Sequence-Specific Actinomycin D Binding to Single-Stranded DNA Inhibits HIV Reverse Transcriptase and Other Polymerases†

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ABSTRACT: Primer extension assays using recombinant templates constructed to contain all 256 possible base quartets in a minimum length sequence were used to examine binding of the anticancer drug actinomycin D to *single-stranded* DNA. Single-stranded templates were generated by digestion of linearized plasmid with the double-strand-specific T7 gene 6 exonuclease. Actinomycin D formed high-affinity, kinetically stable complexes that paused primer elongation at specific sites by HIV-1 reverse transcriptase, Sequenase (modified T4 DNA polymerase), the Klenow fragment of *Escherichia coli* DNA polymerase, and Vent (exo-) DNA polymerase. Pauses occurred most commonly near G+C-rich nucleotide clusters, including GpC steps, the preferred sites of double-stranded DNA binding. Complexes were stable for several minutes at temperatures over 50 °C as determined by their abilities to pause Vent polymerase at elevated temperatures. Significant variations were noted in pause patterns of different polymerases, demonstrating differential responses of polymerases to a bound actinomycin. Covalent adducts formed on template DNA by a photoaffinity analog of actinomycin D completely stopped primer extension. These results support the possibility that actinomycin D inhibits transcription elongation by complexing single-stranded DNA in the open transcription complex. Single-stranded DNA binding by actinomycin D or analogs may also provide routes for combating HIV or other viruses which replicate through single-stranded intermediates.

Actinomycin D (Figure 1) is a well-known anticancer antibiotic that specifically inhibits the elongation phase of transcription (Goldberg et al., 1962; Reich & Goldberg, 1964; Goldberg & Friedman, 1971; Phillips & Crothers, 1986). The drug binds double-stranded DNA with high affinity and strong preferences for GpC and certain GpG sites (Muller & Crothers, 1968; Krugh & Chen, 1975; Aivasashvilli & Beabealashvilli, 1983; Chen, 1988; Rill et al., 1989; White & Phillips, 1989; Goodisman et al., 1992). Binding to GpC sites occurs by intercalation of the phenoxazinone chromophore into the GpC step, with the two cyclic pentapeptide rings snugly fit within the minor groove where they make hydrogen bonding contacts with guanines (Sobell & Jain, 1972; Lybrand et al., 1986; Wilson et al., 1986; Liu et al., 1991; Kamitori & Takusagawa, 1992). For over 20 years, the inhibition of transcription elongation has been attributed to double-stranded DNA binding because of early indications that actinomycin D had little affinity for RNA or single-stranded DNA (Reich & Goldberg, 1964; Müller & Crothers, 1968; Goldberg & Friedman, 1971; Sobell et al., 1971; Sobell & Jain, 1972; Kamitori & Takusagawa, 1992).

There have, however, been observations that actinomycin D inhibits synthesis of double-stranded DNA from RNA tumor viruses (McDonnell et al., 1970; Manly et al., 1971; Novak et al., 1979) and inhibits DNA−RNA and DNA−DNA (but not RNA−RNA) hybridization (Bunte et al., 1980). Recently, the suggestion of Bunte et al. (1980) that single-stranded DNA binds actinomycin D has been confirmed by the discovery of a few single-stranded oligonucleotides that bind the drug with affinities matching or exceeding those for binding to preferred sites in double-

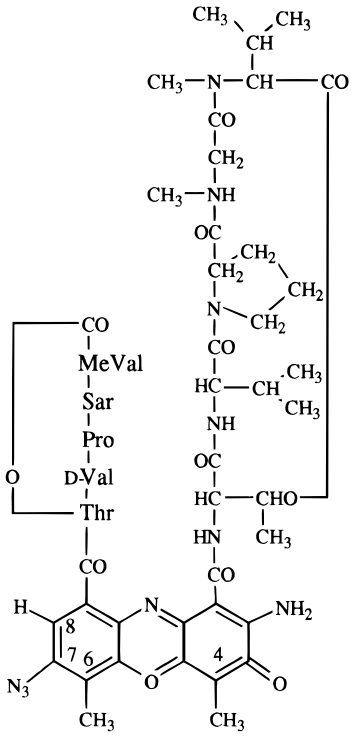

FIGURE 1: Structure of actinomycin D.

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stranded DNA (Wadkins & Jovin, 1991; Crenshaw et al., 1993). The implications of this finding for the mechanism of inhibition of transcription and the development of antiviral and anticancer drugs led us to examine the consequences of actinomycin D binding to single-stranded DNA templates on DNA synthesis.

The discovery of compounds with site-specific affinities for single-stranded DNA may assist development of drugs against HIV and other retroviruses because replication of their single-stranded RNA genomes procedes through a single-stranded DNA intermediate. RNA is first transcribed into an RNA/DNA hybrid by reverse transcriptase, the RNA strand is degraded, and then single-stranded DNA is copied into double-stranded DNA (Mitsuya et al., 1990; Haseltine & Wong-Staal, 1991; Wong-Staal & Haseltine, 1992). Integration of double-stranded DNA provirus into the host genome ensures reproduction of viral RNA. An essential control sequence of a retrovirus genome in any nucleic acid form is a potential Achilles' heel. Drugs aimed at these elements might directly interfere with replication (Zapp et al., 1993) and also promote mutations leading to less competent progeny. Drugs highly selective for single-stranded forms of the HIV genome should cause minimal damage to host genomic DNA.

We are not aware of any drugs designed or proven to act by binding single-stranded nucleic acids. Indeed, few methods have been applied to examining specific drug interactions with these nucleic acid forms. Here we describe the application of primer extension assays using template sequences tailored for high information content to detect sequence-specific binding of actinomycin D to single-stranded DNA, and to characterize the thermal stabilities and polymerase responses to complexes at specific sequences. These techniques are generally applicable to characterizing the binding of drugs to any single-stranded nucleic acid.

