Insertion and Extension of Acyclic, Dideoxy, and Ara Nucleotides by Herpesviridae, Human α and Human β Polymerases

A UNIQUE INHIBITION MECHANISM FOR 9-(1,3-DIHYDROXY-2-PROPOXYMETHYL)GUANINE TRIPHOSPHATE*

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The ability of human α and β DNA polymerases and herpes simplex virus type 2 (HSV-2) and human cytomegalovirus (HCMV) DNA polymerases to insert and extend several nucleotide analogs has been investigated using a variation of Sanger-Coulson DNA sequencing technology. The analogs included the triphosphates of two antiviral nucleotides with complete sugar rings: 9-(1,3-dihydroxy-2-propropyl)guanine (dhpG) and 9-(2-hydroxyethoxy methyl)guanine (acyG) as well as dideoxy and arabinosyl nucleoside triphosphates.

Three pairs of contrasting behaviors were found, each pair distinguishing the two human polymerases from the two viral ones: first, extension behavior with araNTPs; second, insertion/extension behavior with dhpGTP; and third, the relative preference for insertion of ddGTP versus acyGTP. The relative level of insertion of the nucleotide analogs by HCMV and HSV-2 DNA polymerases was dhpGTP > (acyGTP and araNTP) > ddGTP, whereas by human α polymerase it was araATP > ddGTP > (acyGTP and dhpGTP) and by human β polymerase it was (araATP and ddGTP) >> (acyGTP and dhpGTP).

Evidence is presented for three mechanisms of inhibition by extendible nucleotides (of dhp and ara types) exhibiting frequent internalization: araATP acted as a simple pseudoterminator of polymerases and only stalled at sites requiring two or more araATP insertions in a row. Herpesviridae polymerases stalled after adding dhpGMP and one additional nucleotide, suggesting that polymerase translocation problems may be a factor in polymerase inhibition by modified sugar nucleotide analogs.

The amino acid sequence of the human α DNA polymerase, which is acyGTP resistant, was found to vary by one amino acid from the amino acid sequences of the Herpesviridae polymerases in a region of similar significance and probable functional homology. Amino acid differences at that site differentiate acyclovir-resistant HSV-1 mutants from the acyclovir-sensitive HSV-1 wild type.

Several nucleotide analogs based on modification of the

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1 The abbreviations used are: acyGTP, 9-(2-hydroxyethoxy methyl)guanine-triphosphate; dhpGTP, 9-(1,3-dihydroxy-2-propoxymethyl)guanine-triphosphate; HSV, herpes simplex virus; HCMV, human cytomegalovirus; dd, dideoxy; ara, arabinosyl.
Polymerase Inhibition by Nucleotide Sugar Analogs

Enzymes—HCMV DNA polymerase was isolated from infected WI-38 cells as described elsewhere (18). DNA polymerases α and β were purified from uninfected WI-38 cells. DNA polymerase α was isolated as described elsewhere (18). DNA polymerase β was separated from other cellular DNA polymerases as described in Ref. 19 (in which it is eluted from DEAE-cellulose at 0.05 M KCl). It was further purified by elution from a heparin-Sepharose column at 0.18 M KCl and from a single-stranded DNA Sepharose at 0.12 M KCl, as described in Ref. 18.

HSV-2-induced DNA polymerase was purified by successive chromatography on DEAE-cellulose and phosphocellulose, as previously reported (20), and further purified on a single-stranded DNA-agarose

**DNA Polymerase:** None Human β

**dNTP Added:** None ddG acyG

**Reaction:**

![Diagram](image)

**FIG. 1. Illustration of sequencing approach using ddGTP and acyGTP.** The minus reactions were modified from Sanger and Coulson (see "Materials and Methods"). The left-most lane is the labeled template-primer mixture. It was created (with Klenow polymerase) under conditions in which dCTP (including approximately 4–8% [32P]dCTP) was limiting, so the darker bands (such as G3497) tend to represent extended primers waiting for a dCTP. The other lanes contain the results of incubating this template-primer mixture with human β polymerase and a chosen combination of nucleotides (25 μM each of dCTP, dATP, and dTTP plus or minus an analog of ddGTP at 50 μM). In each of the 3 β lanes, a fraction of each primer is extended, creating new dark bands either (2nd and 4th lanes) representing strands requiring a G, or (3rd lane) strands terminated by the analog (ddG). The pattern repeats several times (see arrows); the inferred extensions of one of the primers (the intense band at G3497) are shown below. In the inferred extensions, stall sites are marked by a *single bar*, and a termination site by a *double bar*; the inserted guanosine analog is marked with an asterisk.

