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Reverse Transcription, a Probe by the Immobilized Template Poly(adenylic acid)-Agarose

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SUMMARY

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We have used poly(A)-agarose as an immobilized template to identify certain binding interactions which occur during polymerization of DNA and to analyze the mode of action of several drugs which inhibit oncornavirus DNA synthesis. In addition to demonstrating the binding of Moloney murine leukemia virus DNA polymerase (reverse transcriptase) to this template (both primed and unprimed), we analyzed the effects of nucleotides and several ansamycins (streptoval C, rifamycin SV, rifazone 8_2, and demethyldimethylbenzylrifampicin) on the stability of these binding interactions. The new poly(A)-agarose system allows more precise identification of the steps in reverse transcription which are targets for drugs previously known to inhibit the over-all reaction; thus this novel approach to the study of DNA polymerization on a solid matrix may afford an alternative rationale for designing antiviral drugs.

INTRODUCTION

The ansamycins, which include the streptovaricins (1-3) and rifamycins (4-8), are known to inhibit the RNA-directed DNA polymerase, or reverse transcriptase, of various RNA tumor viruses. In order to understand this mechanism of inhibition, we analyzed the process of DNA polymerization by murine reverse transcriptase in the presence and absence of these drugs. Previously poly(C)-agarose was shown to be an effective affinity ligand in purifying avian myeloblastosis virus DNA polymerase (9); thus we reasoned that analysis of the interaction between reverse transcriptase and an immobilized template might shed some light on the polymerization process itself. We now report the development of poly(A)-agarose as an immobilized template and the isolation of several steps in reverse transcription which are specifically inhibited by certain ansamycin antibiotics.

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METHODS

Materials. The templates and primers poly(dC)·oligo$(dG)_{12-18}$, oligo$(dT)_{12-18}$, and

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oligo(dT)$_4$ were purchased from Collaborative Research, Inc.; poly(A) and poly(dT), from Miles Laboratories; and poly(A) covalently attached to CNBr-activated agarose (1 mg/ml) [poly(A)-agarose], from P-L Biochemicals. Chemicals used for the preparation of buffers and assays were all reagent grade; Triton X-100 was obtained from Beckman Instruments. [^{3}H]dTTP and [^{3}H]dGTP were obtained from New England Nuclear in a solution of ethanol-water (1:1), which was dried and reconstituted with distilled water before use. The nucleotides used in all experiments were products of General Biochemicals. Streptoval C (10) was prepared from streptovaricin complex supplied by the Upjohn Company, and rifamycin SV was obtained from Schwarz/Mann. The rifamycin SV derivative demethyldimethylbenzylrifampicin was supplied by Dr. Robert Gallo (8), and rifazone 8$_2$ and the new derivative rifazacyclo 16 were generous gifts of Dr. Melvin Calvin and his colleagues (5, 11).

MoMuLV[3] *DNA polymerase*. The Moloney murine leukemia virus was purified (12); crude MoMuLV DNA polymerase was obtained by incubating 5 μl (11 mg/ml) of virus in 100 μl of disruption buffer A [10 mM Tris-HCl (pH 7.9), 1 mM dithiothreitol, 0.1 mM EDTA, 0.5 M KCl, 0.05% Triton X-100, and 20% glycerol] for 30 min at 4° and then diluting it with 400 μl of buffer B [50 mM Tris-HCl (pH 7.8), 1 mM EDTA, 1 mM 2-mercaptoethanol, and 20% (v/v) glycerol] (12).

DNA polymerase assays. RNA-directed DNA polymerase was assayed in a 400-μl reaction volume (except where noted) containing 200 μl of polymerase in buffer B and 40 μl of 0.5 M Tris-HCl, pH 8.3, with 0.6 M NaCl, 32 μl of 0.25 M dithiothreitol, 16 μl of oligo(dT)$_{12-18}$ (1.25 A_{260} units/ml), 40 μl of 0.25 M [^{3}H]dTTP or [^{3}H]dGTP (specific activity, 2400 dpm/pmole), 8 μl of 0.05 M $MnCl_2$, and 8 μl of H_2O. Reaction mixtures were incubated (except where noted) for 1 hr at 37° in 2-ml test tubes; trichloracetic acid-precipitable radioactivity was then determined on 50-μl aliquots (13).