Actinomycin D strongly paused the progression of HIV reverse transcriptase and several DNA polymerases at specific sites along single-stranded DNA templates. The lifetimes of complexes were estimated to be many minutes. Use of the thermostable Vent (exo-) polymerase showed that some complexes were stable to 50−60 °C. The high stabilities of actinomycin complexes with single-stranded DNA are consistent with suggestions that the drug inhibits transcription elongation by binding single-stranded DNA in the open complex (Wadkins & Jovin, 1991; Sobell, 1985). Significant differences noted in the positions of pauses of different polymerases indicated that the responses of a polymerase to a bound drug depend on unique combinations of stereochemical and kinetic factors.

MATERIALS AND METHODS

DNA Polynucleotide Synthesis and Cloning. Polynucleotides were synthesized by the phosphoramidite method on an Applied Biosystems Model 391A DNA synthesizer, deblocked and desalted by standard methods. Polynucleotide and M13 primer concentrations were determined from absorbancies at 260 nm (Cantor et al., 1970).

Two polynucleotides were synthesized which together contained all possible base quartets plus other sequences to allow copying and cloning. One was 123 nt and the other 126 nt long (Figure 2). Syntheses (40 nmol) were carried out with double coupling. Full-length products were isolated

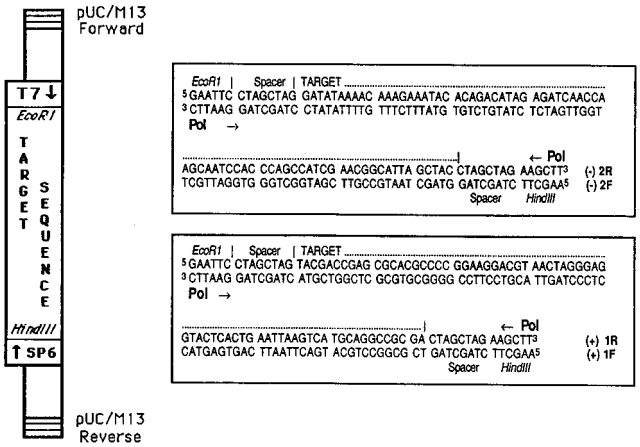

FIGURE 2: Sequences of cloned inserts jointly representing all possible 256 base quartets and schematic of the relevant part of the pGEM vector. The target sequence of each insert is flanked on both ends by a 5′-d(CTAGCTAG)-3′ spacer sequence, and by *Eco*RI or *Hin*dIII sites used for cloning into the pGEM-3Zf(+/−) vectors. The vectors contain counter-directed T7 and SP6 RNA polymerase promoters and pUC/M13 forward and reverse primer binding sites on opposite strands flanking the insert.

from a large excess of early termination products by electrophoresis on a 20% denaturing polyacrylamide gel. Sequences of the two polynucleotides were verified by dideoxy sequencing (Sanger et al., 1977) prior to cloning by using the Sequenase Version 2.0 Kit (Tabor & Richardson, 1987, 1990) (United States Biochemical Corp.) and an M13 primer hybridized to the complement located at the 3′-ends of the synthetic polynucleotides.

Double-stranded inserts for cloning were prepared from the synthetic single-stranded chains by extension of the M13 sequencing primer using ample concentrations of all four dNTPs, followed by consecutive digestion with *Hin*dIII (Promega) and *Eco*RI (Bethesda Research Laboratories). Inserts were cloned into the *Eco*RI/*Hin*dIII polylinker site of pGEM^R-3Zf(+) and pGEM^R-3Zf(−) vectors (Promega), and plasmids were amplified in *E. coli* strain JM109. Plasmids were isolated by standard base/acid extraction and further purified by treatment with RNase H, sodium dodecyl sulfate (SDS), and proteinase K, followed by phenol/chloroform extraction. The resulting plasmids are referred to as pKHQ-(+)1 and pKHQ-(−)2, where (+) and (−) refer to the cloning vector and the number 1 or 2 refers to the original single-stranded polynucleotide.

Primer Extension Assays. Single-stranded DNA templates were prepared using the "Pre-Sequencing Kit for Linear, Double Stranded DNA" from United States Biochemical Corp. The two double-stranded inserts represent a set of four different single-stranded nucleotide sequences which will be referred to as (+)1R and (+)1F, as well as (−)2R and (−)2F, where F and R indicate the accessibilities of the templates from the above vectors for the M13 reverse (R) or forward (F) primers. To prepare (+)1R and (−)2R, plasmids were linearized with the endonuclease *Ssp*I (Promega), which cuts at positions 2142, 2580, and 2604 in the plus vector; and at positions 2142, 2978, and 3002 in the minus vector. For (+)1F and (−)2F, plasmids were linearized by digestion with *Tfi*I (United States Biochemical Corp.), which cuts at positions 280 and 420 in either vector. Products were checked by electrophoresis. Linearized plasmids were used as substrates for treatment with bacteriophage

T7, gene 6 exonuclease, which has a double-strand-dependent, nonprocessive 5′ to 3′ exonuclease activity (Kerr & Sadowski, 1972) and yields single-stranded half-molecules. Inactivation of the exonuclease was achieved by heating of the reactions to 80 °C for 15 min, after which the products were checked by electrophoresis on an agarose gel.

Primer extension reactions involving the enzymes Sequenase (USB), Klenow fragment (Promega), HIV-RT (USB), Tth (USB), and Taq DNA polymerase (Promega) were carried out in Sequenase buffer (40 mM Tris·HCl, pH 7.5 at 25 °C, 20 mM $MgCl_2$, and 50 mM NaCl). The temperature at which the individual reactions were carried out varied for the labeling step as well as for the extending step, depending on the enzyme used, and will be specified below. Reactions in which Vent (exo-) (New England Biolabs) was used were carried out in Vent (exo-) buffer [10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris·HCl, pH 8.8 at 25 °C, 2 mM $MgSO_4$, and 0.1% Triton X-100]. The labeling step in Vent (exo-) reactions was performed at 37 °C, while the extending step was carried out at variable temperatures ranging from 37 to 73 °C (see below).

