Shin, Y.-K., & Moscicki, J. K., & Freed, J. H. (1990) *Biophys. J. 57*, 445–459.
Small, D. M. (1967) *J. Lipid Res. 8*, 551–557.
Smotkin, E. S., Moy, F. T., & Plachy, W. Z. (1991) *Biochim. Biophys. Acta* (in press).
St.-Denis, C. E., & Fell, C. J. D. (1971) *Can. J. Chem. Eng. 49*, 885.
Subczynski, W. K. (1984) *Zeszyty Nauk. Uniw. Jagiellon. 10*, 145–203 (in Polish).
Subczynski, W. K., & Hyde, J. S. (1981) *Biochim. Biophys. Acta 643*, 283–291.
Subczynski, W. K., & Hyde, J. S. (1983) *Biophys. J. 41*, 283–286.
Subczynski, W. K., & Hyde, J. S. (1984) *Biophys. J. 45*, 743–748.
Subczynski, W. K., & Kusumi, A. (1986) *Biochim. Biophys. Acta 854*, 318–320.
Subczynski, W. K., Antholine, W. E., Hyde, J. S., & Petering, D. H. (1987) *J. Am. Chem. Soc. 109*, 46–52.
Subczynski, W. K., Hyde, J. S., & Kusumi, A. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 4474–4478.
Subczynski, W. K., Antholine, W. A., Hyde, J. S., & Kusumi, A. (1990) *Biochemistry 29*, 7936–7945.
Subczynski, W. K., Renk, G., Crouch, R., Hyde, J. S., & Kusumi, A. (1991) *Biophys. J.* (submitted).
Tinker, D. O., Pinteric, L., Hsia, J. C., & Rand, R. P. (1976) *Can. J. Biochem. 54*, 209–218.
Träuble, H. (1971) *J. Membr. Biol. 4*, 193–208.
Träuble, H., & Sackmann, E. (1972) *J. Am. Chem. Soc. 94*, 4499–4510.
Träuble, H., & Eibl, H. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 214–219.
Vaz, W. L. C., Criado, M., Madeira, V. M. C., Schoellmann, G., & Jovin, T. M. (1982) *Biochemistry 21*, 5608–5612.
Vaz, W. L. C., Clegg, R. M., & Hallmann, D. (1985) *Biochemistry 24*, 781–786.
Vist, M. R., & Davis, J. H. (1990) *Biochemistry 29*, 451–464.
Walter, A., & Gutknecht, J. (1986) *J. Membr. Biol. 90*, 207–217.
Wilson, D. F., Erecinska, M., Drown, C., & Silver, J. A. (1979) *Arch. Biochem. Biophys. 195*, 485–493.
Wilson, D. F., Rumsey, W. L., Green, T. J., & Vanderkooi, J. M. (1988) *J. Biol. Chem. 263*, 2712–2718.
Wittenberg, B. A., & Wittenberg, J. B. (1985) *J. Biol. Chem. 260*, 6548–6554.
Yeagle, P. L. (1985) *Biochim. Biophys. Acta 822*, 267–287.
Yeagle, P. L. (1988) *Biology of Cholesterol*, CRC Press, Boca Raton, FL.
Yin, J.-J., & Hyde, J. S. (1987) *Z. Phys. Chem. (Munich) 153*, 541–549.
Yin, J.-J., Pasenkiewicz-Gierula, M., & Hyde, J. S. (1987) *Proc. Natl. Acad. Sci. U.S.A. 79*, 1854–1858.
Zaccai, G., Buldt, G., Seelig, A., & Seelig, J. (1979) *J. Mol. Biol. 134*, 693–706.

Mechanism of DNA Polymerase α Inhibition by Aphidicolin†,‡

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ABSTRACT: Synthetic oligonucleotides of defined sequence were used to examine the mechanism of calf thymus DNA polymerase α inhibition by aphidicolin. Aphidicolin competes with each of the four dNTPs for binding to a pol α·DNA binary complex and thus should not be viewed as a dCTP analogue. Kinetic evidence shows that inhibition proceeds through the formation of a pol α·DNA·aphidicolin ternary complex, while DNase I protection experiments provide direct physical evidence. When deoxyguanosine is the next base to be replicated, K_i = 0.2 μM. In contrast, the K_i is 10-fold higher when the other dNMPs are at this position. Formation of a pol α·DNA·aphidicolin ternary complex did not inhibit the primase activity of the pol α·primase complex. Neither the rate of primer synthesis nor the size distribution of primers 2–10 nucleotides long was changed. Elongation of the primase-synthesized primers by pol α was inhibited both by ternary complex formation using exogenously added DNA and by aphidicolin alone.

Aphidicolin, a tetracyclic diterpenoid isolated from *Cephalosporium aphidicola* (Brundret et al., 1972), is a potent inhibitor of DNA replication (Bucknall et al., 1973). It specifically inhibits polymerase α (Spadari et al., 1982) and was found to inhibit mitotic division of sea urchin embryos while not affecting nondividing cells (Ikegami et al., 1978; Oguro et al., 1979). The drug proved to be instrumental in identifying pol α[1] as a major eukaryotic replicative polymerase (Huberman, 1981). Aphidicolin also specifically inhibits pol δ (Byrnes, 1984), the α-like polymerase of plant cells, and the herpes simplex virus and vaccinia virus encoded DNA polymerases (Pedrali-Noy & Spadari, 1980a; Huberman, 1981; Spadari et al., 1982, 1984, 1985a,b; Fry & Loeb 1986), while not affecting DNA methylation or RNA, protein, and nucleotide biosynthesis (Spadari et al., 1982, 1984, 1985a). This

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‡ This paper is dedicated to Dr. Robert H. Abeles on the occasion of his 65th birthday.

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[1] Abbreviations: Aph, aphidicolin; EDTA, ethylenediaminetetraacetic acid (sodium salt); pol α, DNA polymerase α:primase; pol δ, DNA polymerase δ; Tris, tris(hydroxymethyl)aminomethane (HCl salt).

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high degree of specificity distinguishes aphidicolin from nonspecific inhibitors of replication, such as nucleotide analogues, that affect other metabolic pathways. Aphidicolin is widely used to study DNA repair (Berger, 1979; Pedrali-Noy & Spadari, 1980b; Chang & Trosko, 1989). Given these characteristics, the agent holds potential as an antiviral and anticancer drug (Prasad et al., 1989).