Preparation of immobilized template-enzyme complexes. The purification of DNA polymerase from MoMuLV and determination of its relative binding affinities on poly(A)-agarose were carried out at 4° in a 1-ml plastic tuberculin syringe (Becton-Dickinson) that had been washed with 2 ml of buffer B and contained a glass wool plug and 100 μl of a poly(A)-agarose slurry. The packed columns were washed with 5 ml of buffer B. A template-primer complex was formed by adding buffer containing 355 μl of buffer B, 40 μl of oligo(dT)$_{12-18}$ (1.25 A_{260} units/ml), and 5 μl of 5% Triton X-100 to a poly(A)-agarose-containing column, followed by a 1-ml buffer B wash. An enzyme-template complex was prepared from a poly(A)-agarose column by adding 100 μl of polymerase and then washing with buffer B (3 ml). To form enzyme-template-primer complexes, 400 μl of buffer containing 355 μl of buffer B, 40 μl of oligo(dT)$_{12-18}$ (1.25 A_{260} units/ml), and 5 μl of 5% Triton X-100 were passed through a column already containing the enzyme-template complex; columns were then washed with buffer B (1 ml) immediately before use.

Stability of immobilized template complexes. The affinity of enzyme bound to template-primer in the presence of nucleotides was determined simply by passing a solution (800 μl) consisting of 590 μl of buffer B, 140 μl of poly(dC)·oligo(dG)$_{12-18}$ (1 A_{260} unit/ml), 20 μl of 15 mM nucleotide, 40 μl of water, and 10 μl of 5% Triton X-100 through the column; we obtained four 200-μl fractions and assayed each for polymerase activity (as described above) in the presence of 50 μM poly(A).

Ansamycin effect on immobilized template complexes. The ansamycins were first dissolved in dimethyl sulfoxide and diluted with buffer B (200 μl) to give a final concentration of 2% dimethyl sulfoxide and 100 μg/ml of drug; they were then applied to columns containing either a template-primer, an enzyme-template, or an enzyme-template-primer complex. The column effluents were first assayed for polymerase activity in the presence of 0.05% Triton X-100 and 50 μM poly(A); after these fractions had been taken, all columns received buffer B (1 ml) to remove unbound drug. After the various drug complexes had been formed, we removed

[3] The abbreviation used is: MoMuLV, Moloney murine leukemia virus.

the tips of the syringes, which allowed the agarose to be extruded into a test tube; the syringe interior was then washed with buffer B (200 μl), and the polymerase activity was determined. In each experiment, it was of course necessary to add back any component deleted during complex formation before measuring polymerase; for example, to the template-primer-drug complex, we added back the polymerase itself. Appropriate controls were performed for each experiment in the absence of drug; total trichloracetic acid-precipitable radioactivity was determined after hydrolysis for 2 hr at 37° with 1 N NaOH.

RESULTS

To use poly(A)-agarose as an immobilized template in the study of interactions occurring during polymerization and its inhibition by ansamycins, it was first necessary to show that the polymerase became bound to the poly(A)—i.e., not directly to the matrix—when interacting with poly(A)-agarose. We accomplished this by demonstrating that the MoMuLV DNA polymerase can be bound to poly(A)-agarose and will subsequently synthesize poly(dT) when supplied with appropriate cofactors (Table 1). Detergent-disrupted MoMuLV was first passed through a column containing poly(A)-agarose; the column was then washed and polymerase activity bound to the poly(A)-agarose was measured as described in the legend to Table 1. The polymerization process clearly requires a primer, complementary nucleotides, and the presence of poly(A); it yields products which are both attached to poly(A)-agarose and free in solution. Second, we found polymerase activity with the bound template to be about 20% of the activity obtained with a soluble template (Table 1). Additionally, polymerase binding to poly(A)-agarose can be either markedly stabilized by the addition of primer or actually destabilized by complementary nucleotides (see below), effects not seen with polymerase bound to deactivated CNBr agarose.[4] Since this nonspecific binding of polymerase to deactivated CNBr agarose is not modulated by primer-

TABLE 1

Polymerization with poly(A)-agarose as template

Polymerase was bound as described to poly(A)-agarose or CNBr-activated agarose deactivated in 1 mM HCl. The extruded poly(A)-agarose (or deactivated agarose) was suspended in buffer B (600 μl) and assayed in 1200 μl of a standard reaction mixture (with exceptions as noted). After incubation of the complete mixture at 37° for 15 min, the amount of trichloracetic acid-precipitable radioactivity was determined for a supernatant sample (50 μl) and a mixture sample [100 μl, for poly(A)-agarose-bound incorporation] after hydrolysis in 1 N NaOH (1 ml for 2 hr at 37°).