**MATERIALS AND METHODS**

Nucleotides—AraATP and araGTP were purchased from Behring Diagnostics. DideoxyGTP and normal dNTPs were purchased from Pharmacia LKB Biotechnology Inc. AcyGTP and dhGTP were generous gifts from W. Miller and R. Miller at Burroughs Wellcome Co.
Polymerase Inhibition by Nucleotide Sugar Analogs

RESULTS

DNA Sequencing Assay—A DNA sequencing approach based on the Sanger method (21, 25, 26) was used to directly study nucleotide analog insertion. The template-primer concentration was often kept low in our approach, compared to normal DNA sequencing, to enable use of polymerase preparations (human α and HCMV) at activities orders of magnitude below those (e.g. of Klenow DNA polymerase I) normally used for DNA sequencing. The primer/polymerase ratio was still high enough for α and HCMV polymerases so that almost all active primers (that is, primers which were extended at least one nucleotide) were expected to have only one encounter with a polymerase (one cycle of processive synthesis).

The sequencing approach is illustrated using ddGTP and acyGTP with human β DNA polymerase (Fig. 1). A spectrum of different length DNA primers (shown in the "no-polymerase" lane) was offered to β polymerase for extension, either in the absence of dGTP (the second lane) or with dGTP replaced by ddGTP or acyGTP (the next two lanes). In each case, the remaining three normal nucleoside triphosphates were present. In the absence of dGTP, primers were extended to the sites before G sites in the growing-strand sequence. The polymerase could extend only a fraction of the primers represented in each band, evidenced by intensity remaining at original band positions. The strongest original bands correspond to high-frequency primers (e.g. position G3497) produced under the C-limiting conditions used when the primer mixture was made (see "Materials and Methods"). Added ddGTP was efficiently inserted in place of dGTP at many sites, terminating replication at those sites. One particular sequencing gel area (shown by brackets in Fig. 1) was chosen

![Fig. 2. Insertion/extension with araATP, ddGTP, and acyGTP: a comparison of human DNA polymerases α and β and the viral DNA polymerase of HCMV. A DNA template-primer mixture prepared as in the legend to Fig. 1 (C-limiting reactions) was offered to DNA polymerases α, β, and HCMV. Only the portion of the sequence near 3498 is shown but, as in Fig. 1, the behavior illustrated in the schematic is repeated at several other parts of the sequence. Reactions were minus-adenine reactions with and without araATP and minus-guanine reactions with and without ddGTP and acyGTP. All analogs when present are at 50 μM. Inferred extensions are shown below as in Fig. 1.](image-url)
Polymerase Inhibition by Nucleotide Sugar Analogs

1.0
0.8
0.6
0.4
0.2
0.0

0
20
40
DISTANCE MIGRATED (cm)

FIG. 3. Densitometric scans of selected lanes of autoradiogram of the gel of Fig. 2; a lighter exposure was used for densitometry. a, an overlay of scans of two lanes showing α polymerase use of araATP. One scan is of the "-A" lane, which shows the results of a 1-h incubation with human α polymerase and 25 μM each of three normal triphosphates (dGTP, dCTP, and dTTP); the other is the same, except that araATP is present at 50 μM. The peak, which is more intense in the minus-adenosine scan (C3498), is shaded where it exceeds the equivalent peak of the scan of the araATP lane; the peak more intense in the araATP lane scan (where araATP has inserted at the position of A3499) is hatched. b, an overlay of scans of two lanes showing HCMV polymerase use of araATP. As in a, the lanes show the results of incubating the polymerase for 1 h with reaction mixtures that include three normal triphosphates at 25 μM (dGTP, dCTP, and dTTP), but either, first, no analog for dATP, or second, 50 μM araATP. Again, the araATP-induced decrease in intensity (at C3498) is shown by shading, and the increases are hatched. In the region shown, most strands used primers at G3497; above certain peaks are shown the inferred extensions. Note that each insertion of an ara-nucleotide retards the migration rate (which is left to right) slightly more than insertion of a deoxy-nucleotide.

for illustration purposes; an interpretation of its bands is included at the bottom of the figure. AcyGTP did not insert (lane 4 versus lane 3) or inhibit (lane 4 and lane 2 versus lane 1) DNA elongation by human β polymerase under the conditions of Fig. 1.