Single-stranded DNA templates for primer extension assays were generated in ca. 1 pmol aliquots in 10 μL reaction volumes, which were split into two portions for samples and controls. The appropriate primer was added (1:1 with template), M13Forward or M13Reverse depending on the template strand to be transcribed, and the primer was annealed by heating of the samples to 65 °C and slow-cooling to 35 °C, after which the samples were put on ice. A known concentration of actinomycin D in water, diluted from a 0.3 mM stock solution, was added to one portion, and the other was brought to the same volume with water. Both samples were incubated on ice for 30−45 min. Actinomycin D from Sigma was used without further purification. Concentrations were determined using an extinction coefficient of 24 500 M^{-1} cm^{-1} at 440 nm (Müller & Crothers, 1968). All steps which involved actinomycin D were carried out in amber microfuge tubes and under dimmed lights.

To the annealed template-primer on ice, in the presence and absence of actinomycin D, were added 1 μL of 0.1 M DTT, 2 μL of diluted (1:5) Labeling Mix (5× concentrate: dGTP, dCTP, and dTTP, 7.5 μM each), and 1 μL of [α-32P]-dATP (ca. 2.5 μCi). The reaction volume at this point was 15 μL. DNA polymerase (ca. 1.6 units/reaction) was added, and samples were incubated at room temperature for 2−3 min. After this labeling reaction, 2 μL of Sequence Extending Mix (all four dNTPs at 180 μM concentration in 50 mM NaCl) was added, and samples were incubated at 37 °C. Aliquots were removed at various time points, and DNA synthesis was terminated by adding two-thirds volume of Stop Solution (95% formamide, 20 mM EDTA, 0.05% Bromophenol Blue, and 0.05% Xylene Cyanol FF).

Standard sequencing reactions were carried out according to the supplier's protocol and loaded in lanes adjacent to drug reactions. Drug-induced pause or halt sites were assigned by comparison with sequencing lanes. Samples were electrophoresed on 8% denaturing acrylamide gels [18:1 acrylamide/bis(acrylamide)] in 1× TBE buffer [89 mM Tris−borate, 2 mM EDTA (pH 8.0)], 7 M urea. Autoradiograms were made using Kodak XAR-5 film (35 × 43 cm). Densitometer scans were obtained on a DeskTop scanner, Model DNA 35, analyzed, and quantitated using Quantity One One Dimensional Analysis Version 2.2 software from PDI, Inc.

Covalent Adduct Formation of 7-Azidoactinomycin D to Single-Stranded DNA. A stock solution of 5 mM 7-azidoactinomycin D (kindly provided by David Graves, University of Mississippi) was prepared in dimethyl sulfoxide. Single-stranded template for primer extension reactions was prepared as described above. Reactions in which 7-azidoactinomycin D was used were performed in Sequenase buffer (see above). Concentrations of the photoaffinity analogue used ranged from 62.5 μM to as low as 4.0 μM final concentration. All reactions were done in amber microfuge tubes, and all steps involving unphotolyzed 7-azidoactinomycin D were carried out under dimmed lights to prevent premature photolysis and random adduct formation.

Single-stranded template and the appropriate primer were annealed as described above. Samples were incubated with 7-azidoactinomycin D in the dark at 0 °C, and then photolyzed for 30 min by placing two General Electric F15T8/Daylight fluorescent bulbs 1 cm above the opened microfuge tubes kept on ice. Unreacted 7-azidoactinomycin D was extracted twice with water-saturated 1-butanol; then samples were dried and redissolved in water. Primer extension assays were performed on these samples as above.

RESULTS

Inhibition of Primer Extension by Actinomycin D. Binding of actinomycin D to single-stranded DNA was probed by extending a short primer with HIV-1 reverse transcriptase or other DNA polymerases on single-stranded templates in the presence of micromolar concentrations of the drug. The target sequences inserted into the templates used in these assays are unique, being specifically designed for high information content and minimum potential for formation of stable, local secondary structure. Two polynucleotides were synthesized that together, in double-strand form, contain all possible base quartets in a minimum length. Of the 256 possible base quartets, 240 define a group of 120 nonidentical complement pairs. The remaining 16 quartets define a group with identical complements; thus, there are 136 unique quartets in double-stranded DNA [see also Yanagi et al. (1991)]. Several "target" sequences representing all quartets were designed by a computer-aided approach minimizing redundancy. The shortest duplex lengths achieved, 144 bp, represented all 120 quartet pairs of the first group at least once (5 were represented twice), and the 16 quartet pairs of the second group only once. One strand of such a target sequence was chemically synthesized in two parts. Each part was flanked by sequences permitting synthesis of the opposite strand by extension of M13 primers and cloning as a double-stranded insert into pGEM-3Zf(+) or -3Zf(−) vectors (Figure 2). One of these inserts, KH(+)1, is G+C-rich, while the other, KH(−)2 is A+T-rich. A total of four single-stranded DNA templates, two from each of these inserts, were utilized for primer extension assays.