Aphidicolin has been studied extensively in vivo with the use of a variety of cell lines, and in all cases it interferes with cell growth. Because of its specificity and reversibility, the drug has been used as a tool to synchronize HeLa cells and SV40 replicons (Pedrali-Noy et al., 1981; Dinter-Gottlieb & Kaufman, 1982; Spadari et al., 1984). In the presence of aphidicolin, cells that have entered S phase can no longer synthesize DNA whereas cells in other phases continue the cell cycle and stop at the G1/S border (Pedrali-Noy et al., 1981; Chang & Trosko, 1989). The accumulation of early replication intermediates in SV40 systems can also be detected (Decker et al., 1986).

Data regarding the mechanism of inhibition comes primarily from studies using homopolymeric DNA, DNase I activated calf thymus DNA, and in vivo cell systems. Studies utilizing purified pol α from various species are generally in agreement that inhibition is competitive with respect to dCTP (Dicioccio et al., 1980; Pedrali-Noy & Spadari, 1980a; Huberman, 1981; Chang & Trosko, 1989). However, with respect to the other dNTPs and DNA, inhibition has been interpreted as noncompetitive, uncompetitive, and mixed (Oguro et al., 1979; Pedrali-Noy & Spadari, 1980a; Huberman, 1981; Spadari et al., 1984; Chang & Trosko, 1989). Pedrali-Noy and Spadari (1980a) found that the inhibition of herpes simplex virus DNA polymerase was primarily noncompetitive with respect to dGTP, dTTP, and dATP but competitive with dCTP. Using the same enzyme, Frank et al. (1984) reported that the incorporation of dATP, dTTP, and dCTP was inhibited competitively while dGTP incorporation was inhibited noncompetitively. These results suggest that inhibition is a complex process and may occur differently depending upon the nucleotide to be inserted by the polymerase. Most of the DNAs that have been utilized as substrates for pol α are complex in terms of structure, template composition, and length. These factors, combined with the relative complexity of DNA replication itself, probably account for the different descriptions of how inhibition occurs.

Deducing the mechanism of inhibition requires an understanding of what step(s) of the polymerization process is affected. No evidence exists for insertion of aphidicolin into the 3′ end of the growing primer strand (chain termination), and there are no apparent interactions between aphidicolin and free DNA or with free dNTPs (Dicioccio et al., 1980; Spadari et al., 1984). Rather, aphidicolin is thought to bind at or near the nucleotide binding site(s) on pol α and prevent dNTP binding (Huberman, 1981). However, direct evidence supporting this idea has yet to be presented.

Several aphidicolin-resistant cell lines contain mutations that are consistent with the afore-mentioned in vitro studies (Chang & Trosko, 1989). Chinese hamster V79 and *Drosophila* cell lines contain an aphidicolin-resistant DNA pol α (Chang & Trosko, 1989; Sugino & Nakayama, 1980). In the former case, pol α had an increased affinity for dCTP while in the latter the apparent K_i for aphidicolin increased 8-fold. Other resistant cell lines contain increased dCTP and/or dNTP concentrations (Chang & Trosko, 1989).

Given variances in the data and the fact that it is not immediately obvious how the structure of aphidicolin could mimic

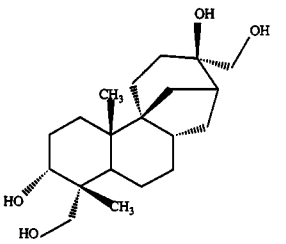


FIGURE 1: Structure of aphidicolin.

Table I: Primer–Templates Constructed from Synthetic Oligonucleotides

DNA_C	TCC ATA TCA CAT-3′
	AGG TAT AGT GTA CAT CTT ATC ATC T
DNA_G	TCC ATA TCA CAT-3′
	AGG TAT AGT GTA GAT CTT ATC ATC T
DNA_{22}	TCC ATA TCA CAT-3′
	AGG TAT AGT GTA GGG TGG GTG G
DNA_{BIII}	GAT TCA GCT AGT CCA-3′
	CTA AGT CGA TCA GGT AGC ATA GTG TCA AAC

that of dCTP (Figure 1), we undertook an in vitro kinetic analysis of the mechanism of inhibition. Our approach utilized synthetic oligonucleotides to generate primer–templates of defined length and sequence. A kinetic scheme describing inhibition was derived that allows for further investigation into the structural and functional relationships of pol α and the potential for aphidicolin to act as an antitumor and antiviral drug.

Experimental Procedures

Materials

Unless noted, all materials and methods were as described previously (Kuchta et al., 1990; Kuchta & Willhelm, 1991). Aphidicolin and DNase I were from Sigma. DNA pol α·primase complex was purified from calf thymus with use of immunoaffinity chromatography as described previously (Kuchta et al., 1990).

Aphidicolin was dissolved in 100% ethanol. The final concentration of EtOH in the reactions was no more than 2%. This concentration of EtOH did not affect pol α activity. Fresh dilutions were made daily as the drug precipitated when stored at low EtOH concentrations. DNase I was dissolved and stored according to Maniatis et al. (1982).

Synthetic Oligonucleotides. Poly(dT) was from U.S. Biochemicals and oligo$(rA)_{12-18}$ was from Pharmacia. Other synthetic deoxyoligonucleotides were purchased from Oligos Etc. and stored in 10 mM Tris, pH 7.5. They were annealed by heating to 75 °C, followed by a slow cool (Table I). Poly(dT)·oligo(rA) was annealed at a 20:1 ratio (total nucleotide). Labeled DNAs were constructed by incubating 5′-^{32}P-labeled template or primer strand with a 10% excess of its unlabeled partner so that all of the labeled strand was annealed.

Methods

All reactions were performed at 37 °C and initiated by the addition of pol α unless noted otherwise.

Pol α Activity. Assays (10–20 μL) contained 50 mM Tris, pH 7.5, 5 mM $MgCl_2$, DNA, 1 unit of pol α, 10–20 μM dNTPS, and aphidicolin. One dNTP was radiolabeled ([α-^{32}P]dATP or [α-^{32}P]dCTP) at a specific activity of ≅1000 cpm pmol^{-1}. Activated calf thymus DNA was used at 0.5 mg mL^{-1}. Poly(dT)·oligo(rA) was used at 50 μM poly(dT) total nucleotide. Direct competition assays contained 1–5 μM substrate DNA (measured as 3′ termini). The equilibrium in-

hibition assays contained 1 μM substrate DNA and various inhibitor DNA concentrations. Aliquots were quenched at single time points with 2.5 volumes of 50 mM EDTA, pH 8.0. The assays were linear over the time points taken. Radioactivity incorporated into oligonucleotides was determined as described previously (Kuchta et al., 1990) except the DE81 filters were washed with 0.1 M Na_2HPO_4. Radioactivity in samples was measured with use of a Beckman LS7000 scintillation counter and scintillation fluid as described previously (Kuchta et al., 1990). In some cases we measured polymerase activity using DNA containing a 5′-^{32}P-labeled primer strand. The products were quantified by gel electrophoresis and phosphorimagery.