Nucleotide Analog Insertion and Extension—The abilities of four DNA polymerases (polymerase α, polymerase β, HSV-2 polymerase, and HCMV polymerase) to insert acyGTP and ddGTP and to insert and possibly extend dhpGTP, araATP, and araGTP were compared (Figs. 2–5) as described above. In each gel, the most prominent primer (before extension

FIG. 4. Insertion/extension with dhpGTP: a comparison of human DNA polymerases α and β and the viral DNA polymerase of HCMV. a, minus-guanine reactions with and without dhpGTP. Conditions were as described in legend to Fig. 2, b, densitometric scans of selected lanes of the autoradiogram of the gel; a lighter exposure was used for densitometry. An overlay of scans of two lanes shows HCMV polymerase use of dhpGTP. One scan is of the "-G" lane, which shows the results of a 1-h incubation with human HCMV polymerase and 25 μM each of three normal triphosphates (dATP, dCTP, and dTTP); the other is the same, except that dhpGTP is present at 50 μM. The peak that is more intense in the first scan (A3499) is shaded where it exceeds the equivalent peak of the scan of the dhpGTP lane; the increased intensities in the second scan are hatched.

FIG. 5. HSV-2 DNA polymerase insertion/extension of dhpGTP, ddGTP, acyGTP, araGTP, and araATP. Reaction conditions are as described in Fig. 1. As in Fig. 2, only the portion of the autoradiogram near 3500 is shown, but the behavior illustrated is repeated at several parts of the sequence on the original gel. Inferred extensions from G3497 are shown below the figure as in Fig. 1.
begins) corresponds to the band at G3497; only the region immediately above it is shown, but the behaviors described are repeated at other sites, as exemplified in Fig. 1.

The α and HCMV polymerases were at low specific activity, so long exposure was used to make the bands to the C3498 to A3502 region more easily visible (Fig. 2). For each of α and HCMV polymerases, the peaks at A3499 in the −G lanes, which serve as a measure of the original C3497 primers that were extended, are much less than the residual peaks at G3497.

Qualitatively, band appearance at G3500 (Figs. 2 and 5) demonstrated, for example, that ddGTP was incorporated by all polymerases studied, as indicated schematically at the bottom of the figures. For quantitative comparisons, less exposed autoradiograms of the gels were analyzed by densitometry (Figs. 3 and 4; Tables I and II). Scans of the araA and dhpG lanes are shown in the figures; the ddG and acyG lanes are simpler, because insertion implies termination, whereas for araA and dhpG both insertion and extension must be followed.

Ara Nucleotide Insertion and Extension—The behavior of human α polymerase in minus reactions without analog was to add one dCMP subunit to a rather small fraction of the primers ending with G3497 to produce a shoulder at C3498 (Fig. 3). In the parallel reaction with 50 mM araATP, the area of the shoulder was decreased by more than half (the shaded region), and a new peak appeared (the hatched peak) near (but slightly to the left of) the A3499 site (Fig. 3). The fraction of G3497 primers that inserted (at least) the dCMP at C3498 (which we will call the “active” primers) should be the same in the two reactions, so the decrease in the C3498 shoulder is a measure of the active primers that are further extended by araATP insertion. Although half of the active primers (about 70%, by densitometry; see Table I) extended well over half of the active primers, as shown by the decrease in the C3498 shoulder from the −A lane scan to the −A plus araA scan. More than half of these extended primers have been further extended by (presumably) adding G at the G3500 site and then (usually) a second araA at the A3501 site. There, however, the enzyme apparently pauses, as no insertion corresponding to A3502 can be detected (it should run between the normal A3502 and G3503 peaks). This represents a significant blockage, since about half of the active G3497 primers (as measured by the sum of the hatched areas in Fig. 3, plus the residue remaining at C3498) are extended to the A3501 nucleotide position (Table I). As in the α case, the large proportion of unextended G3497 primers implies that the extensions shown represent single encounters of polymerase with template-primer (single processivity events).

