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The relationship between the antiviral activity of 5′-amino-5′-deoxythymidine and its incorporation into herpes simplex virus type 1 DNA

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Summary

As part of our studies on the molecular basis for the antiherpes activity of 5′-AdThd (5′-amino-5′-deoxythymidine), a study of the HSV-1 DNA synthesized in infected Vero cells exposed to 5′-AdThd was undertaken. Unlike many other antiviral nucleoside analogs, 5′-AdThd did not inhibit HSV-1 DNA synthesis. Analysis of the DNA synthesized in the presence of [^{14}C]5′-AdThd revealed that the analog was incorporated into the viral DNA in a dose-dependent manner and that the degree of incorporation correlated with the antiviral activity as measured by yield reduction assays. Analysis of the 5′-AdThd substituted DNA by centrifugation in neutral and alkaline sucrose gradients revealed no double-stranded breaks but an increase in single-stranded breaks, at very high concentrations of the analog. Analysis of HSV-1-specific RNAs revealed a shift from poly(A$^+$) to poly(A$^-$) RNA. The degree of this shift paralleled the substitution of 5′-AdThd for thymidine in the HSV-1 DNA.

HSV-1 DNA; HSV-1 RNA; thymidine analog; antiviral activity

Introduction

The antiherpes activity of 5′-AdThd (5′-amino-5′-deoxythymidine) in cell culture has been established [9,11]. However, the molecular basis for the antiviral activity is yet to be fully understood. Like many other nucleoside analogs of thymidine, 5′-AdThd is specifically phosphorylated by the herpesvirus thymidine kinase [3] and it is presumed, but until now not established, that after conversion to the triphosphate it is incorporated into viral DNA.

Previous studies have shown that the incorporation of the related compounds AIdUrd (5-iodo-5′-amino-2′,5′-dideoxyuridine) and IdUrd (5-iodo-2′-deoxyuridine) into HSV-1 DNA is quantitatively related to the antiviral activity [6]. However, other studies in our laboratory on the effects of these three compounds, 5′-AdThd, AIdUrd

and IdUrd on HSV-1-induced beta and gamma proteins [13] as well as studies concerning the properties of the drug-substituted virions indicated that 5'-AdThd might be different in its mode of action. Therefore, the object of this study was to confirm the incorporation of 5'-AdThd into viral DNA and correlate the incorporation with the antiviral activity.

Materials and Methods

Cells and virus

Vero cells were grown in Dulbecco's modified minimal essential medium supplemented with 10% calf serum (Grand Island Biological Co.). The C1-101 strain of HSV-1 [4] was passaged at a multiplicity of 0.01 PFU/cell and plaque assayed on Vero cells.

Chemicals

5'-Amino-5'-deoxythymidine (5'-AdThd) was synthesized by Dr. T.S. Lin [9]. Ortho[^{32}P]phosphate (carrier-free) was obtained from Amersham Corporation. 5'-[2-^{14}C]AdThd was synthesized by Dr. G. Shiau using [2-^{14}C]thymidine (50 Ci/mol) as starting material and the method of Lin et al. [9]. Pronase (nuclease-free), ribonuclease A, alkaline phosphatase, snake venom phosphodiesterase and optical grade CsCl were obtained from Calbiochem and Sigma Chemical Co.; [2,8-^{3}H]deoxyadenosine (6.8 Ci/mmol) and deoxy[8-^{14}C]adenosine (30 Ci/mol) were purchased from ICN Pharmaceuticals, Inc.

Virus infection

Vero cells were grown to confluency before being exposed to 10 PFU/cell of HSV-1. Following a 1 h absorption at 37°C, the remaining virus was removed by aspiration and the monolayer was washed twice with phosphate-buffered saline (137 mM NaCl, 2.6 mM KCl, 8.1 mM Na_2HPO_4 and 1.4 mM KH_2PO_4, pH 7.4) before adding fresh medium (37°C) containing the desired concentration of 5'-AdThd. Radiolabeled deoxy[^{3}H]adenosine (10 μCi/ml) or [^{32}P]phosphate (20 μCi/ml) was added to the medium 3.5 h post-infection and the cultures were incubated for an additional 20 h before being harvested.

In preparations for sucrose gradient centrifugation 5.9 μM [8-^{14}C]dAdo was added to the control cultures and 0.27 μM [2,8-^{3}H]dAdo was used for the 5'-AdThd-treated cultures. The HSV-infected cells were harvested by scraping the monolayer with a rubber policeman followed by centrifugation to pellet the cells.

CsCl gradient centrifugation

The procedure used was essentially as previously described [6] except that 1% sarkosyl, 3 mg/ml pronase (autodigested at 37°C for 60 min) and 500 μg/ml heat-inactivated (80°C for 15 min) ribonuclease A were used to digest the samples. The samples were adjusted with CsCl to a density of 1.71 g/ml before centrifugation at 34 000 rpm for 64 h at 18°C in a 50 Ti rotor. Fractions were collected from the bottom of the

gradients and the density of representative fractions was immediately determined using a Bausch and Lomb refractometer. A portion of each fraction was spotted on a Whatman No. 1 disc before being washed twice in 5% trichloroacetic acid and once in 95% ethanol. The discs were dried and the amount of radiolabel was determined by liquid scintillation spectrometry.

