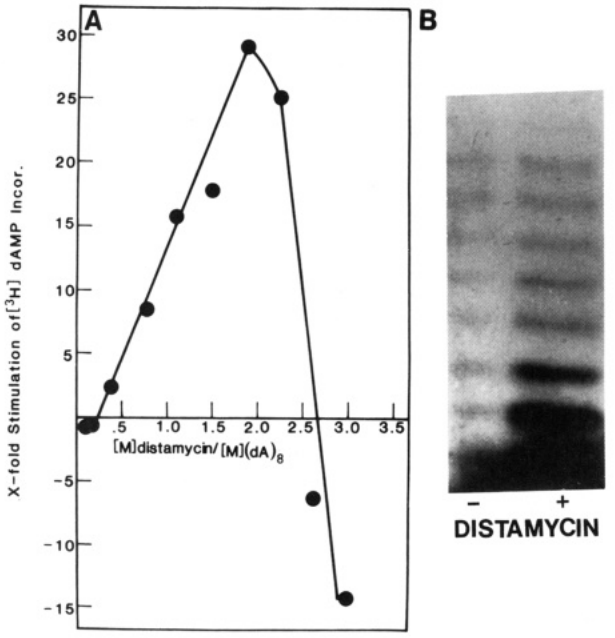


FIGURE 1: Stoichiometry of distamycin stimulation of oligo(dA) extension and electrophoretic separation of products synthesized with and without distamycin. (A) Pol I (2.0 units/mL) catalyzed the copying of $(dA)_8$·poly(dT) [40 μg/mL; primer to template ratio (w/w) 1:5] at 37 °C for 30 min under standard assay conditions. Incorporation of [$^3$H]dAMP into acid-insoluble material was determined in reaction mixtures that contained increasing amounts of distamycin. Without distamycin, pol I incorporated 2.0 pmol of [$^3$H]dAMP. Data points are averages of duplicate determinations. (B) Pol I (2.2 units/mL) without or with distamycin (2.0 μg/mL) copied at 37 °C for 2 min [5′-$^{32}$P]$(dA)_9$·poly(dT). Extension of the end-labeled primer was terminated, and the denatured products were separated by sequencing gel electrophoresis as described under Materials and Methods.

molecule itself but rather that (A + T)-rich templates serve as the target for binding and inhibition by the drug (Muller et al., 1974; Wahnert et al., 1975; Wilkins, 1982; Grehn et al., 1983). Surprisingly, however, we found distamycin to greatly stimulate the copying of $(dA)_8$·poly(dT) by pol I (Table I). Distamycin also exerted similar specific stimulation on the copying of oligo(dA)·poly(dT) by the large (Klenow) fragment of pol I and by DNA polymerase from bacteriophage T4 (results not shown). Since stimulation was indistinguishable for pol I and its Klenow fragment, both enzymes were used interchangeably in this work. It is notable, however, that distamycin does not universally stimulate every DNA polymerase and it suppressed the activity of calf thymus polymerase-α with every tested template including oligo(dA)·poly(dT) (data not presented; see Discussion).

To study the stoichiometry of distamycin stimulation, the antibiotic was added at increasing concentrations to the oligo(dA)·poly(dT) polymerization reaction mixture, and rates of extension of $(dA)_8$ were determined. A seen in Figure 1A, a maximum 30-fold stimulation of the activity of pol I was attained at a distamycin to primer nucleotide ratio of 0.25 whereas at a ratio of 0.37 the drug already inhibited synthesis 15-fold. Since a distamycin molecule binds four (dA)·(dT) base pairs (Van Dyke et al., 1982; Portugal & Waring, 1987), it appears that maximum stimulation was reached upon saturation of the 8-mer primer stem by the antibiotic and that even a moderate excess of the drug became inhibitory (see Discussion). The greatly increased polymerization of dAMP in the presence of distamycin was visualized directly following electrophoresis of products of the extension of [5′-$^{32}$P]$(dA)_9$ by pol I (Figure 1B). It is notable that the antibiotic mostly enhanced the addition of only the first three bases to the primer, suggesting that it mainly increased the rate of initiation of synthesis (Figure 1B).