Inhibition of HCMV and HSV-2 DNA Polymerases by dhpGTP—The experiments with dhpGTP provided a surprising result. When HCMV or HSV-2 DNA polymerases were used, a band appeared at a level two nucleotides above the −G band (A3499) (see Figs. 2 and 5). This could be due to insertion of dhpGTP alone, if the inserted nucleotide retarded primer electrophoretic mobility twice as much as inserted dGTP or acyGTP alone. To test this possibility, dGTP, ddGTP, araGTP, and dhpGTP were all added to the end of a primer by avian myeloblastosis virus reverse transcriptase in the absence of dNTP needed for additional extension. Electrophoresis of the products on a denaturing DNA sequencing gel indicated that the greatest retardation occurred in the primer capped by araGMP; no discernible difference was seen between the effect of dGMP and that of dhpGMP. Thus, insertion of dhpGTP at G3500 by HCMV or HSV-2 polymerases allowed insertion of one additional nucleotide (in Fig. 4, this would be dATP) followed by stalling. The stalling is not absolute, as can be seen by densitometric overlay (Fig. 4). Here the active extensions of the G3497 primer by HCMV polymerases are shown by the peak at A3501 in the scan of the −G lane, the result of adding dCMP and then dAMP to the primer. The shaded decrease in the A3499 peak, in the parallel reaction with dhpGTP, measures the insertion of dhpGTP at the G3500 sites, whether further extended or not. The hatched area at A3501 and the smaller one at T3504 show the preferred pause or termination sites for a processive cycle. Each follows a G site; the peak at T3504 shows the degree to which the polymerase can overcome the first block and extend to a second similar one.

**Table I**

| Polymerase | C3498 (blocked) | A3499 (araA inserted) | A3501 (extended to AA) |
|------------|----------------|-----------------------|------------------------|
| HCMV pol   | 0.3            | 0.2                   | 0.5                    |
| HSV-2 pol* | 0.1            | 0.10                  | 0.7                    |
| β          | 0.5            | 0.5                   | 0.0                    |
| α          | 0.3            | 0.7                   | 0.0                    |

*The experiment with HSV-2 polymerase used a different extended primer mixture, the 10% not accounted for in the HSV-2 lane is at the G3500 site.

**Table II**

| Polymerase | Analog (50 μM) | A3499 (blocked) | G3500 (extended) |
|------------|---------------|----------------|------------------|
| HCMV pol   | None (=G)     | 1.0            | 0.3              |
| ddGTP      | 0.7           | 0.2            | (0.1 at T3504)   |
| acyGTP     | 0.5           | 0.2            | (0.1 at A3501)   |
| dhpGTP     | (0.1 at A3501)| (0.1 at T3504) |                  |
| β          | None (=G)     | 1.0            | 0.6              |
| ddGTP      | 0.4           | 1.0            |                  |
| acyGTP     | 1.0           | 1.0            |                  |
| dhpGTP     | 1.0           | 1.0            |                  |
| α          | None (=G)     | 1.0            | 0.4              |
| ddGTP      | 0.6           | 1.0            |                  |
| acyGTP     | 1.0           | 1.0            |                  |
| dhpGTP     | 1.0           | 1.0            |                  |

*The dhpGTP data is from a separate experiment (see Fig. 4).
between the A3499 band (if not further extended) and the G3500 band (if the analog has been inserted). The table gives the fraction at each site. β polymerase shows a higher ddGTP incorporation than α or HCMV, but for all three the incorporation is significant (0.6, 0.4, and 0.3, by densitometric estimate (Table II)).