Analysis of 5'-[^{14}C]AdThd-labeled DNA

CsCl gradient fractions containing 5'-[^{14}C]AdThd-labeled HSV DNA were pooled and dialyzed against 75 mM Tris-HCl, pH 8.8, and 50 mM NaCl. Dialyzed samples were adjusted to 20 mM $MgCl_2$, 15 units/ml snake venom phosphodiesterase and 1 unit/ml alkaline phosphatase and incubated for 90 min at 37°C. The digestion was terminated by the addition of equal volumes of 0.75 M $HClO_4$ on ice for 15 min. The samples were neutralized by the addition of 1 M KOH and the resulting precipitate removed by low speed centrifugation. Aliquots of the supernatant were spotted on either PEI or silica TLC plates. PEI plates were developed with 0.5 M LiCl/2 M acetic acid and the silica plates with chloroform/ethanol (2 : 1). TLC plates were cut and the slices counted by liquid scintillation spectrometry. The degree of substitution of 5'-AdThd for dThd was determined using a specific activity of 1.4 Ci/mol for 5'-[^{14}C]AdThd, a composition of 16.5% dThd in HSV-1 DNA and a known amount of digested HSV-1 DNA.

Sucrose gradient analyses

The procedure was a modification of a previously described method [10]. The cells were washed with buffer (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 1 mM $MgCl_2$ and 5 mM EDTA), collected by centrifugation and resuspended in a small amount of the same buffer containing 0.5% NP-40. After standing at 5°C for 15 min the cells were Dounce-homogenized and the nuclei were removed by centrifugation at 3000 rpm for 15 min at 5°C in a Sorvall SA 600 rotor. The supernatant was layered onto a 15% (w/v) sucrose solution containing the above buffer. The herpes virions were pelleted at 15 000 rpm for 45 min at 5°C in a SW 50.1 rotor. The supernatant was removed by aspiration and the pelleted virions were resuspended in a small amount of buffer (40 mM Tris-HCl, pH 7.5, and 2 mM EDTA). In experiments using neutral sucrose gradients the above suspension was treated for 30 min at 37°C with 1 mg/ml ribonuclease A which had been heated at 80°C for 30 min. Control and 5'-AdThd-treated virions were mixed and the solution was adjusted to contain 20 mM Tris-HCl, pH 7.5, 1 M NaCl, 1 mM EDTA, 0.5% sodium dodecyl sulfate and 2% sarkosyl before being heated at 60°C for 2 min. The lysate was cooled at 24°C for 10 min before gently layering onto a 15–40% (w/v) neutral sucrose gradient containing 50 mM Tris-HCl, pH 7.5, 1 M NaCl and 1 mM EDTA. The samples were sedimented at 35 000 rpm for 2.5 h at 20°C in a SW 50.1 rotor.

In experiments using alkaline sucrose gradients the resuspended control and 5'-AdThd-treated virions were mixed as above except that lysis was performed on top of a 15–40% (w/v) alkaline (adjusted to pH 12.1 with 2 M NaOH) sucrose gradient containing 1 M NaCl and 1 mM EDTA. The samples were sedimented at 35 000 rpm for 2.5 h at 20°C in a SW 50.1 rotor.

Isolation and analysis of HSV-1 RNA

Vero cells infected at 10 PFU/cell were incubated in the presence of [^{3}H]uridine and various concentrations of 5'-AdThd. After 18 h the cells were lysed in 1.0% SDS, 10 mM Tris-HCl, pH 7.2, and 1 mg/ml proteinase K. The RNA was extracted with phenol/chloroform/isopentanol (25 : 25 : 1). RNA was separated into poly(A^+) and poly(A^-) fractions using an oligo(dT) Sepharose column as described by Anderson et al. [1]. The RNA fractions were precipitated with ethanol and resuspended in 50% formamide, 4 × SSC (1 × SSC = 1.15 M NaCl, 0.015 M sodium citrate, pH 7.0) with 5 mM EDTA for hybridization to purified HSV-1 DNA bound to nitrocellulose filters (10 μg/filter) which had been washed with unlabeled yeast tRNA. This RNA wash reduced the non-specific binding of radioactivity to the filters to a background of less than 25 cpm/filter. Hybridization was performed at 40°C for 48 h, filters were incubated with RNAse for 1 h at 37°C and washed in 1 × SSC before being counted for bound RNA.