If a drug binds with high affinity and dissociates slowly from a single-stranded DNA template, then chain elongation should be paused or terminated when polymerase encounters an occupied site. In agreement with this expectation, actinomycin D caused accumulation of several discrete length, prematurely terminated DNA chains when a primer was extended on single-stranded DNA by HIV reverse

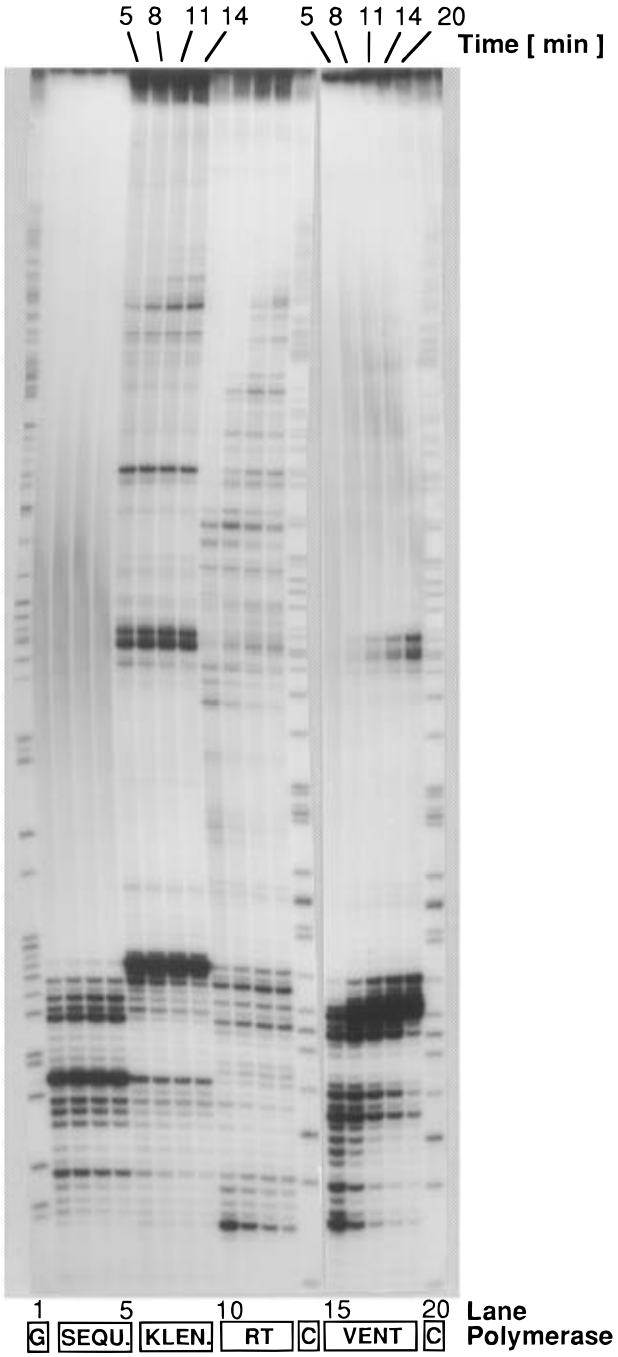

FIGURE 3: Pausing of primer extension on a single-stranded DNA template by actinomycin D. Single-stranded template DNA was prepared by digesting the pGEM-3Zf(+) vector (Promega) carrying the (+)1F/(+)1R target sequence insert (Figure 2) with *Ssp*I, followed by 5′ to 3′ digestion with T7 gene 6 exonuclease. Template was annealed with pUC/M13 forward primer, and then incubated with actinomycin D (30 μM) at 0 °C. Chain extension was performed as described under Materials and Methods at 50 °C for Vent (exo-) and at 37 °C for other polymerases. Aliquots were taken at the indicated times after initiating the extending step. Lane 1, dideoxy sequencing using ddCTP. Lanes 14, 20, sequencing using ddGTP. Set I (lanes 2−5, Sequenase), set II (lanes 6−9, Klenow fragment), set III (lanes 10−13, HIV-RT), and set IV [lanes 15−19, Vent (exo-)]. Reactions in sets I−III were performed in Sequenase buffer; the reaction of set IV was carried out in Vent (exo-) buffer.

transcriptase, the Klenow fragment of *E. coli* DNA polymerase I, modified T7 polymerase (Sequenase), or Vent (exo-) DNA polymerase (Figure 3). All four polymerases were paused at a small number of discrete sites at 30 μM concentrations of actinomycin D, and pauses were observed at a subset of these sites at an actinomycin D concentration of 7.5 μM (not shown, but see related data in Figure 7), demonstrating that the drug binds single-stranded DNA with high affinity (K_d in the micromolar range) and sequence selectivity.

The pauses induced by actinomycin D, with rare exceptions, did not correspond to natural pauses of the polymerases. This is illustrated for Sequenase in Figure 7, described below in more detail. Extensive studies of the intrinsic pause sites of Sequenase in the absence of drug yielded background pause patterns of low intensity and almost complete readthrough of the target sequences with all templates. While some propensities for pausing at G+C-rich tracts were noted, the pause patterns observed were weak and distinct from those observed in the presence of actinomycin. Like Sequenase, all other polymerases synthesized efficiently in the absence of drug and yielded predominantly long products remaining near the top of the gel. No discernible background bands due to natural pauses were observed for Vent (exo-) reactions, presumably because of the elevated temperature. Only faint backgrounds corresponding to a few weak pauses were noted in the target sequences of interest for reactions performed with Klenow fragment or HIV RT (not shown).

Actinomycin D must dissociate very slowly from preferred sites because pauses persisted for extension times of over 14 min. This was the case even for Vent (exo-), which was assayed at elevated temperature (≥50 °C) (Figures 3 and 4). Detailed analyses to extract site-specific rates of drug dissociation constants by analogy to prior studies of transcription inhibition by actinomycin D (Phillips & Crothers, 1986; White & Phillips, 1988) were not performed, but inspection of the amount of extension achieved over 14−20 min by stable polymerases such as Klenow or Vent indicates that half-lives of actinomycin D complexes at some preferred sites are at least 10 min, comparable to half-lives observed for binding to double-stranded DNA sites (Phillips & Crothers, 1986; Chen, 1988).

The activity of Vent polymerase at high temperature and over a wide temperature range allowed investigation of the thermal stabilities of site-specific complexes by primer extension. A single prominent actinomycin-induced pause of Vent polymerase on the (+)2R template was observed at 37 and 53 °C, but not at 65 °C (Figure 4), indicating that this complex was stable to well over 50 °C. A few downstream pause sites persisted to 65 °C.