Relative Insertion of Nucleotide Analogs—For each polymerase, the gels also provide a rank ordering of the insertion rates of the nucleotide analogs. In particular, they show dhpGTP to be a slightly more effective substrate for insertion than is acyGTP for each of the two Herpesviridae polymerases (Table II), despite the fact that in each case acyGTP has the lower $K_I$ (reviewed in Ref. 8) by a factor of 2.5 to 10. For HCMV, this can be seen by comparing the 50 mM acyGTP lane in Fig. 2 to the 5 and 50 mM lanes in Fig. 4; the 50 mM acyGTP extended about half the available primers, the 5 mM dhpGTP nearly half, and the 50 mM dhpGTP well over half.

For HSV-2 (Fig. 5), the acyGTP lanes show that at 500 nM acyGTP the ratio of the intensity at the G3500 site (insertion of acyGTP) to that at the A3499 site (waiting for extension by dGTP analog) is just over 1:1, while at 50 nM acyGTP the ratio is much less. For dhpGTP insertion, the ratio of the intensity at A3501 to that at A3499 (Fig. 5) show that, at 20 nM dhpGTP, insertion is at least comparable to acyGTP insertion at 50 mM. At 500 mM dhpGTP, enough strands are extended past the A3501 stall point that the next dhpGTP-specific stall point (at T3504, after G3503; compare Fig. 4) becomes the most intense of the extensions (results not shown); the sum of the bands representing insertion of dhpGTP at the G3500 site (plus further extensions) is then significantly greater than the band at A3499 representing failure to insert.

For each of the HCMV and HSV-2 polymerases, the level of insertion could be ordered as follows: dhpGTP > (acyGTP and araNTP) > ddGTP. This order is not the same as the order by inhibition strength as measured by ($K_I$)−1, which is acyGTP > dhpGTP > araNTP > ddNTP (8).

Although the cellular polymerases differ in sensitivity to ddGTP ($β$ is well known to be more sensitive to ddNTP inhibition than α), they each insert ddGTP significantly at concentrations at which acyGTP insertion is not detectable on the gels. The order for level of insertion is araATP > ddGTP > (acyGTP and dhpGTP) > araATP and (araATP and dGTP) > (acyGTP and dhpGTP) for α polymerase and (araATP and ddGTP) > (acyGTP and dhpGTP) for β polymerase.

DISCUSSION

For each of the cases (ara and dhp) in which termination is not forced, the mechanistic possibilities considered by previous workers have included competitive inhibition (i.e. tight binding but low or slow insertion) and pseudotermination (i.e. slow extension after insertion) (8, 27, 28). In this work, three types of nonterminating inhibition were seen, exhibited respectively by: (i) Herpesviridae polymerases with dhpGTP, (ii) Herpesviridae polymerases with ara nucleotides (araATP and araGTP), and (iii) α and β polymerases with ara nucleotide (araATP).

AraATP with α and β acted as a simple pseudoterminator, with slow extension after any insertion. (Although both α and β exhibit the same mechanism, pseudotermination, with araATP, they are distinguishable from each other by a lower $K_I$ for β than for α polymerases (9).) Herpesviridae polymerases, however, easily extended ara nucleotides at singlet sites, but stalled when attempting to insert an additional ara nucleotide at runs of two or more consecutive ara sites or in other ara-rich regions.

In the third type of inhibition, the Herpesviridae polymerases were stalled after adding dhpGMP and one additional nucleotide. This suggests that in vivo, and in assays where dhpGTP and following nucleotides are present, most dhpGMP subunits that are incorporated by Herpesviridae polymerases should be internal rather than at termini. This is consistent with studies (4) that found that at least half of the dhpGMP subunits incorporated into activated calf thymus DNA (by HSV-1 polymerase) were internal. (This amount may be too low, as any phosphatase contamination in the assay (as discussed in Ref. 14) would have lowered the result.)