Table II: Synergistic Stimulation by Factor D and Distamycin of the Copying of Oligo(dA)·Poly(dT)[a]

| reagent added | [$^3$H]dAMP incorporated (pmol) | x-fold stimulation |
|---|---|---|
| none | 7.7 | 1.0 |
| distamycin | 82.3 | 10.7 |
| factor D | 16.6 | 2.2 |
| distamycin + factor D | 190.8 | 24.9 |

[a] Copying of $(dA)_8$·poly(dT) (1:5 w/w) was conducted by pol I for 30 min at 37 °C under standard assay conditions without or in the presence of distamycin (3.0 μg/mL), phosphocellulose-purified rabbit liver factor D, or both. Incorporation of [$^3$H]dAMP (200 cpm/pmol) into acid-insoluble material was measured as described under Materials and Methods.

The effect of distamycin on the kinetic parameters of the extension of oligo(dA) by pol I was examined next. Incorporation of [$^3$H]dAMP was conducted in the presence of saturating amounts of poly(dT) template which was primed with diminishing amounts of $(dA)_8$. Results obtained show that in the presence of distamycin the maximum velocity of synthesis ($V_{max}$) increased 7.7-fold from 18.1 to 140.0 pmol of [$^3$H]dAMP incorporated in 10 min. By contrast, the Michaelis constant ($K_m$) remained unchanged at 142.8 μg of $(dA)_8$ mL$^{-1}$ (data not shown). These results suggest that distamycin has no effect on the affinity of the polymerase to the primer but, rather, that it enhances synthesis by increasing the rate of primer extension.

*Distamycin and Factor D Act Synergistically To Stimulate Oligo(dA)·Poly(dT) Synthesis.* We next sought to further demonstrate the distamycin enhances synthesis by augmenting the initiation of oligo(dA) extension. In previous works, we have described an oligo(dT)-binding protein, termed factor D, that specifically increases the affinity of DNA polymerases to oligo(dT) template stretches and thus augments the processivity of elongation (Fry et al., 1987a,b, 1988; Sharf et al., 1988). If the enhancement by distamycin of the copying of poly(dT) is indeed due to an increased rate of initiation of synthesis rather than to an augmented elongation, the stimulatory effect of the drug should complement that of factor D. Data presented in Table II show that such indeed is the case: Whereas distamycin and factor D increased the rates of copying of $(dA)_9$·poly(dT) 10.7- and 2.2-fold, respectively, synthesis increased synergistically 24.9-fold in the presence of both agents. The synergistic increase of synthesis in the presence of both stimulators was visualized by electrophoresis of products of the polymerization of [$^{32}$P]dAMP residues added to a $(dA)_9$ primer. As seen in Figure 2, distamycin increased significantly the amount of labeled oligo(dA) chains without affecting their size distribution. Although its stimulatory effect was less pronounced than that of distamycin, factor D increased the amount of longer product oligomers. Most interestingly, however, in the presence of both agents, the amount of the products as well as their maximum length increased (Figure 2). Results shown in Table II and in Figure 2 indicate, therefore, that the mechanism of stimulation by distamycin differs from that of the processivity-augmenting factor D. This result, together with the observed increase in $V_{max}$ and the data shown in Figure 1B, suggests that distamycin has no effect on the processivity of synthesis but, rather, that it enhances primer extension by increasing the rate of initiation of dAMP polymerization.