Differential Responses of Polymerases to Bound Actinomycin D. Differences were noted in the abilities of polymerases to read through actinomycin binding sites, as well as in the locations and intensities of bands associated with specific pause sites (Figures 3 and 5). Actinomycin at a concentration of 30 μM was sufficient to strongly pause all four polymerases and limit production of long extension products, but modest binding site read-through was observed for all polymerases except Sequenase (Figure 3). The failure of Sequenase to extend past several prominent pauses early in the target can be rationalized in terms of the instability of the polymerase after dissociation from the template (Pisa-Williamson & Fuller, 1992) promoted by a drug encounter. The other polymerases are sufficiently stable, either as a template complex or in solution, to reinitiate synthesis after a pause, presumably when the drug dissociates.

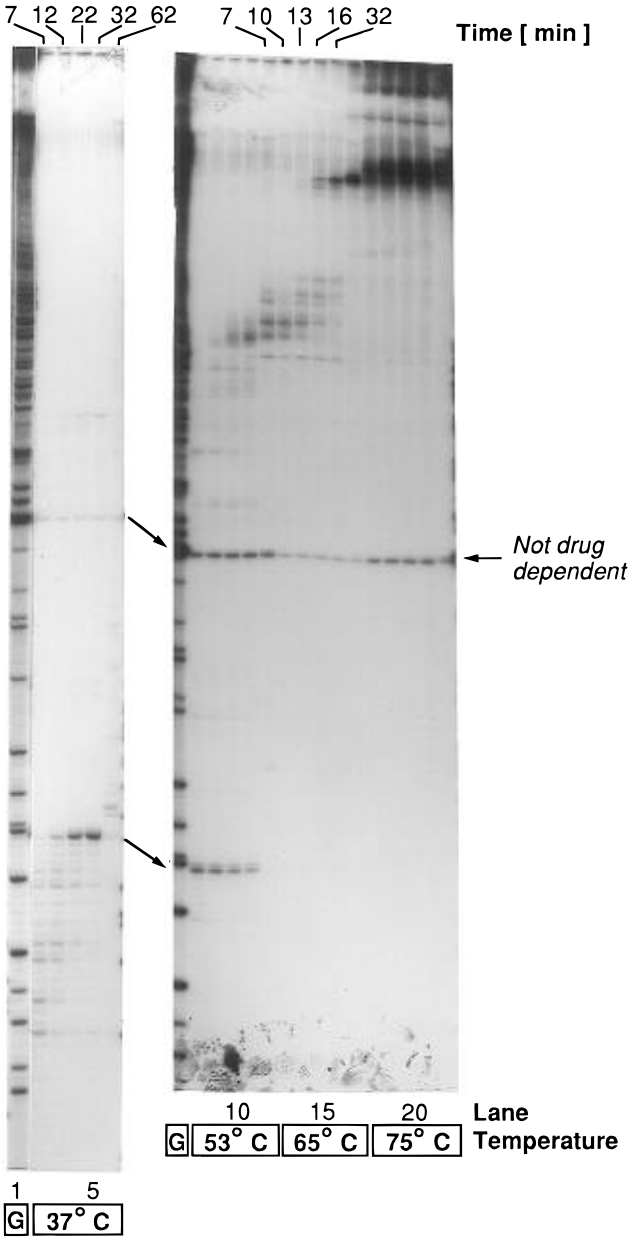

FIGURE 4: Temperature dependence of the pausing of Vent (exo-) polymerase by actinomycin D. Single-stranded DNA containing the (−)2R insert was prepared using endonuclease *Tfi*I and T7 exonuclease, annealed with pUC/M13 reverse primer, and then extended in the presence of 30 μM actinomycin D as described in Figure 3 for Vent (exo-) polymerase except for variations in the incubation temperature. Lanes 1, 6, dideoxy sequencing using ddCTP. Samples in the right plate were withdrawn after 7, 10, 13, 16, and 32 min incubation at the indicated temperatures. Many fewer (predominately one) pause sites were observed in the A+T-rich target sequence of this template (compare with Figure 3, see also Figure 5).

Remarkable variations were observed in the pause patterns of different polymerases. Particularly striking are the large number of pauses of HIV-RT on the (+)1F template, relative to the smaller number of pauses of Klenow and Vent polymerases (Figure 3). Some differences were also noted in the pauses of these polymerases in the absence of drug. These differences are not considered here since bands corresponding to natural pauses were much weaker than, and had little direct correlation with, the pauses induced by actinomycin D.

In general terms, there are at least two possible contributions to these differential responses. (i) If each pause is associated with a distinct actinomycin D binding site, then HIV-RT must be more sensitive to short-lived, presumably weaker, complexes than Klenow or Vent (exo-) polymerases. Such sensitivity could arise from a greater tendency of HIV-RT to dissociate from the template upon a direct encounter with the drug. (ii) Alternatively, several pauses could be associated with a single actinomycin D binding site if, as seems likely, the template wraps around the drug in a stem−loop or other configuration (see also below). The pattern of pauses in this case would depend on the steric constraints and relative dynamics of several processes including the residence time of the stalled polymerase, the ability of the polymerase to disrupt or otherwise read-through drug-induced secondary structures, the precise features and "breathing" rate of these structures, and the rate of drug dissociation.

Closer examination of the pause patterns suggests that both mechanisms may be operative. For example, Sequenase, HIV-RT, and Vent paused rather cleanly after incorporation of the complement to a C prior to two G's early in the (−)-2R template (Figure 5), but Klenow paused more or less evenly at both the C and the successive two G's, suggesting an ability to penetrate into the putative secondary structure surrounding the binding site. A second prominent, well-isolated, single base pause site further downstream in this template was observed for Sequenase and HIV-RT, but not for Klenow or Vent polymerases, suggesting that this is a weak, isolated binding site. Curiously, an additional isolated pause was observed for Klenow, but not the other polymerases, at an earlier (upstream) site. This observation negates any conclusion that Klenow is paused only at the highest affinity sites and draws attention to the complexity of factors governing polymerase pauses. Polymerases clearly differ in response to specific sequences or geometric features of a complex.