Incubation mixture	dTTP incorporated	
	In supernatant	Bound to template
	pmoles/15 min	
Complete	40	190
Omit oligo(dT)$_{12-18}$	0	0
Omit enzyme	0	0
Substitute [^{3}H]GTP for [^{3}H]TTP	5	0
Use CNBr-activated agarose deactivated in 1 mM HCl	0	0
Substitute 50 μM poly(A) for poly(A)-agarose	1100	

template or nucleotides, it has no impact on the interpretation of modulatory effects seen upon binding of polymerase to poly(A)-agarose. Thus we concluded that poly(A)-agarose is a template for reverse transcriptase and that this interaction can be specifically modulated by primer and nucleotides.

To determine whether nucleotides or polynucleotides compete with the poly(A)-agarose for bound polymerase activity, we then analyzed the conditions of displacement of polymerase from poly(A)-agarose (Table 2). Once again, polymerase was first bound to poly(A)-agarose by passing MoMuLV (detergent-disrupted) through a poly(A)-agarose column; a buffer wash then removed any unbound polymerase. Next a nucleotide or polynucleotide solution was passed through the column, after which we could measure the amount of polymerase activity which had been displaced. At relatively high concentrations, neither dGTP, dATP, ATP, dTTP, oligo(dT)$_4$, oligo(dT)$_{12-18}$, nor poly(dT) alone was capable of displacing significant

[4] Unpublished observations.

TABLE 2

Displacement of enzyme from poly(A)-agarose by various nucleotides

After the preparation of complexes as described in METHODS, enzyme was eluted with a solution (400 μl) consisting of buffer B (345 μl) and either nucleotide (10 μl, 15 mM) and/or primer (40 μl, 1 unit/ml) and 5 μl of 5% Triton X-100. A fraction (200 μl) was taken and assayed for polymerase activity as described in the text.

Column washed with	Polymerase activity of wash
	pmoles dTTP incorporated
Buffer B (400 μl) with 0.05% Triton	0
0.3 M KCl in buffer B with 0.05% Triton	1200
Oligo(dT)$_4$, 10 μM	0
Oligo(dT)$_{12-18}$, 10 μM	0.5
Poly(dT), 10 μM	2
dGTP, 360 μM	7.5
dATP, 360 μM	2.5
ATP, 360 μM	2.5
dTTP, 360 μM	1.3
dT$_{12-18}$, 10μM, + dTTP, 360 μM	2
dT$_{12-18}$, 10 μM, + dTTP, 360 μM, + Mn^{++}, 1 mM	0
Poly(dC)·oligo(dG)$_{12-18}$, 10 μM	75

amounts of enzyme; similarly, a complete polymerization mixture was unable to displace the polymerase. As expected, 0.3 M KCl removed all bound enzyme. Poly(dC)·oligo(dG)$_{12-18}$, which is an alternative template-primer for this DNA polymerase, was also capable of removing a portion (about 5–10%) of polymerase already bound to poly(A)-agarose, even when the alternative template was present at a 5-fold lower concentration than the poly(A).

Since poly(dC)·oligo(dG)$_{12-18}$ could compete for polymerase, this gave us an opportunity to determine the effects of nucleotides on polymerase displacement by the alternative template. We anticipated that binding of certain nucleotides to an enzyme-template-primer complex might lead to a more stable complex and hence to less displacement by the alternative template poly(dC)·oligo(dG)$_{12-18}$. The enzyme-template complexes were prepared by passing MoMuLV (detergent-disrupted) through a poly(A)-agarose column, followed by a buffer wash. We then prepared enzyme-template-primer complexes from these enzyme-template complexes by passing an oligo(dT)$_{12-18}$ solution through each column and washing with buffer. Nucleotides were then passed through each column, and the eluents were assayed for polymerase. Since these experiments were performed in the absence of manganese ion to prevent polymerization, we refer to these complexes as abortive complexes.