*Stimulation by Distamycin Is Inversely Related to the Stability of the Oligo(dA) Primer Stem.* One possible

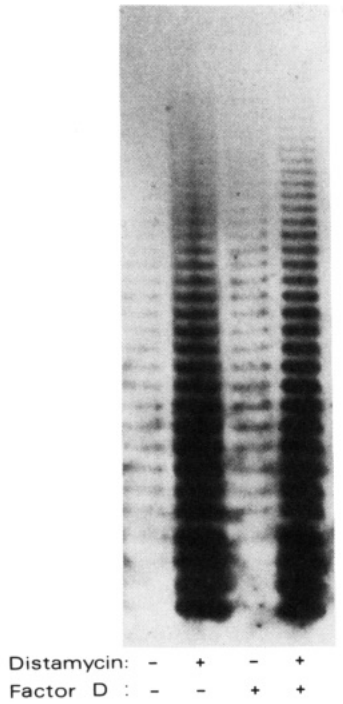


FIGURE 2: Electrophoretic separation of products of oligo(dA)·poly(dT) extension in the presence of factor D, distamycin, or both. Pol I (2.0 units/mL) extended $(dA)_9$·poly(dT) (40 μg/mL) by polymerization of [$^{32}$P]dAMP with or without distamycin (3.0 μg/mL), factor D, or both. Conditions of the polymerization reaction and gel electrophoresis were as described under Materials and Methods.

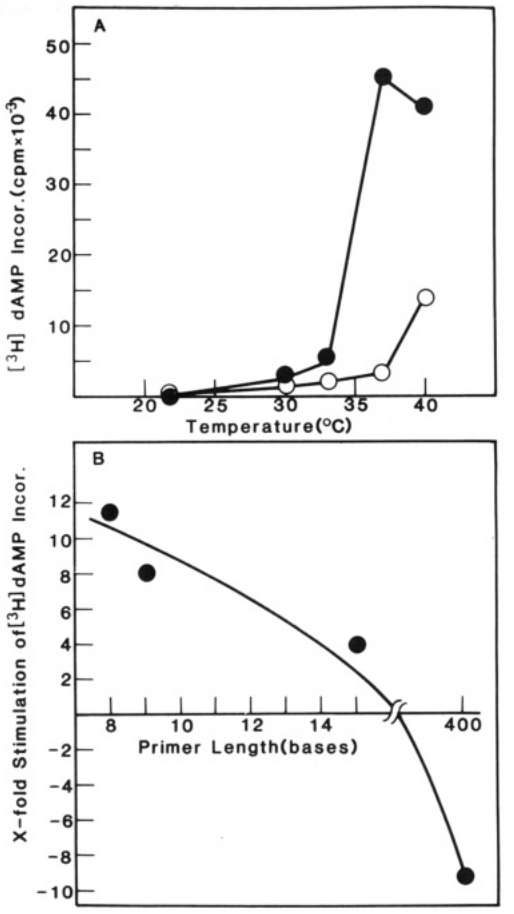


FIGURE 3: Effects of temperature and primer length on stimulation by distamycin of poly(dT) copying. (A) Pol I (5.2 units/mL) copied $(dA)_8$·poly(dT) (40 μg/mL) at the indicated temperatures for 30 min with or without added distamycin (3.0 μg/mL). Incorporation of [$^3$H]dAMP (560 cpm/pmol) into acid-insoluble material served to measure the rate of synthesis, and presented values are averages of duplicate determinations. (B) Pol I (5.2 units/mL) copied poly(dT) (40 μg/mL) which was annealed to oligo(dA) primers of different lengths at a primer to template ratio of 1:5 (w/w). Polymerization of [$^3$H]dAMP (560 cpm/pmol) was conducted at 37 °C for 30 min. The extent of stimulation is the ratio of activity in the presence of distamycin relative to the activity of polymerase without distamycin which ranged for the different primers between 3.7 and 23.0 pmol of dAMP incorporated. The primer marked $(dA)_{15}$ is the average of a $(dA)_{12-18}$ primer which was actually used. Incorporation with $(dA)_{400}$ was obtained with a 1:1 (w/w) hybrid poly(dA)·poly(dT) primer-template. Values presented are averages of duplicate determinations.

mechanism by which distamycin might increase the rate of initiation of polymerization is by providing the polymerase with an increased amount of available 3′-OH primer termini. Such might be the case if distamycin stabilized the association of the short oligo(dA) primer with the poly(dT) template. To investigate this possibility, we examined the effect of distamycin on the polymerization of [$^3$H]dAMP under conditions of variable stability of the primer–template complex.