Cases analogous to those described for the (−)2R template were also observed on the (+)1F template, but are more difficult to analyze because of the greater complexity of pause sites. One additional feature of the pauses observed for the (+1)F template is noteworthy, namely, the smeared patterns near the middle or top of the lanes in the Sequenase and Vent reactions, respectively. Such smeared patterns were not highly reproducible in detail, but were commonly obtained at high drug concentrations leading to few fully extended chains. The simplest interpretation is that band spreading of the longest extension products results from copy infidelities, perhaps due to drug-induced frame shifts.

Sequence Selectivity of Actinomycin D Binding. A high degree of sequence selectivity was observed in the patterns of polymerase pauses. While several pauses were observed within the G+C-rich target sequence of the (+)1F template (Figure 3) and its complement (not shown, but see summary in Figure 6), no pauses were observed in the ≈60 nt preceding the targets, and few pauses were noted after the targets. Only two or three prominent pauses occurred on the A+T-rich (−)2R template (Figures 5 and 6), while no pauses of Sequenase were observed on its complement (−)-2F (not shown).

Most pauses occurred within or prior to G+C-rich clusters, as shown in summary form in Figure 6. A majority occurred at or 1−2 nucleotides upstream of GC steps, a theme

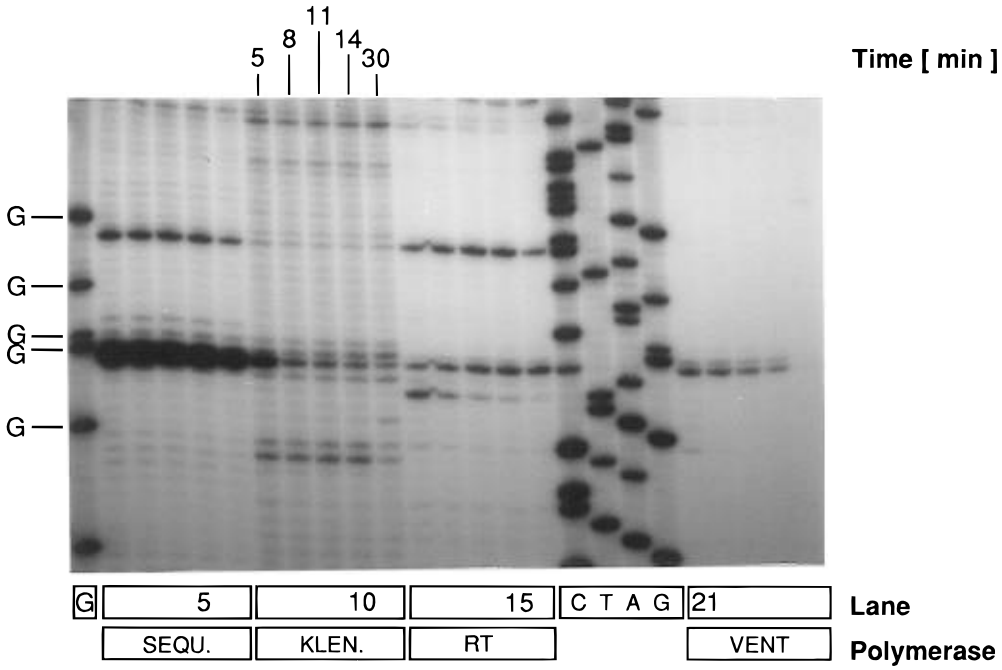

FIGURE 5: Polymerase-dependent pausing of primer extension. Reactions were performed using the (−)2R template and pUC/M13 reverse primer as described in Figure 3. Lanes 1 and 17−20 are sequencing lanes. Lanes 2−6, Sequenase; lanes 7−11, Klenow fragment; lanes 12−16, HIV-RT; lanes 21−25, Vent (exo-). Aliquots were taken at 5, 8, 11, 14, and 30 min after addition of enzyme.

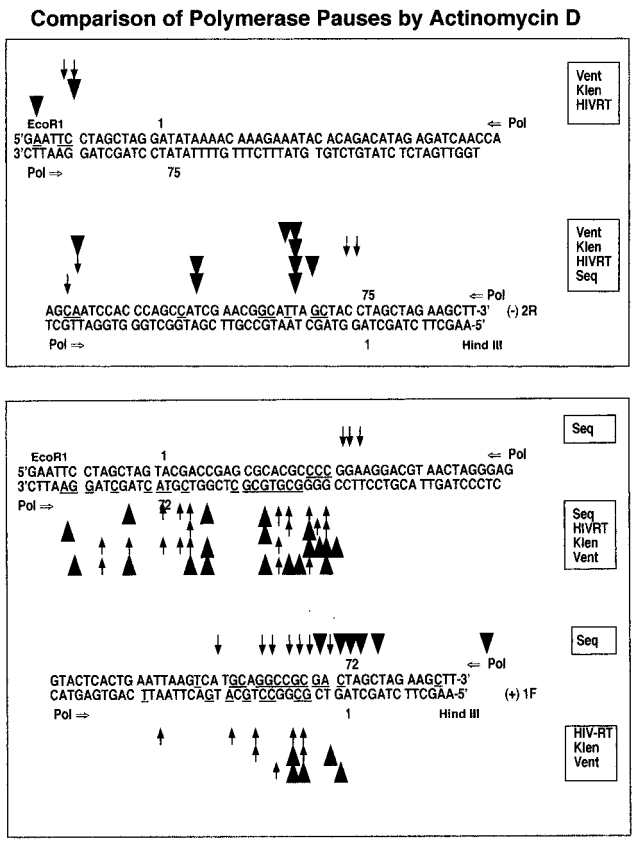

FIGURE 6: Summary of actinomycin D-dependent polymerase pause sites on the A+T-rich KH2 target sequence (top panel) and G+C-rich KH1 target sequence (bottom panel). Data for all polymerases were obtained with 30 μM actinomycin D. Strong pause sites are marked by large inverted triangles and other pause sites by arrows. Primer extension on the (−2)F template by Sequenase was not paused by actinomycin D. Experiments with other polymerases were not performed on this template.