Stalling of polymerization after internalization in the growing strand may explain the apparent contradiction (7, 27) that in certain cases polymerases find it easy to internalize a nucleotide analog, while at the same time total polymerization is strongly inhibited. One mechanism for such stalling would be a delayed fraying of the template-primer; dhpG-C base pairs might initially be held together with the help of the polymerase, but then induce fraying after two polymerase translocations. Perhaps more likely, interaction with the missing portion of the sugar may be required for efficiency in some step in the polymerase's normal sequence of activities, the most obvious possibility being the second translocation after insertion. This model, then, proposes that the polymerase inserts a dhpGMP subunit, translocates to the next site, inserts the appropriate next nucleotide, and then is strongly inhibited (but not totally prevented) from proceeding through the next translocation, that is, that Herpesviridae DNA polymerases depend for normal translocation on a correct interaction with the second sugar from the terminus. In fact, it is possible that this model is more general among polymerases. Since the concentrations used here were not high enough to allow detection of insertion of dhp nucleotides by the cellular polymerases, their extension behavior after insertion of dhpG remains untested.

It might be mentioned that translocation problems may contribute to inhibition mechanisms of terminators. For acyGTP, there may be two separate modes of inhibition by analog-terminated template-primer, the first characterized by $K_I$ values in the low nanomolar range (4), and the second by even lower (or nonexistent) off rates, which has led to the suggestion that suicide inhibition might be involved (see Ref. 5 and compare also Ref. 17). The results presented here give evidence against inactivation by formation of a covalent bond between primer and DNA polymerase, since the mobility of the analog-terminated primer in sequencing gels was not disturbed. The sequencing gels show (a) acyGTP attached to primers and (b) the enzyme not attached to primers. A likely explanation for the appearance of suicide inhibition might involve the fact that polymerases bind template-primers in two modes: pretranslocation (after insertion) and post-translocation (or equivalently the initial binding mode). The appearance of suicide inhibition might then be due to binding in the first mode, reached through an insertion event rather than through initial binding, with an inhibition of translocation after insertion being a potential source of slower dissociation.

Relationship between Polymerase Amino Acid Sequence Homology and Acyclovir Triphosphate Sensitivity—Recent sequencing results make it apparent that α polymerases and all Herpesviridae polymerases belong to a polymerase superfamily (29–39). Members of this family have discernible sequence homology throughout a series of regions defining a large probable nucleotide/template-primer binding region. Since

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2 K. Frank, personal communication.
3 Spicer, E. K., Rush, J., Fung, C., Reha-Krantz, L. J., Karam, J. D., and Konigsberg, W. H. (1988) J Biol. Chem., submitted for publication.
of the superfamily. The human α has especially high homology, with a match of 14/15 in a stretch that varies only at the site of the HSV-1 A → V mutation; the vaccinia virus (Poxviridae) sequence matches the consensus at 10/13, or 11/13 if the highly homologous F → V match is counted, the two clear mismatches being at the two mutant sites.

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TABLE III

Amino acid homology at a putative portion of the nucleotide binding site that in HSV-1 is important to acyclovir triphosphate sensitivity

The polymersases belong to a superfamily characterized by sequence homology that seems to include all polymerases sensitive to aphidicolin. In the consensus sequence, "h" (for "hydrophobic") is used for I, L, V, M, A, G, F, and Y. The A → V and S → N mutations indicated have each been identified (see Ref. 38, in whose terminology this is a portion of Region II) in acyclovir-resistant HSV-1 mutants. Variation from A and S at these sites is marked with a double underline; variation from the consensus at other sites is marked with a single underline. The partial sequence from human α polymerase was provided by Teresa Wang (Stanford Univ.); also included are five sequences from the herpesvirus family (31-34, 37, 41), one from a poxvirus (35), one from an adenovirus (29), and two from bacteriophages (phi29 and T4).

| HSVI mutants | V | N |
|--------------|---|---|
| Consensus:   | PhhhhDFAFLSPShhQAHHN |
| VZV          | PPVVVDASFLPSIIQAHLN |
| HSV-1        | PPVVVDASFLPSIIQAHLN |
| HSV-2        | PPVVVDASFLPSIIQAHLN |
| EBV          | PVLVVDASFLPSIIQAHLN |
| HCMV         | PLYFVDASFLPSIIQAHLN |
| α            | FILLDFNSLPSIIQDFN1 |
| VaccV        | NLFLPDFNSLPSINVCIFGML |
| T4           | YIMSFDIRSLPSIIROQNI |
| PH129        | EGMVFDNSLPSLPMYSRLL |
| Ad2          | PLYVYDICQMYASALT(-)HP |