Primase Activity. Primase activity was measured as described previously (Kuchta et al., 1990). During the assay for primase activity in the presence of DNA_G (Table I) and aphidicolin, pol α (1–2 units), DNA_G (10 μM), and aphidicolin were preincubated for 2–5 min at room temperature. The reaction was initiated by the addition of poly(dT) and quenched as described above.

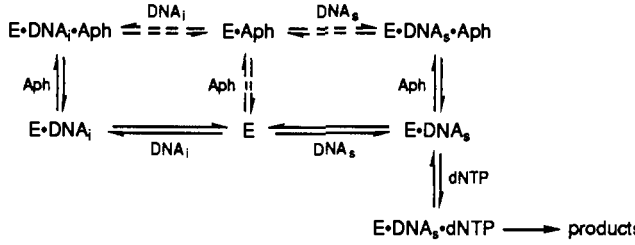
Coupled Primase–Pol α Assay. Assays (10 μL) to measure the fraction of primase-synthesized primers elongated by pol α contained 50 mM Tris, pH 7.5, 5 mM $MgCl_2$, 100 μM [α-^{32}P]ATP (2000 cpm pmol^{-1}), 2.5 μM dATP, 25 μM poly(dT) (total nucleotide), 1–2 units of pol α, and various aphidicolin concentrations. Reactions were quenched by the addition of 2.5 volumes of gel loading buffer (90% formamide), and products were separated by gel electrophoresis. Gels were exposed to phosphorimager screens, and the amount of radioactive product was quantified. The fraction of primers elongated was determined by (α-^{32}P-labeled polymerase products)/(α-^{32}P-labeled polymerase products + α-^{32}P-labeled unit length primase products) (Kuchta et al., 1990). Primase-coupled pol α activity was measured as described previously (Kuchta & Willhelm, 1991). Assays contained 100 μM ATP, 50 μM poly(dT), and various concentrations of [α-^{32}P]dATP.

K_m and K_D Determinations. The K_ms for the DNAs were determined by Lineweaver–Burk plots using 0.5–3 μM DNA and 20 μM dNTPs. The K_Ds of various DNAs were measured as a K_i. Assays contained one DNA (e.g., DNA_C) that served as substrate and then varying concentrations of a second DNA (e.g., DNA_G) that was the inhibitor. dNTPs present in the assay were adjusted such that no synthesis could occur on the inhibitor DNA. Data were analyzed via Dixon plots. The concentrations of substrate DNA were 0.5, 1.5, and 3 μM while the inhibitor DNA varied from 0 to 3 μM.

Gel Electrophoresis and Phosphorimagery. Gel electrophoresis was as described previously, except that Tris–borate–EDTA buffer in the gel was increased 1.5-fold to reduce the run time (Kuchta et al., 1990). Autoradiograms were developed with use of Kodak X-ray film and intensifying screens. Alternatively, the amount of radioactivity was quantitated with the use of a Molecular Dynamics phosphorimager. In all cases the amount of radioactivity was within the linear range of the phosphorimager. Background levels were determined by integrating identical volumes of a reaction containing either no enzyme (processivity experiments) or an equal quantity of [^{32}P]ATP but no 5′-^{32}P-labeled DNA (DNase I protection experiments).

DNase I Protection. Reactions containing 50 mM Tris, pH 7.5, 5 mM $MgCl_2$, 50 nM 5′-^{32}P-labeled DNA, 2 units of pol α, and various concentrations of aphidicolin were incubated for 2–7 min at room temperature. DNase I was added to a final concentration of 0.03 mg mL^{-1}, and the reactions were incubated at room temperature for a further 5 min. Reactions were quenched by the addition of 2.5 volumes of gel loading buffer (90% formamide). The amount of radioactive products was quantified by phosphorimagery. The total amount of product was determined by quantifying products 12 nucleotides long and longer.

Scheme I: Kinetic Scheme of Aphidicolin Inhibition



Processivity Measurements. Reactions were performed in the same manner as filter binding assays but were quenched at 3–10 min with 2.5 volumes of gel loading buffer. The labeled dNTP was chosen so that all polymerization products would be visualized. The relative molar amount of each length product was calculated by correcting for the number of [^{32}P]dNMPs incorporated.

Kinetics

The following velocity equation for Scheme I (eq 1) was derived using the King–Altman method (1956) and assuming (i) dNTP polymerization is irreversible and (ii) aphidicolin binding to free enzyme is insignificant:

$$V = V_{max}[DNA_S][dNTP]/\left([DNA_S][dNTP] + K_{mN}[DNA_S]\left(1 + \frac{[A]}{K_{A1}}\right) + K_{mN}K_S\left(1 + \frac{[DNA_i]}{K_I}\left(1 + \frac{[A]}{K_A}\right)\right) + K_{mS}[dNTP]\left(1 + \frac{[DNA_i]}{K_I}\left(1 + \frac{[A]}{K_A}\right)\right)\right) \quad (1)$$

where DNA_S = substrate DNA, DNA_i = inhibitor DNA, A = aphidicolin, K_I = dissociation constant of DNA_i, K_S = dissociation constant of DNA_S, K_{mN} = K_m dNTPs, K_{mS} = K_m substrate DNA, K_A = dissociation constant of aphidicolin from E•DNA_i•Aph, and K_{A1} = dissociation constant of aphidicolin from E•DNA_S•Aph. Inverting this equation yields

$$\frac{1}{V} = \left(\frac{1}{V_{max}}\right)\left[\frac{K_{mN}}{K_{A1}[dNTP]} + \frac{[DNA_i]}{K_AK_I[DNA_S]}\left(\frac{K_{mN}K_S}{[dNTP]} + K_{mS}\right)\right][A] + \left(\frac{1}{V_{max}}\right)\left[1 + \frac{K_{mN}}{[dNTP]}\left(1 + \frac{K_S}{[DNA_S]}\left(1 + \frac{[DNA_i]}{K_I}\right)\right) + \frac{K_{mS}}{[DNA_S]}\left(1 + \frac{[DNA_i]}{K_I}\right)\right] \quad (2)$$

To determine the K_i for aphidicolin in a Dixon plot ($1/v$ vs [A]) via direct competition between aphidicolin and dNTPs (i.e., [DNA_i = 0]), the value of $1/v$ at different dNTP concentrations becomes equal when $-[A] = K_{A1}(1 + K_S/[DNA_S])$.