observed by transcription footprinting on *double-stranded* DNA templates (Aivasashvilli & Beabealashvilli, 1983; White & Phillips, 1989). A GC step was neither necessary nor sufficient to stall polymerase, however, as some pauses occurred far from GC steps in the (+)1F templates, and several GC steps were bypassed, particularly in the (−)2R templates. Because the mechanism of actinomycin D binding to the template is unknown, there is a fundamental uncertainty in identifying the binding site of the drug with respect to the end of the terminated chain. Possible binding mechanisms are described under Discussion.

Photoaffinity Labeling of Single-Stranded DNA with an Analog of Actinomycin D. Inhibition of transcription or replication would be maximized if a sequence-specific drug could be covalently linked to the template, preventing polymerase action past the adduct. This effect was demonstrated using 7-azidoactinomycin D, a photoaffinity analog of actinomycin D (Graves & Wadkins, 1989; Rill et al., 1989). Photolysis of 7-azidoactinomycin D noncovalently bound to a single-stranded DNA template led to covalent adducts at a few sites [not shown, but see Rill et al. (1989)]. Elongation of a primer by Sequenase was completely blocked at specific sites on the covalently modified template at drug concentrations that allowed synthesis of some full-length products in the absence of photolysis (covalent binding) (Figure 7). The pause pattern observed for *noncovalently* bound azidoactinomycin D closely paralleled that of the parent actinomycin D (compare with Figure 3). There were, however, significant differences in patterns observed for noncovalently bound azidoactinomycin D and drug covalently bound after photolysis. In some, but not all, instances the pauses at covalent adducts were 1−2 nucleotides downstream of pauses at noncovalent complexes (arrows, Figure 7), as expected if induced secondary or tertiary structure halts the polymerase prior to the actual binding site.

Two other distinctions between these pause patterns are noteworthy. Pauses at noncovalent binding sites were not equally represented by pauses at covalent adducts, indicating

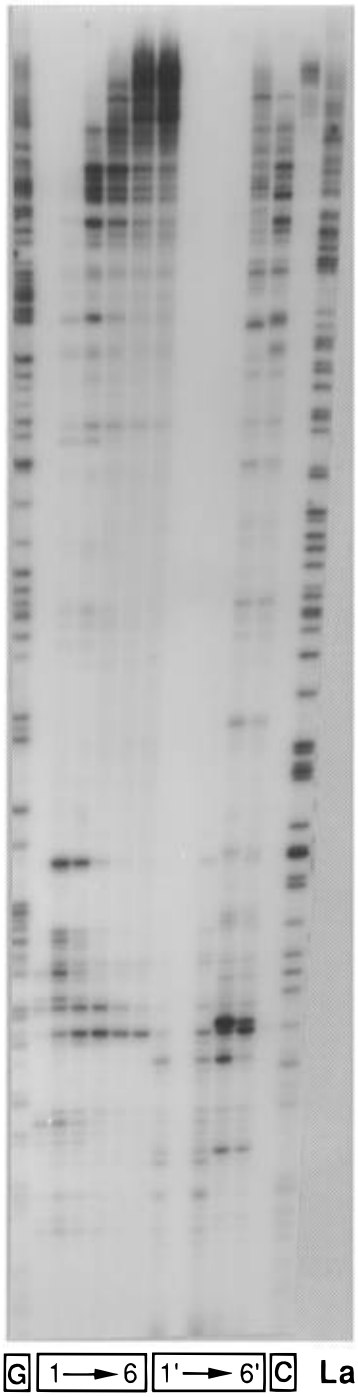

FIGURE 7: Termination of primer extension by covalent adducts formed after photoaffinity labeling of single-stranded DNA with 7-azidoactinomycin D (generously provided by David E. Graves, University of Mississippi). The template (+)1F was prepared and annealed with primer, and the primer was extended with Sequenase as described in Figure 3. 7-Azidoactinomycin D reactions were performed in Sequenase buffer using drug concentrations of 63 μM (lanes 1 and 1′), 31 μM (lanes 2 and 2′), 16 μM (lanes 3 and 3′), 8 μM (lanes 4 and 4′), 4 μM (lanes 5 and 5′), and 0 μM (lanes 6 and 6′). (No samples was loaded in lane 2′.) Reactions in lanes 1−6 were carried out in the dark to prevent photolysis and covalent adduct formation; hence, pauses of Sequenase were due to noncovalent complexes. Significant amounts of full-length extension products were apparent below 31 μM drug. Reactions in lanes 1′−6′ were photolyzed, and drug not covalently bound to DNA was extracted with 1-butanol before primer extension. No full-length extension products were observed for 62.5 μM (lane 1′) and 15.6 μM (lane 3′) 7-azidoactinomycin D, and long extension products were significantly reduced for drug concentrations down to 4.0 μM (lane 5′).

either differences in photolabeling efficiency or differences in the responses of polymerase to adducts at these sites. Both interpretations imply site-specific variations in the actinomycin binding mechanism. In addition, covalent adducts caused pauses at sites not observed in the absence of photolysis. Since such pauses were observed at low drug doses (4, 8, and 16 μM), there must be sites where actinomycin binds noncovalently with high affinity, yet does not inhibit the polymerase, perhaps because the drug dissociation rates are high at these sites. However, the time that the drug spends in association with the template must be sufficient to allow for photoinduced cross-linking leading to adducts that pause polymerization.