Figure 1 shows the modulatory effect of nucleotides on this displacement reaction. Enzyme displacement, in the absence of divalent cation, increases linearly with all nucleotides used. We presume that the displacement of bound polymerase from poly(A)-agarose depends on both the concentration and affinity of the competing ligand; thus it seems likely that the slopes of these lines are a function of the binding interaction between polymerase and poly(A)-agarose in the presence of the nucleotides; i.e., a greater slope corresponds to a weaker interaction. A number of conclusions are immediately evident from Fig. 1: (a) the interaction between polymerase and template is markedly stabilized by addition of primer [oligo(dT)] (Fig.1A); (b) the addition of nucleotides which are complementary to the immobilized template—like dTTP, dTDP, and dTMP—has a substantial effect (4–6-fold) in accelerating the displacement of bound polymerase (Fig. 1B) while the addition of noncomplementary nucleotides affects polymerase-template stability no more than 1.5–2.5-fold (Fig. 1C and D); (c) thymidine itself has little effect (Fig. 1D) while manganese ion causes rapid displacement of polymerase from the immobilized template (Fig. 1A).

On the basis of these results, we carried out a series of experiments with certain ansamycins to determine at which step of DNA polymerase complex formation they might exert their effects. Enzyme-template and enzyme-template-primer complexes were prepared from detergent-disrupted MoMuLV, poly(A)-agarose, and oligo(dT)$_{12-18}$ (where appropriate) as described above. The drugs (in buffer) were then passed through each column and the eluents were assayed for polymerase activ-

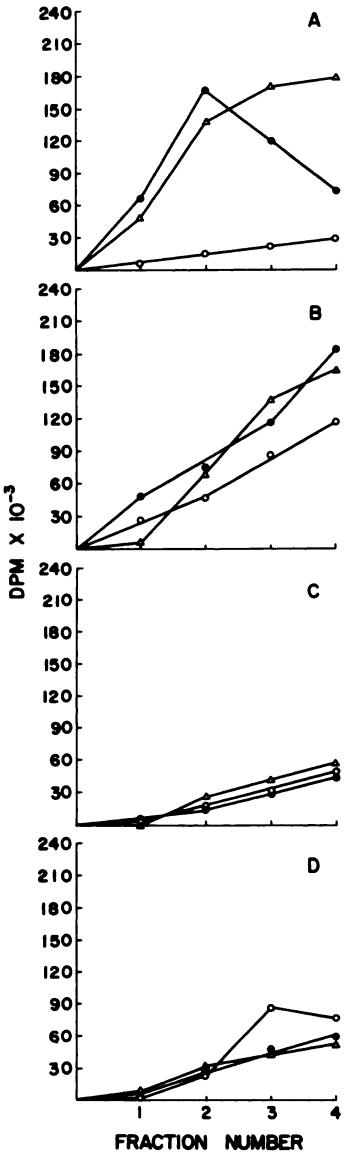

FIG. 1. *Displacement of reverse transcriptase bound to poly(A)·oligo(dT)-agarose columns by poly(dC)·oligo(dG)$_{12-18}$ in the presence of nucleotides and nucleosides*

The enzyme and template were first bound to agarose columns as described. The enzyme was then eluted with buffer B (800 μl) containing 0.05% Triton X-100, 10 μM poly(dC)·oligo(dG)$_{12-18}$, and the appropriate nucleotide (360 μM). Fractions (200 μl) were collected, and the amount of polymerase released was determined. A. ●——●, no primer or nucleotide added; ○——○, primer but no nucleotide; △——△, 1 mM $MnCl_2$ and no nucleotide. B. ●——●, dTMP; ○——○, dTTP; △——△, dTDP. C. ●——●, dCTP; ○——○, dATP; △——△, dCMP. D. ●——●, ATP; ○——○, UTP; △——△, thymidine. Measurements were performed in triplicate, and the results proved reproducible to within 10% [they were also corrected for polymerase inhibition by poly(dC)·oligo(dG)$_{12-18}$ and the various nucleotides or nucleosides].

ity; after a buffer wash of each column, the amount of remaining bound polymerase could be determined. The principal step in complex formation affected by each drug was determined by observing the amount of polymerizing activity remaining after the drug interacted with a template-primer (Table 3, column 2), polymerase-template (column 3), or polymerase-template-primer complex (column 4) and comparing this with the relative amount of polymerase displaced from a polymerase-template (column 5) or polymerase-template-primer complex (column 6). Additionally, we measured the amount of polymerase activity when unbound drug was still present in the column bed (column 1).