In the equilibrium inhibition assay, $1/v$ was again measured as a function of aphidicolin concentration except that DNA_i was now present. All assays contained identical dNTP concentrations. The value of $1/v$ at different DNA_i concentrations becomes equal when $-[A] = K_A$. If aphidicolin or an equivalent ligand does not bind to the E•DNA_i complex ($K_A \rightarrow \infty$), then parallel lines will be obtained at different DNA_i con-

centrations. Thus, by adding a second DNA and complicating the experimental scheme, we simplify the kinetic analysis.

Results and Discussion

Our initial studies found that aphidicolin inhibition of DNA synthesis on activated calf thymus DNA appeared competitive with dCTP (data not shown), consistent with previous studies using pol α from different sources. Similar to the results of Prasad et al. (1989), we observed less potent inhibition of synthesis on poly(dT)·oligo(rA), suggesting that aphidicolin inhibition does not proceed solely through competition with dCTP. In light of these and previous data, and to analyze inhibition in greater detail, we constructed primer–templates from synthetic oligonucleotides of defined length and sequence (Table I). Advantages for using these synthetic templates include synthesis in relatively large amounts, direct control of the sequence, and greater resemblance to "natural" DNA than homopolymeric DNA. The composition of the template strands was chosen to determine if the nucleotide being replicated dictates how inhibition occurs. For example, in the case of DNA_C and DNA_G, the only difference is the first nucleotide to be replicated.

Despite their short length, all of the DNAs are good substrates for pol α. The rates of dNTP polymerization on 1 μM DNA_C and 1 μM DNA_G were 17 pmol min^{-1} and 15 pmol min^{-1}, respectively, similar to the rate of 13 pmol min^{-1} obtained on 0.5 mg mL^{-1} calf thymus DNA. Comparable rates were also obtained on DNA_{22} and DNA_{Bill}. The apparent K_ms for DNA_C and DNA_G are 3 μM, and the K_Ds are 0.9 μM and 0.6 μM, respectively. The K_m values for dNTPs on DNA_C and DNA_G were determined by varying the concentration of all dNTPs simultaneously, which gave a value of 5 μM.

Aphidicolin Competes with All dNTPs. Initial experiments showed that aphidicolin was a more potent inhibitor of polymerization on DNA_G than on DNA_C. At 10 μM dNTPs and 5 μM aphidicolin, 65% inhibition of polymerization was observed on DNA_G, while the same conditions only gave 40% inhibition on DNA_C. These results confirmed that a requirement for dCTP polymerization is not necessary for inhibition by aphidicolin. Importantly, the presence of up to 300 μM dCTP had no effect upon the amount of inhibition observed on DNA_C, indicating that aphidicolin was not binding to an allosteric site specific for dCTP. We attributed the greater amount of inhibition on DNA_G to the presence of a template deoxyguanosine on this substrate.

To determine whether aphidicolin was competing with nucleotides in addition to dCTP, we examined the effect of increasing the dNTP concentration on inhibition with the respective substrates. On DNA_C, overcoming inhibition necessitated increasing simultaneously the concentration of all three dNTPs required for polymerization, consistent with a competition for binding between aphidicolin and all three dNTPs.

With DNA_G and 10 μM dNTPs, the addition of 5 μM aphidicolin resulted in 65% inhibition of synthesis. Again, completely overcoming inhibition required increasing the concentration of all the dNTPs required for polymerization. Increasing the dCTP concentration alone, however, reduced the extent of inhibition 42%, supporting the hypothesis that template deoxyguanosines give more potent inhibition.

We therefore examined the relative importance of the template nucleotides by determining an apparent K_i for aphidicolin on DNA_G and DNA_C. With 5 μM DNA_G, direct competition (Scheme I) between aphidicolin and dCTP gave K_i = 0.2 μM (data not shown). Inhibition appeared purely competitive with dCTP since the other three dNTPs were held constant at a level (15 μM) sufficient to prevent aphidicolin inhibition with respect to these dNTPs. We also determined an apparent K_i for aphidicolin on DNA_C by varying the concentration of all required dNTPs. This value was approximately 8-fold higher (1.5 μM) than that measured on DNA_G, again showing that the presence of a template deoxyguanosine promoted tighter aphidicolin binding to the E·DNA complex. It must be stressed that these are only apparent K_is since incorporation of one dNMP generates a new aphidicolin binding site at the next template position to be polymerized. This method of K_i determination is potentially further complicated by a dependence on DNA concentration (see Experimental Procedures).

The data demonstrate that the apparent type of inhibition (competitive, noncompetitive, etc.) can be varied by simply changing the template composition or the ratio of dNTPs and suggest the following explanation for earlier reports of uncompetitive or noncompetitive inhibition with respect to nucleotides other than dCTP. On a heterogeneous DNA (e.g., activated calf thymus DNA), pol α activity is generally measured as the summation of the polymerization of all four dNTPs. Increasing the concentration of one dNTP in the presence of aphidicolin only overcomes inhibition due to competition with that dNTP. Inhibition at other template positions is unaffected, giving un- or noncompetitive inhibition with respect to that nucleotide in standard plots used to determine the K_i. In the case of dCTP, however, inhibition appears competitive because aphidicolin competes 10-fold more effectively with dCTP than with the other dNTPs (see below). This competition will be magnified if the concentration of dCTP (the varied substrate) is less than the concentration of the other three dNTPs. Consistent with this interpretation, aphidicolin inhibition on poly(dT)·oligo(rA) was purely competitive with respect to dATP (K_i = 11 μM, data not shown). With heterogeneous DNAs, the type and potency of inhibition depends upon the relative concentration of each dNTP as well as the template composition.

Equilibrium Inhibition Assay. Given the potential difficulties in obtaining a true K_i in the direct competition assays, we developed a novel method for determining the dissociation constant for aphidicolin at a single template nucleotide (Scheme I). Both DNA_s (substrate DNA) and DNA_i (inhibitor DNA) were present in the same reaction.[2] The dNTPs present could be adjusted such that no synthesis occurred on DNA_i; thus, DNA_i acted as a simple competitive inhibitor with respect to DNA_s. Pol α was in equilibrium between the two primer–templates; hence, aphidicolin binding to the E·DNA_i complex would shift the equilibrium via formation of an E·DNA_i·Aph ternary complex, further reducing the activity observed on DNA_s. Measured inhibition thus becomes a matter of binding events representing the true dissociation constant for aphidicolin from the E·DNA_i complex (see eq 2, Experimental Procedures). Importantly, this K_i reflects aphidicolin binding to just one E·DNA species. In direct competition assays, one measures aphidicolin binding to a multitude of E·DNA species due to dNTP polymerization on the substrate DNA. In contrast, since the dNTPs present in the equilibrium inhibition assay preclude synthesis on DNA_i, we measure aphidicolin binding to a single E·DNA_i complex. Table II summarizes the dissociation constants of aphidicolin obtained with each DNA using the equilibrium inhibition assay.