DISCUSSION

Possible Mechanisms of Single-Stranded DNA Template Binding of Actinomycin D. We have demonstrated that actinomycin D forms complexes at specific sites on single-stranded DNA templates with sufficient stability to inhibit DNA polymerases and HIV reverse transcriptase. It is most reasonable to suppose that single-stranded DNA binding by actinomycin occurs in a manner retaining major features of the double-strand complex, with the actinomycin chromophore intercalated between two bases on at least one strand, and additional parts of the strand shielding the hydrophobic polypeptide chains from solvent (Sobell & Jain, 1972; Kamitori & Takusagawa, 1992). That is, the DNA chain is likely to wrap in some fashion about the polypeptides with the chromophore intercalated between bases or base pairs.

There are two general approaches to wrapping DNA around the drug—one places actinomycin in a more or less "normal" double-stranded context, and the other does not. The former is most consistent with known features of actinomycin D, and is most easily conceptualized. In this case, actinomycin must strongly induce double-helix formation. Actinomycin intercalation into local stem−loop structures is one possibility which we strongly favor for certain sites. It should be noted, however, that the construction of the target sequences examined assures that stems cannot contain more than three, and seldom more than two, Watson−Crick base pairs. In experiments to be described elsewhere, we have shown that oligonucleotides homologous to sequences downstream of certain prominent pause sites tightly bind actinomycin D ($K_a > 10^6$ M^{-1}). Electrophoretic and preliminary two-dimensional NMR structure analyses strongly suggest that these oligomers form stem−loop structures involving non-Watson−Crick base pairs *in the absence of actinomycin D*, and that the drug binds to and stabilizes these structures (Leila Su and Rill, in preparation).

Duplex formation between short regions far removed in the primary sequence could also be induced by actinomycin D. Such occurrences would be consistent with the high incidence of GC steps at or downstream of pause sites, but there are no long regions of homology between the targets and downstream sequences in the vector. An ability of actinomycin to induce formation of short duplexes when the individual sequences are present at only ≈1pM concentration would be extraordinary and of potential physiological significance. We cannot rule out this possibility, but consider it unlikely as a universal binding mechanism because of the strong 1:1 binding of actinomycin to single-stranded oligo-

nucleotides demonstrated in our laboratory and elsewhere (Wadkins & Jovin, 1991; Crenshaw et al. 1993).

The occurrences of multiple binding mechanisms would not be surprising considering the great conformational variability of single-stranded DNA, and would be consistent with differences observed in pauses induced by covalent vs noncovalent binding by azidoactinomycin D. Induced secondary structures could stall the polymerase some distance from the binding site center defined by the putative pseudo-intercalation site. It is premature to conclude that GC steps are the actual actinomycin binding sites when they might instead assist in stabilizing secondary structure. More detailed analyses of the sequence contexts and structures of pause sites are in progress.

Primer Extension and Related Assays Using Defined Target Sequences. The target sequences of these experiments represent all base quartets in a minimal sequence length. They provide templates for comprehensive examination of the influences of nearest and next-nearest base neighbors on the binding of small molecule drug candidates to any nucleic acid—single- or double-stranded—by numerous assay methods (Tullius, 1989; Goodisman et al. 1992). Since the target sequences have minimal complementarity within each strand, interactions of single-stranded RNA or DNA can be studied with few complications from stable secondary structures with Watson−Crick base pairs. The pGEM vectors permit amplification of the target sequences in any nucleic acid form. Flanking T7 and SP6 promoters allow production of single-stranded RNA from either strand. Double-stranded RNA can be obtained by annealing the complements. M13 primer binding sites allow primer-initiated reverse transcription to form RNA/DNA hybrids from RNA templates. Single-stranded DNA can be obtained in several ways.

The primer extension assay provides a systematic *in vitro* approach to determining whether drugs bind site-specifically to single-stranded DNA or RNA with sufficient stability to inhibit copying of the genetic information. Variations in the assay conditions allow investigations of site-specific thermal stabilities (using Vent polymerase) or dissociation kinetics of complexes. If bidirectional transcription footprinting (White & Phillips, 1989) is also performed with RNA polymerase on the same target sequences in double-stranded DNA form, then the efficiencies and consequences of binding to single- and double-stranded nucleic acids can be compared.

Functional Significance of Actinomycin Binding to Single-Stranded Templates. The observation that actinomycin D can pause DNA polymerases acting on a single-stranded template is consistent with prior suggestions that the drug may inhibit RNA transcript elongation by binding to the open complex (Sobell, 1985; Wadkins & Jovin, 1991). A more definitive statement cannot be made without understanding the mechanisms of drug binding to these templates. These observations also provide an explanation for inhibition of retroviral replication (McDonnell et al., 1970; Manly et al., 1971; Novak et al., 1979) and DNA hybridization (Bunte et al., 1980) by the drug.

To our knowledge, actinomycin D is the first "small molecule" drug demonstrated to bind single-stranded DNA with high affinity [$K_a = 10^6-10^7$ M^{-1} oligomer (Wadkins & Jovin, 1991; Crenshaw et al., 1993)] and sequence selectivity. Regardless of the mechanism, the inhibition of DNA polymerases by actinomycin D or related compounds may provide an approach to combating HIV and other viruses that replicate through a single-stranded DNA intermediate. The observation that DNA polymerases differ in their responses to an encounter with actinomycin D as they travel along the template opens the possibility that drugs may discriminate between viral and host polymerases. Identification of drugs with selective affinities for single-stranded DNA unique to viral infections should minimize cellular toxicity and mutagenicity. Screening of small molecules that bind double-stranded DNA for unsuspected high affinities for other nucleic acid forms is one starting point in identifying lead compounds for drug development. Illustrating the point are observations that the anticancer antibiotic actinomycin D, which was discovered over 50 years ago (Waksman & Woodruff, 1940; Brockmann, 1974) and subjected to intense scrutiny, possesses a previously unsuspected strong affinity for a few sites in single-stranded DNA and effectively blocks replication through these sites.

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