First, the stability of association between $(dA)_8$ and poly(dT) was varied by progressive elevation of temperature. As seen in Figure 3A, distamycin had no significant effect on the rate of $(dA)_8$·poly(dT) copying at 22 and 30 °C, and it stimulated synthesis only slightly at 33 °C. By contrast, the antibiotic increased synthesis more than 10-fold at 37 °C and a similar enhanced rate of polymerization was maintained at 40 °C (Figure 3A). Second, primers of increasing length were used to attain different stabilities of primer–template association at 37 °C. Data presented in Figure 3B show that the stimulation by distamycin of poly(dT) copying was inversely related to the length of the primer stem. Extension of primers that contained 8, 9, and 15 nucleotides was stimulated 12-, 8-, and 4-fold, respectively, in the presence of the antibiotic, whereas with a poly(dA) primer that contained approximately 400 nucleotides, a 10-fold inhibition of incorporation was observed (Figure 3B).

*Distamycin Stabilizes $(dA)_9$ in Its Hybrid with Poly(dT).* The above presented results indicate that the extent of stimulation by distamycin is directly related to the degree of instability of the primer–template complex. It is thus likely that distamycin stimulates the copying of poly(dT) by stabilizing its complex with the oligo(dA) primer. To examine directly whether distamycin affects the stability of a $(dA)_9$·poly(dT) hybrid, [5′-$^{32}$P]$(dA)_9$ was annealed to poly(dT), the hybrid was heated at 37 °C for 20 min with or without distamycin, and the dissociated primer was separated electrophoretically from annealed primer as described under Materials and Methods. As seen in Figure 4, distamycin had no effect on the electrophoretic mobility of free $(dA)_9$. Also, when $(dA)_9$·poly(dT) was heated at 37 °C without distamycin, the hybrid was quantitatively denatured, and the labeled $(dA)_9$ migrated as a free oligomer (Figure 4). By contrast, however, when distamycin was present during the thermal treatment, some of the labeled $(dA)_9$ remained in high molecular size hybrid which did not penetrate the gel. In addition, gradual dissociation of the primer during electrophoresis resulted in retardation of the oligomer so that it streaked along the full length of the gel (Figure 4). It appears, therefore, that distamycin maintains an increased proportion of $(dA)_9$ in hybrid with poly(dT).

*Stimulation by Distamycin Requires Initiation To Be Rate Limiting.* The preferential binding of distamycin to (A + T)-rich DNA and the proposed mechanism of stimulation of oligo(dA) extension by the drug suggest that it should similarly

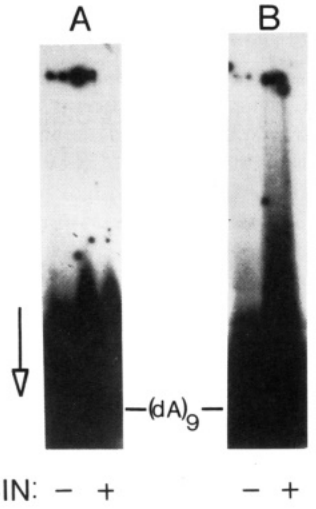


FIGURE 4: Effect of distamycin on the stability of [$^{32}$P](dA)$_9$·poly(dT). Free [5′-$^{32}$P](dA)$_9$ and [5′-$^{32}$P](dA)$_9$·poly(dT) hybrid were incubated at 37 °C for 20 min with or without distamycin (3.0 μg/mL) and electrophoresed in a 4.0% polyacrylamide gel as described under Materials and Methods. The arrow indicates the direction of electrophoretic migration, and the position of free (dA)$_9$ is indicated. (A) Free (dA)$_9$ without and with distamycin. (B) (dA)$_9$·poly(dT) without and with distamycin.