[2] The concentrations of DNA_s, DNA_i, and aphidicolin do not affect the K_i in the equilibrium inhibition assay (see Experimental Procedures). The only requirement in choosing concentrations is that sufficient pol α activity be obtained on DNA_s.

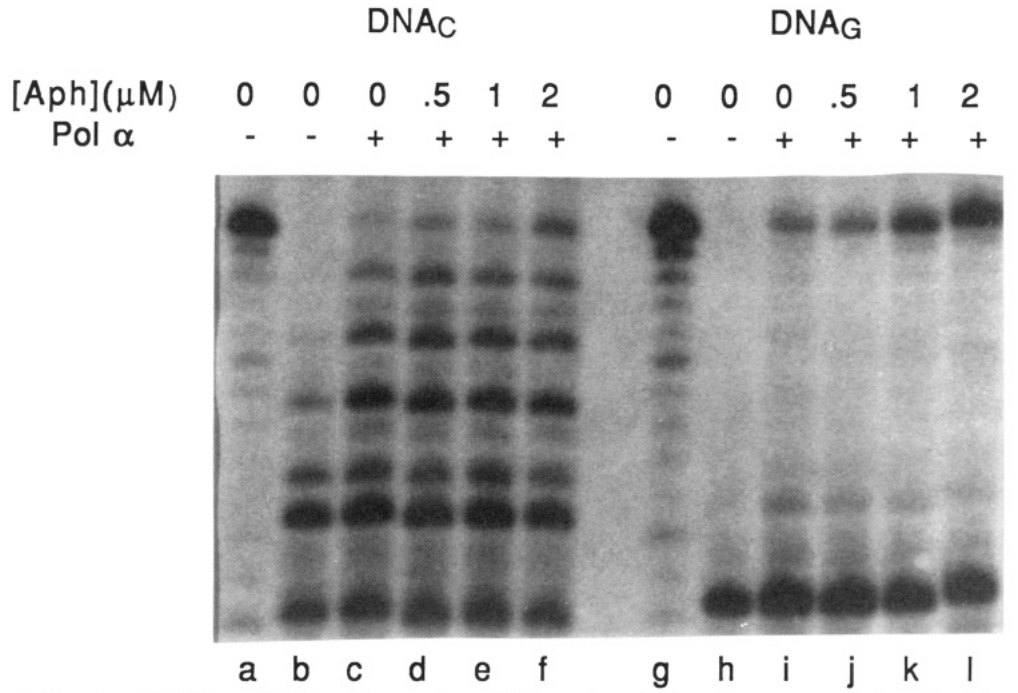


FIGURE 2: DNase I digestion of DNA_C and DNA_G. Lane a shows DNA_C prior to DNase I treatment. In lanes b–f, the presence of pol α and aphidicolin is as indicated. Lanes g–l correspond to lanes a–f except that DNA_C was replaced with DNA_G. The amounts of DNA_C and DNA_G protected by addition of pol α alone were similar (comparing lanes a and c, and lanes g and i). The 5′-^{32}P-labeling of the DNAs resulted in greater labeling of DNA_G than DNA_C.

Table II: Dissociation Constants for Aphidicolin[a]

DNAs	DNA_i	nucleotide[b]	K_i[c] (μM)
DNA_C	DNA_G	G	0.20
poly(dT)·(rA)	DNA_G	G	0.22
DNA_C	DNA_{22}	G	0.15
poly(dT)·(rA)	DNA_{22}	G	0.22
poly(dT·(rA)	DNA_C	C	2.0
poly(dT)·(rA)	DNA_{Bill}	A	1.6
DNA_C	oligo$(dG)_{10}$		7.5

[a] K_is of aphidicolin for various E·DNA complexes were measured with use of the equilibrium inhibition assay (Experimental Procedures). [b] Nucleotide refers to the nucleotide at the primer–template junction of DNA_i. [c] Margin of error was less than 15%.

The data showed that DNA_G and increasing concentrations of aphidicolin inhibited polymerization on DNA_C synergistically, confirming that inhibition proceeds through the formation of an E·DNA_i·Aph ternary complex. The dissociation constant for aphidicolin from the ternary complex formed at the primer–template junction of DNA_G was 0.20 μM (Table II). Using poly(dT)·oligo(rA) as DNA_s gave K_i = 0.22 μM, indicating that the DNA used to measure activity does not affect the K_i. Since aphidicolin also inhibited polymerization on DNA_C, we investigated ternary complex formation with this DNA. The K_i for aphidicolin on DNA_C (which contains a deoxycytosine at the primer–template junction) was 2 μM, 10-fold higher than with DNA_G (Table II).

We further investigated the importance of a template deoxyguanosine at the primer–template junction. With DNA_{Bill}, deoxyguanosine is the second base from the primer–template junction (Table I) while the first base to be replicated is deoxyadenosine. For DNA_{Bill}, K_i = 1.6 μM, similar to the value obtained on DNA_C. Adding dTTP to the equilibrium inhibition assay containing DNA_{Bill} decreased the K_i to 0.15 μM, since deoxyguanosine became the first base at the primer–template junction due to polymerization of a dTMP.

We examined DNA_{22} (Table I) to determine if adding deoxyguanosines on the template strand contributed additional binding energy to the ternary complex. Using poly(dT)·oligo(rA) as the substrate, K_i = 0.22 μM. The presence of additional and adjacent deoxyguanosines had no effect on the K_i for aphidicolin, i.e., a template deoxyguanosine at the primer–template junction is both necessary and sufficient for strong inhibition. We utilized single-stranded oligo$(dG)_{10}$ to examine the importance of the primer in ternary complex formation. Surprisingly, the single-stranded DNA supported ternary complex formation, albeit with an increased K_i (7.5 μM). Given that dNTPs do not bind to a pol α·single-stranded DNA complex (Fisher & Korn, 1981), this result suggests that aphidicolin interacts with pol α in a fundamentally different manner than does a dNTP. As a control, we examined dCTP binding to a pol α·oligo$(dG)_{10}$ complex and observed no measurable pol α·dCTP·oligo(dG) complex formation (data not shown).