From our results (Table 3) one immediately notes that levels of inhibition of the MoMuLV reverse transcriptase, as displayed in our new solid phase (immobilized template) assay (column 1), are quite similar to those reported by others using these compounds in a totally soluble assay (column 7). The effects of these drugs on complexes of a template and primer, a polymerase and template, and a polymerase, template, and primer are shown in columns 2–4 of Table 3; columns 5 and 6 indicate the amount of polymerase activity displaced from the polymerase-template and polymerase-template-primer complexes, respectively, by the inhibitor. (In the absence of inhibitor, less than 1500 dpm of polymerase activity could be displaced.)

Streptoval C appears to inhibit reverse transcription only when added to a polymerase-template complex prior to primer addition (cf. columns 3 and 4); in addition, it has no apparent ability to cause displacement of the polymerase (columns 5 and 6). Rifamycin SV appears to have a similar mode of action although it can affect other complexes to a slight degree; for example, it inhibits (about 25%) when the polymerase is already bound to a template-primer complex (column 4). Although demethyldi-

TABLE 3

Inhibition by ansamycins of specific complexes formed during reverse transcription

The preparation of complexes and assays were performed as described in METHODS. Inhibition is reported as a percentage of control. The background of displacement (columns 5 and 6) was 1500 dpm. Controls lacking drug (columns 2-4) yielded 225,000 dpm.

Drug (100 μg/ml)	1. Solid phase assay for over-all reaction	2. Effect on template and primer	3. Effect on polymerase and template	4. Effect on polymerase, template, and primer	5. Polymerase activity displaced from poly(A)-agarose	6. Polymerase activity displaced from poly(A)-agarose·oligo (dT)	7. Previously reported inhibition
	% control				*dpm*	*dpm*	*% control*
Streptoval C	18	83	1	86	1,500	900	20[a]
Rifamycin SV	49	64	38	75	3,300	2,100	61 (8)
Demethyldimethylbenzylrifampicin	20	102	79	93	4,500	3,300	1 (8)
Rifazone 8_2	0	47	0	0	18,000	7,800	50[b] (5)

[a] Unpublished data.

[b] Fifty per cent inhibition at 2.5 μg/ml.

methylbenzylrifampicin is an extremely active inhibitor of the over-all reaction (columns 1 and 7), interestingly, it does not appear to affect substantially any of the complexes which we measured. Since the assay measures only effects on previously formed complexes, we can postulate that this derivative actually blocks the formation of one (or more) of these complexes. Finally, rifazone 8_2 is a particularly interesting derivative because it is a potent inhibitor of all the complexes; it can inhibit polymerization when added to either previously formed template-primer complexes, polymerase-template complexes, or polymerase-template-primer complexes. A portion of this inhibition may be due to the fact that this inhibitor causes the displacement of significant amounts of polymerase (approximately 18,000 dpm, column 5).

DISCUSSION

Polynucleotides covalently attached to agarose have been shown previously to be effective tools for purification of certain proteins (9, 14, 15). We now report their first use as a tool to dissect the process of DNA polymerization by a murine reverse transcriptase. Additionally, we used this immobilized template approach to analyze a series of interactions which may occur during the over-all reaction of DNA synthesis, and to determine which of these interactions are blocked by various ansamycin inhibitors. We can also show that the immobilized interaction clearly simulates a natural DNA polymerase-template reaction, since polymerization occurs only when necessary cofactors are present. Therefore poly(A)-agarose can be used as a new probe of the polymerization process itself because it permits the ready isolation of intermediate structures in the polymerization process.

Features of the binding interaction between the DNA polymerase and the immobilized template include the observation that the binding cannot be substantially weakened by the addition of nucleotides or primer but can be reversed with poly(dC)·oligo$(dG)_{12-18}$, which is an alternative template. Subsequently we utilized the competition by poly(dC)·oligo$(dG)_{12-18}$ for DNA polymerase already bound to an immobilized template to measure the destabilizing or stabilizing effects of nucleotides.