Table III: Effect of Distamycin on Rates of Copying of Oligo(dA)·Poly(dT) and Oligo(dT)·Poly(dA)[a]

| template-primer | primer: template ratio (w/w) | [$^3$H]dXMP incorporated (pmol) −dist | [$^3$H]dXMP incorporated (pmol) +dist | stimulation (*x*-fold) |
|---|---|---|---|---|
| (dA)$_8$·poly(dT) | 2.0 | 5.6 | 54.5 | 9.7 |
| | 0.5 | 3.5 | 45.0 | 12.9 |
| | 0.2 | 3.8 | 126.7 | 33.4 |
| (dT)$_8$·poly(dA) | 2.0 | 84.3 | 0.45 | 0.005 |
| | 0.5 | 113.0 | 0.96 | 0.008 |
| | 0.2 | 95.2 | 0.36 | 0.004 |

[a] The two templates (40 μg/mL), primed by their respective complementary primers at the indicated primer to template ratios, were copied at 37 °C for 30 min by pol I (2.0 units/mL) under standard assay conditions. Incorporation of [$^3$H]dAMP or [$^3$H]dTMP (205 and 295 cpm/pmol, respectively) was conducted without or with distamycin (3.0 μg/mL).

enhance the copying of oligo(dA)·poly(dT) and of oligo(dT)·poly(dA). We have compared, therefore, the effect of distamycin on the utilization of these two primer-templates.

Results summarized in Table III show that the rate of copying of (dA)$_8$·poly(dT) was increased by distamycin 6.0–33.0-fold at primer to template weight ratios ranging between 2.0 and 0.2. Surprisingly, however, extension from an oligo(dT) primer was inhibited by distamycin 125–250-fold (Table III).

The asymmetric effect of the antibiotic can be understood in light of the different efficiencies at which poly(dA) and poly(dT) templates are copied by pol I. In the absence of distamycin, rates of copying of oligo(dT)·poly(dA) were on average 24-fold higher than those of oligo(dA)·poly(dT) at similar primer to template ratios (Table III). Moreover, copying of (dA)$_8$·poly(dT) was undetectably low at primer to template ratios lower than 0.025 whereas the rate of copying of (dT)$_8$·poly(dA) remained almost unaltered down to a primer to template ratio of 0.001 (data not shown). These results are in line with previous reports on the highly frequent pausing by pol I during is advancement along a poly(dT) template (Fry et al., 1987a,b). Due to the frequent events of dissociation and reassociation of the polymerase with this template, availability of primer termini might thus become rate limiting. Stabilization by distamycin of the bonding of oligo(dA) to the poly(dT) template is likely, therefore, to be the basis to the observed enhancement of synthesis. With a poly(dA) template, however, processivity of polymerization is high, and availability of the primer is not rate limiting. Moreover, with this template, the rapid accumulation of a double-stranded poly(dA)·poly(dT) product and the binding of distamycin to the polymeric duplex result in inhibition of synthesis by the antibiotic (see Discussion).

## Discussion

The major observation described in this paper is that distamycin A, an extensively characterized inhibitor of DNA synthesis (Zimmer, 1975; Zimmer & Wahnert 1986), unexpectedly stimulates copying in vitro of oligo(dA)·poly(dT) by DNA polymerases. We propose that the antibiotic exerts this primer-template-preferential stimulation by stabilizing the association between the short oligo(dA) primer stem and the poly(dT) template.

The primer-template-specific stimulation of polymerase activity by distamycin is found to depend on two elements.