These results indicated that additional deoxyguanosines in close proximity to the primer–template junction do not affect aphidicolin binding. Only the next template nucleotide to be replicated is important, consistent with pol α reading a single template base and containing a single dNTP binding site. The E·DNA·Aph complex still formed in the absence of a primer but was much less stable, showing that the primer was important for formation of a stable ternary complex. The shorter length of the oligo$(dG)_{10}$ may also have affected the stability of the complex. Under the conditions tested, the tightest ternary complex formed when there was a deoxyguanosine at the primer–template junction.

DNase I Protection Assays. We have shown kinetically that aphidicolin inhibits pol α activity by forming an E·DNA·Aph ternary complex. We then employed DNase I to provide physical evidence for ternary complex formation by measuring the amount of DNA bound to pol α (and hence resistant to DNase I cleavage) as a function of aphidicolin concentration.

DNA_C and DNA_G containing 5′-^{32}P-labeled template strands were treated with DNase I (Figure 2, lanes b and h). The difference in cleavage patterns on DNA_C and DNA_G likely reflects the different base at the primer–template junction. Aphidicolin alone did not alter the cleavage pattern,

Table III: DNase I Protection

[Aph] (μM)	amount protected[a]			
	DNA_C	DNA_G	DNA_{BIII}	DNA_{BIII} + dTTP[b]
0	1.0	1.0	1.0	1.0
0.5	1.2	2.5	1.0	1.2
1.0	1.7	3.6	1.0	1.8
2.0	2.5	5.3		
5			1.3	2.6

[a] The amount protected in the presence of pol α alone (0 aphidicolin) was normalized to 1.0 for each DNA. [b] 50 μM dTTP was added to these reactions before the addition of DNase I.

suggesting that aphidicolin does not interact with DNA in solution (data not shown). Addition of pol α (no aphidicolin) resulted in protection of a small amount of the entire template strand (lanes c and i). Now, addition of aphidicolin resulted in the protection of more template molecules (lanes d–f and j–l), consistent with pol α·DNA·Aph ternary complex formation. Importantly, addition of aphidicolin resulted in a greater enhancement of protection with DNA_G than with DNA_C (Figure 2, Table III).

We also examined DNA_{BIII} to confirm the importance of the template deoxyguanosine as the next base to be replicated. Addition of aphidicolin to reactions containing DNA_{BIII} and pol α only modestly increased the amount of protection. However, incorporation of dTMP into DNA_{BIII} such that deoxyguanosine became the base at the primer–template junction resulted in enhanced protection by aphidicolin (Table III). DNase I experiments thus support the kinetic results. Aphidicolin can bind to any E·DNA binary complex regardless of the identity of the next nucleotide to be replicated, but deoxyguanosine imparts a lower dissociation constant.

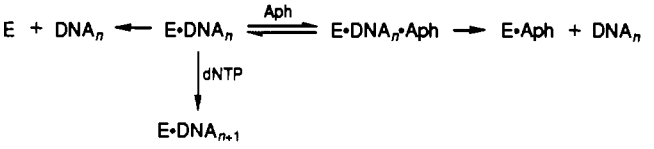
With each DNA, pol α binding resulted in the protection of the entire template strand, indicating that pol α likely contains a large DNA binding domain. Similarly, the footprinting data of Tsurimoto and Stillman (1991) indicate that human pol α binds to an extended region of the DNA.

Aphidicolin Primarily Binds an E·DNA Complex. Earlier work reported both uncompetitive and noncompetitive inhibition with respect to the DNA (Oguro et al., 1979; Huberman, 1981; Spadari et al., 1984). The distinction is an important one, since in the former case aphidicolin does not bind free enzyme while in the latter it does. Using our synthetic templates, we observed apparent uncompetitive inhibition with respect to both DNA_G and DNA_C (parallel lines in a v^{-1} vs [aphidicolin] plot, data not shown). If aphidicolin binding to free enzyme was very weak, however, this method may not have been sensitive enough to measure its occurrence. We therefore utilized a more sensitive method—the effect of aphidicolin on pol α processivity—to examine binding to free enzyme.

Processivity is a competition between dissociation and addition of the next correct nucleotide (Bryant et al., 1983). For aphidicolin to decrease processivity, it must increase the rate of DNA dissociation relative to polymerization. Our data showed that aphidicolin converts an E·DNA binary complex to an E·DNA·Aph ternary complex that cannot polymerize dNTPs. Two possible fates exist for this complex: aphidicolin could dissociate, leaving an E·DNA complex capable of further elongation, or DNA could dissociate, leaving an E·Aph complex (Scheme II). The latter event could be measured as a decrease in processivity and would suggest that aphidicolin can bind free enzyme.

Polymerization products from DNA_{22} and DNA_C at various aphidicolin concentrations were quantified. The amount of

Scheme II: Kinetic Scheme Depicting the Effect of Aphidicolin on Pol α Processivity



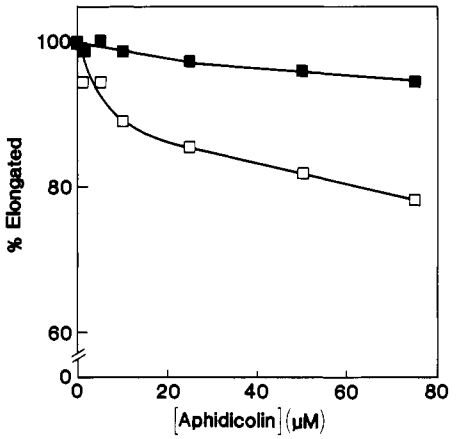


FIGURE 3: Effect of aphidicolin on pol α processivity. Reactions contained DNA_{22}, [α-^{32}P]dCTP, and dATP. Shown are the effects of aphidicolin on the fraction of the 13-mer (□) and the 14-mer (■) elongated. The 13-mer and the 14-mer are the species generated after polymerization of one dCTP and two dCTPs, respectively. The amount elongated for each species was normalized to 100 in the absence of aphidicolin. (In the absence of aphidicolin, 55% of the 13-mer and 75% of the 14-mer were elongated.)

each product represents pol α dissociation after the insertion of a nucleotide at that position. The fraction of each species elongated therefore reflects the processivity of pol α. The percent of any length species (y) that is elongated is given by eq 3. The presence of excess original primer–template (i.e., steady-state conditions) assured that once pol α dissociated from the DNA it could not rebind products. This was confirmed in experiments showing that the distribution of products did not change with time (data not shown).

$$\text{percent elongated} = (\text{products} > y/\text{products} \geq y) \times 100 \quad (3)$$

Upon the addition of aphidicolin, there was no change in processivity at any template position on DNA_C even at greater than 90% inhibition of pol α activity. On DNA_{22}, however, the fraction of the 13-mer elongated decreased 20% when pol α was inhibited 93% (Figure 3). At other template positions, the processivity changed less than 5%. Thus, DNA can dissociate from the ternary complex under the proper conditions, suggesting that aphidicolin can weakly bind to pol α.