By this method, we can show that the interaction between DNA polymerase and its immobilized template is markedly stabilized by the presence of an appropriate primer; no further stabilization can be observed by the addition of either nucleotides complementary or noncomplementary to the poly(A). To the contrary, complementary nucleotides actually destabilize the enzyme-template-primer complex signifi-

cantly (6–8-fold); noncomplementary nucleotides, however, tend to destabilize the enzyme-template-primer complex minimally (1–2-fold). These results are consistent with other observations (16).

A model for polymerization for *Escherichia coli* polymerase I has been proposed by Kornberg (17), and we found it useful to analyze our data on agarose-bound DNA synthesis by assuming that the MoMuLV DNA polymerase may have similar binding sites. The *E. coli* polymerase model postulates binding between the enzyme and a partially double-stranded segment of DNA at internal free 3′-hydroxyl groups (17); binding at the triphosphate binding site of a nucleoside triphosphate complementary to the available base on the template strand of the DNA then leads to polymerization and movement along the template (17). Consistent with this mode are our observations that (a) the polymerase binds more tightly to a template-primer complex than to a template alone, (b) phosphate groups are required for the nucleotide interaction (also see ref. 18), and (c) complementary nucleotides modulate the polymerase-template-primer interaction. This modulation suggests an interaction between the nucleotide binding site and the primer and template binding sites. The destabilization of polymerase-template-primer complexes, which we observed to be induced by complementary nucleotides under nonpolymerizing conditions (no manganese present), may be the cause of the dissociation of polymerase from its template-primer seen under normal polymerizing conditions and characterized by Chang (19) and McClure and Jovin (20) as distributive synthesis.

The use of poly(A)-agarose as an immobilized template also promises to yield new information on the modes of action of ansamycin inhibitors of reverse transcriptase. For example, from our working model of polymerization, we can propose that these drugs may be effective at one of many steps, by preventing the formation of polymerase-template or polymerase-template-primer complexes, by causing their premature dissociation, or by stabilizing them so that subsequent polymerization is effectively blocked. By observing the effects of selected ansamycins on complexes (of either template and primer, or polymerase and template, or polymerase, template, and primer), we can determine which complex is primarily affected and whether the effect is due to the binding of inhibitor in a manner which causes stabilization, or dissociation, of that particular complex. For example, the unsubstituted ansamycins, streptoval C and rifamycin SV, both appear to affect a polymerase-template complex, with the former having the greatest specificity for this complex (Table 3). Since a polymerase-template-primer complex is relatively resistant to the action of these drugs (Table 3, column 4), and the drugs do not dissociate any bound polymerase activity (columns 5 and 6), these ansamycins appear to act by binding to a polymerase-template complex in a manner which stabilizes the complex. In short, the resultant complex cannot dissociate to re-form as a polymerase-template-primer complex, a requirement for polymerization. Alternatively, the drug may block a primer binding site on the enzyme. Demethyldimethylbenzylrifampicin, a rifamycin SV derivative substituted with a bulky aromatic side chain, does not appear to affect any of the previously formed complexes which we have measured, although it is clearly a potent inhibitory agent. Apparently, the addition of the side chain makes the derivative too bulky to bind any of the complexes in a manner similar to its parent compound, rifamycin SV. Thus we propose, and can test, the hypothesis that this derivative may act by preventing the formation of one or more of these complexes. Rifazone 8_2, a derivative of rifamycin SV characterized by the addition of two hydrophobic side chains, is a potent inhibitor of all the complexes, especially those two in which polymerase is present initially (Table 3). Again, the addition of the hydrophobic side chains clearly affects the mode of action of this derivative, by making it behave differently from any of the other derivatives. Significant amounts of polymerase (18,000 dpm) are also displaced by rifazone 8_2 (Table 3, columns 5 and 6). For comparison, we have recently observed[4] that rifazacyclo 16 (11) causes the displacement of approximately 180,000

dpm of polymerase activity; we are now in a position to refine the understanding of this class of inhibitor, by determining whether binding is to the complexes or to the polymerase itself. It is clear that the modulation of the activity of these rifamycin SV derivatives by structural modification is due to the modification of their mode of action.

Further analysis of these compounds, and other potent inhibitors of reverse transcriptase, by our solid phase assay should allow us to correlate some structural parameters of the ansamycins with their mode of action; this line of investigation would encourage the possibility of the ultimate synthesis of an agent both potent and selective.

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