(A) Stimulation by distamycin is exerted only if initiation of nucleotide addition to the primer stem is the rate-limiting step of the polymerization reaction. Several lines of evidence indicate that distamycin stimulates the copying of oligo(dA)·poly(dT) by enhancing initiation of synthesis. First, a synergistic stimulation of the copying of oligo(dA)·poly(dT) is obtained when distamycin is added together with factor D, a protein which specifically increases the processivity of poly(dT) elongation (Fry et al., 1987a,b; Sharf et al., 1988). Thus, stimulation by distamycin of poly(dT) copying is not due to an increase in the processivity of polymerization (Table II and Figure 3). Second, the antibiotic increases the maximum velocity of polymerization without affecting the affinity of the polymerase to the primer stem (see Results). Lastly, electrophoresis of labeled oligo(dA) chains synthesized in the presence of distamycin provides a direct demonstration for a preferential increase in the rate of addition of the very first dAMP residues to the primer (Figure 1B). For distamycin to be an effective stimulator, however, initiation of primer extension must be the rate-limiting step of synthesis. It was previously shown that pol I is blocked frequently in the course of its advancement along a poly(dT) template and that synthesis is terminated at each succeeding dT template residue (Fry et al., 1987a,b; Table III in this work). With this template, the rate-limiting step of synthesis is plausibly the productive reassociation of the enzyme with the primer-template, and thus distamycin acts to enhance synthesis. By contrast, the copying of poly(A) is highly processive, and initiation is not rate limiting. Therefore, upon the copying of oligo(dT)·poly(dA), a duplex poly(dA)·poly(dT) product is rapidly accumulated, and distamycin becomes inhibitory (Table III, see below). In this context, it is notable that the copying of oligo(dA)·poly(dT) by polymerase-α, which terminates synthesis along a poly(dT) template less frequently than pol I (Fry et al., 1987b), is not stimulated by distamycin (see Results).

(B) The extent of enhancement of polymerase activity is directly related to the degree of instability of the primer–template complex. Hence, with an octameric dA primer, the antibiotic enhances synthesis at elevated temperatures that promote dissociation of the primer stem (Figure 3A). Further, the observed inverse relation between the length of the primer and the extent of stimulation of its extension in the presence of distamycin (Figure 3B) also indicates that the stimulatory

action of the antibiotic depends on the degree of instability of the primer–template complex. This indirect evidence suggests that distamycin might increase the stability of the complex of oligo(dA) with poly(dT). Direct physical evidence for such stabilization was provided by electrophoretic demonstration that $(dA)_9$·poly(dT) has an increased thermal stability in the presence of distamycin (Figure 4).

Present knowledge on the binding of distamycin to DNA allows one to speculate on the mechanism of stabilization of the oligo(dA)·poly(dT) complex by the antibiotic. Binding of netropsin, an antibiotic structurally similar to distamycin, to octameric or dodecameric duplexes that contain four dA·dT pairs was studied in detail (Patel, 1979, 1982; Kopka et al., 1985). Specificity of binding to dA·dT pairs originates from close van der Waals contacts between adenine C(2) hydrogens and CH groups on the pyrrole rings of the antibiotic (Kopka et al., 1985). The correct positioning of netropsin within the minor groove of the duplex is provided by replacement of water molecules of the hydration spine. Specifically, the NH groups of the antibiotic form bifurcated hydrogen bonds with N(3) of adenines and O(2) of thymines, thus bridging the same sites occupied by water molecules in the hydration spine (Kopka et al., 1985). It was proposed that the drug plays the role of the "hydration shell", thus stabilizing the B geometry of the duplex (Kopka et al., 1985; Zimmer & Wahnert, 1986). Reasonably, therefore, similar hydrogen and van der Waals bonds which are formed upon the binding of distamycin to the short duplex stretches of oligo(dA)·poly(dT) are responsible for fixation of the dissociable primer. However, after the binding of distamycin by a long poly(dA)·poly(dT) duplex, its effectiveness as primer-template diminishes—probably as a result of changes in the geometry of double-stranded polymer (Kopka et al., 1985; Ekambareswara et al., 1988).

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