Two additional explanations must be considered. Pol α could dissociate leaving a DNA·Aph binary complex, but earlier work (Dicioccio et al., 1980; Spadari et al., 1984) and our DNase I experiments suggest that aphidicolin does not interact with free DNA. Alternatively, the change in processivity could reflect the simultaneous dissociation of aphidicolin, DNA, and pol α. This requires, however, that ternary binding also occur, an extremely unlikely event. The rather modest decrease in processivity relative to the extent of inhibition requires that aphidicolin bind very weakly to free enzyme, hence the data are consistent with the observed uncompetitive inhibition with respect to DNA. This decreased processivity may also be important for the accumulation of shorter replication products that have been observed upon the addition of aphidicolin to SV40 replication systems (Decker et al., 1986).

Table IV: Effect of Aphidicolin on the Fraction of Primase-Synthesized Primers Elongated by Pol α

[Aph] (μM)	fraction elongated[a] 25 °C	37 °C
0	0.87	0.41
10	0.80	0.34
50	0.70	0.31
100	0.58	0.27
200	0.42	0.18
400	0.20	0.08

[a] The fraction elongated was measured as described under Experimental Procedures. Pol α can only elongate primers at least seven nucleotides long (Kuchta et al., 1990), and hence only these primers were considered.

Primase Activity. The pol α complex, consisting of 4 subunits, has an associated primase activity found in the 48- and 58-kDa subunits (Kaguni et al., 1983; Tseng & Ahlem, 1983; Grosse & Nasheuer, 1988). At aphidicolin concentrations sufficient to completely inhibit pol α activity (50 μM), we observed no effect on either the rate of primer synthesis or the size distribution of primers 2–10 nucleotides long using a poly(dT) template, consistent with previous data (Tseng & Ahlem, 1983; Cotterill et al., 1987). Very high aphidicolin concentrations (200 μM) slightly inhibited the rate of primer synthesis (20%), but again did not change the size distribution.

We examined more closely the relationship between the polymerase and primase DNA binding domains by forming an E·DNA_G·Aph ternary complex and then measuring primase activity. Surprisingly, the ternary complex exhibited normal primase activity with no effect on either the rate or size distribution of primers 2–10 nucleotides long (data not shown). No primers were elongated upon the addition of 50 μM dATP, showing that the polymerase site was >98% blocked. The primase subunit was therefore able to synthesize RNA primers even though aphidicolin and DNA_G were bound at the polymerase active site, supporting the idea that the primase and polymerase functional domains are completely distinct.

Earlier reports have postulated that the polymerization of the first 30 dNTPs onto a primase-synthesized primer is aphidicolin resistant (Decker et al., 1986). This was explicitly examined by measuring [α-^{32}P]dATP polymerization onto a poly(dT) template. Poly(dT) lacks endogenous primers; hence, pol α activity is strictly dependent upon primase-catalyzed primer synthesis. Aphidicolin inhibited polymerization competitively with dATP (K_i = 4 μM). This value is similar to the K_i measured for inhibition of polymerization on poly(dT)·oligo(rA) (K_i = 11 μM). However, for primase-coupled pol α activity, K_m(dATP) = 75 nM, whereas the use of poly(dT)·oligo(rA) as the substrate gave K_m = 3 μM (Kuchta & Willhelm, 1991).[3] Thus, while aphidicolin is an inhibitor of dNTP polymerization onto primase-synthesized primers, polymerization may appear aphidicolin resistant due to the low K_m for dNTPs.

Aphidicolin inhibition of DNA synthesis onto primase-synthesized primers predicts that the fraction of primers elongated by pol α should decrease in the presence of aphidicolin. As shown in Table IV, aphidicolin greatly decreased the fraction of primers elongated at both 25 and 37 °C. This provides further support for both the formation of an E·DNA·Aph complex with primase-synthesized primers and the presence of primase activity when the polymerase is blocked.

[3] The low K_m for dATP during in vitro primase-coupled pol α activity likely reflects rate-limiting primer synthesis (data not shown). It is unknown if this is also true in vivo.

Conclusions

The use of synthetic oligonucleotides to construct well-defined DNAs has proven fruitful in deducing the kinetic mechanism of aphidicolin inhibition. Aphidicolin binds primarily to the E·DNA complex, and binding is competitive with respect to all four dNTPs. Processivity results suggest that aphidicolin weakly binds free enzyme, although this pathway may become more significant with different polymerases or DNAs.

Previous work has resulted in varied, and often contradictory, data with respect to aphidicolin inhibition. It has been suggested that inhibition occurs differently within the cell than with the use of purified enzyme. However, both the in vitro and in vivo data are consistent with a mechanism in which inhibition occurs at every template position through ternary complex formation, but with more potent inhibition at template deoxyguanosines. Thus, the amount and type of inhibition observed in any system is dependent on the ratio of deoxyguanosines to other nucleotides in the DNA as well as the concentrations of dNTPs. Interpreting the in vivo effect of aphidicolin on DNA replication is likely to be further complicated by inhibition of both pol α and pol δ.

It has been suggested that aphidicolin may interfere with the binding of nucleotides by sterically blocking part of the nucleotide binding site (Huberman, 1981; Spadari et al., 1984). Alternatively, aphidicolin could bind in an entirely different location on pol α and inhibit the binding of nucleotides by an allosteric mechanism. This seems unlikely, however, since increasing the dCTP concentration on DNA_C (where no template deoxyguanosines are present) had no effect on aphidicolin inhibition. Other researchers have speculated that multiple distinct dNTP binding sites exist and that aphidicolin binds tighter at the binding site for dCTP (Oguro et al., 1979). Although we cannot rule out this hypothesis, its invocation is not required to explain our results. Aphidicolin binding to pol α at a single dNTP binding domain is sufficient to account for the observed competition with each dNTP.

The dissociation constant for aphidicolin is 10-fold lower when the ternary complex forms at a deoxyguanosine template nucleotide. Interestingly, a factor of 10 corresponds to 1.4 kcal, the approximate energy of one hydrogen bond. The hydroxyl groups on aphidicolin are the only functional groups that could participate in hydrogen-bond formation; hence, tighter binding could be explained if an additional hydrogen bond was being formed between aphidicolin and an altered conformation of the enzyme when a dCTP is required for polymerization. Alternatively, aphidicolin could form a hydrogen bond with O-6 of the template deoxyguanosine. The O-6 is attractive since it is the only hydrogen-bond acceptor present on deoxyguanosine but not deoxyadenosine. The hydroxyl groups in aphidicolin have been selectively modified and the relative importance to inhibition determined (Prasad et al., 1989). With use of the equilibrium inhibition assay and the modified aphidicolin compounds, it may be possible to determine which hydroxyl groups contribute additional binding energy when template deoxyguanosines are present.

On the basis of our results and various computer modeling studies, aphidicolin should not be viewed as a dCTP or nucleotide analogue (Prasad et al., 1989). In addition to lacking a phosphate moiety, aphidicolin bears little structural resemblance to nucleosides. Aphidicolin binds remarkably well regardless of the next base to be replicated, while correct base pairing appears to be very important for dNTP binding (Boosalis et al., 1987). Additionally, whereas aphidicolin can bind to an E·single-stranded DNA binary complex, dNTPs

cannot (Fisher & Korn, 1981; and data herein). Thus, there appear to be distinct differences between aphidicolin and dNTPs.

Inhibition of pol α by ternary complex formation will also prove useful in the study of primase activity. Physical separation of pol α and primase often results in the loss of primase stability and activity. Inhibiting pol α with DNA_G and aphidicolin permits the study of primase activity while in the pol α·primase complex. Experiments using aphidicolin are currently underway to understand how the newly synthesized primers are translocated from the primase site to the polymerase site.

Aphidicolin was extremely important in establishing DNA pol α as a eukaryotic replicative polymerase. With a better understanding of the mechanism of inhibition, it promises to reveal much more about how pol α orchestrates the complex task of DNA replication.

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References

Berger, N. A., Kurohara, K. K., Petzold, S. J., & Sikorski, G. W. (1979) *Biochem. Biophys. Res. Commun. 89*, 218–225.

Boosalis, M. S., Petruska, J., & Goodman, M. F. (1987) *J. Biol. Chem. 262*, 14689–14696.

Brundret, K. M., Dalziel, W., Hesp, B., Javis, J. A. J., & Neidle, S. (1972) *J. Chem. Soc., Chem. Commun. 18*, 1027–1028.

Bryant, F. R., Johnson, K. A., & Benkovic, S. J. (1983) *Biochemistry 22*, 3537–3546.

Bucknall, R. A., Moores, H., Simms, R., & Hesp, B. (1973) *Antimicrob. Agents Chemother. 4*, 294–298.

Byrnes, J. J. (1984) *Mol. Cell. Biochem. 62*, 13–24.

Chang, C. C., & Trosko, J. E. (1989) in *Drug Resistance in Mammalian Cells*, pp 29–43, CRC Press Inc., Boca Raton, FL.

Cotterill, S., Chui, G., & Lehman, I. R. (1987) *J. Biol. Chem. 262*, 16105–16108.

Decker, R. S., Yamaguchi, M., Possenti, R., & DePamphilis, M. (1986) *Mol. Cell. Biol. 6*, 3815–3825.

Dicioccio, R. A., Chadha, K., & Srivastava, B. I. (1980) *Biochim. Biophys. Acta 609*, 224–231.

Dinter-Gottlieb, G., & Kaufmann, G. (1982) *Nucleic Acids Res. 10*, 763–773

Fisher, P. A., & Korn, D. (1981) *Biochemistry 20*, 4560–4569.

Frank, K. B., Derse, D. D., Bastow, K. F., & Cheng, Y.-C. (1984) *J. Biol. Chem. 259*, 13282–13286.

Fry, M., & Loeb, A. (1986) in *Animal Cell Polymerases*, CRC Press Inc., Boca Raton, FL.

Grosse, F., & Nasheuer, H. P. (1988) *Cancer Cells 6*, 397–402.

Huberman, J. (1981) *Cell 23*, 647–648.

Ikegami, S., Taguchi, T., Ohashi, M., Oguro, M., Nagano, H., & Mano, Y. (1978) *Nature 275*, 458–460.

Kaguni, L. S., Rossignol, J.-M., Conaway, R. C., Banks, G. R., & Lehman, I. R. (1983) *J. Biol. Chem. 258*, 9037–9039.

King, E. L., & Altman, C. (1956) *J. Phys. Chem. 60*, 1375–1385.

Kuchta, R. D., & Willhelm, L. (1991) *Biochemistry 30*, 797–803.

Kuchta, R. D., Reid, B., & Chang, L. M. S. (1990) *J. Biol. Chem. 265*, 16158–16165.

Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Oguro, M., Suzuki-Hori, C., Nagano, H., Mano, Y., & Ikegami, S. (1979) *Eur. J. Biochem. 97*, 603–607.

Pedrali-Noy, G., & Spadari, S. (1980a) *J. Virol. 36*, 457–464.

Pedrali-Noy, G., & Spadari, S. (1980b) *Mutat. Res. 70*, 389–394.

Pedrali-Noy, G., & Spadari, S. (1979) *Biochem. Biophys. Res. Commun. 88*, 1194–2002.

Pedrali-Noy, G., Kuenzle, C. G., Focher, F., & Spadari, S. (1981) *J. Biochem. Biophys. Methods 4*, 113–121.

Prasad, G., Edelson, R. A., Gorycki, P. D., & MacDonald, T. L. (1989) *Nucleic Acids Res. 17*, 6339–6349.

Spadari, S., Sala, F., & Pedrali-Noy, G. (1984) *Adv. Exp. Med. Biol. 178*, 169–181.

Spadari, S., Sala, F., & Pedrali-Noy, G. (1982) *Trends Biochem. Sci. 7*, 29–32.

Spadari, S., Focher, S., Sala, S., Ciarricchi, G., Koch, G., Falaschi, A., & Pedrali-Noy, G. (1985a) *Arzneim. Forsch. Drug Res. 3*, 1108–1116.

Spadari, S., Focher, F., Kuenzle, G., Corey, E. J., Meyers, A. G., Hardt, N., Rebuzzini, A., Ciarricchi, G., & Pedrali-Noy, G. (1985b) *Antiviral Res. 5*, 93–102.

Sugino, A., & Nakayama, K. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 7049–7053.

Tseng, B. Y., & Ahlem, C. N. (1983) *J. Biol. Chem. 258*, 9845–9849.

Tsurimoto, T., & Stillman, B. (1991) *J. Biol. Chem. 266*, 1950–1960.