Results

Effect of 5'-AdThd on the amount of HSV-1 DNA synthesized

The synthesis of HSV-1 DNA in the presence of increasing concentrations of 5'-AdThd was determined. The incorporation of ortho[^{32}P]phosphate into HSV-1 DNA in infected cells was determined by banding the DNA in CsCl gradients and determining the radioactivity in the DNA at a density of approx. 1.726 g/ml. As indicated in Table 1, 5'-AdThd had no significant effect on the incorporation of ^{32}P into HSV-1 DNA. Analysis of HSV-1 DNA from 5'-AdThd-treated or untreated cells by Eco R-1 restriction endonuclease digestion revealed no difference between the DNA fragments with respect to their restriction patterns on agarose gels (data not shown).

Incorporation of 5'-[^{14}C]AdThd into HSV-1 DNA

In order to establish that 5'-AdThd becomes internally incorporated into HSV-1

TABLE 1

HSV-1 DNA synthesis in the presence of 5'-AdThd and ortho[^{32}P]phosphate

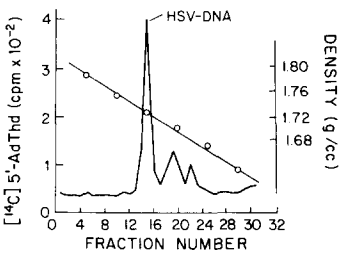
Sample	Expt. 1	Expt. 2
No drug	46 000	59 600
5'-AdThd		
200 μM	40 000	59 800
400 μM	41 000	60 300
800 μM	42 000	60 000

Vero cells were infected as described in Materials and Methods in the presence of the indicated concentration of 5'-AdThd. $^{32}P_i$ was added 3.5 h after infection at 20 μCi/ml and virions were harvested 20 h later. Viral DNA was isolated and analyzed as described in Methods. Numbers are the average of two determinations in a given experiment.

DNA, cells were infected with HSV-1 and exposed to 400 μM 5′-[^{14}C]AdThd for 23 h. Virions labeled with 5′-[^{14}C]AdThd were obtained as described in Methods and the DNA was analyzed on CsCl gradients. The incorporation of 5′-AdThd did not effect a change in the density of the HSV-1 DNA as shown in Fig. 1. The 5′-[^{14}C]AdThd-substituted HSV-1 DNA was digested with snake venom phosphodiesterase and alkaline phosphatase. The resulting nucleosides were analyzed by thin-layer chromatography (Fig. 2). Using appropriate markers it was determined that 5′-[^{14}C]AdThd could be recovered from the labeled HSV-1 DNA. These data along with the absence of detectable breaks in the substituted viral DNA indicate that the drug had been internally incorporated into the DNA. It should be noted that the 5′-[^{14}C]AdThd contained a small amount of contaminating [^{14}C]dThd (less than 0.5%). A preferential utilization of this contaminant is the most likely explanation for the [^{14}C]dThd in the HSV-1 DNA since a biochemical deamination of 5′-AdThd is unlikely.

Relationship between degree of incorporation of 5′-AdThd and the antiviral activity

Drug-substituted virions were prepared at various concentrations of 5′-AdThd and the yield of the virus (indicating the antiviral effect) and the degree of substitution of 5′-[^{14}C]AdThd for thymidine determined. Fig. 3 shows the results of these analyses. There appears to be a good correlation between the antiviral effect, the concentration of 5′-AdThd and the degree of substitution for thymidine in the HSV-1 DNA.



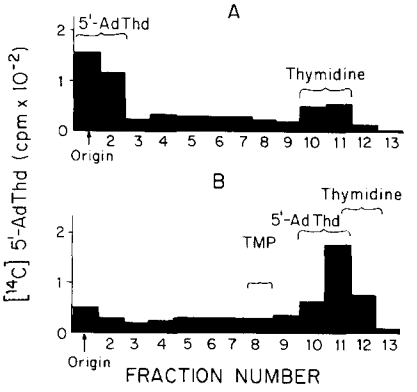


Fig. 1. Incorporation of 5′-[^{14}C]AdThd into HSV-1 DNA. Vero cells were infected at 10 PFU/cell for 1 h and exposed to 400 μM 5′-[^{14}C]AdThd (1.4 Ci/mol) for 23 h. Total DNA from infected cells was analyzed by CsCl gradient centrifugation. An aliquot of each fraction was TCA precipitated and counted.

Fig. 2. Confirmation of the incorporation of 5′-[^{14}C]AdThd by thin-layer chromatography of digested labeled HSV-1 DNA. HSV-1 DNA from CsCl gradients similar to that in Fig. 1 was analyzed. The DNA was dialyzed against 50 mM NaCl, 75 mM Tris-HCl, pH 8.8, and adjusted to 20 mM $MgCl_2$, 15 units/ml snake venom phosphodiesterase and 1 unit/ml alkaline phosphatase. After incubation for 90 min at 37°C, equal volumes of 0.75 N $HClO_4$ were added and samples were placed on ice. KOH neutralized samples were spotted on TLC plates with markers and developed. (A) Silica plate developed with chloroform/ethanol (2:1). (B) PEI plate developed with 0.5 M LiCl and 2.0 N acetic acid.

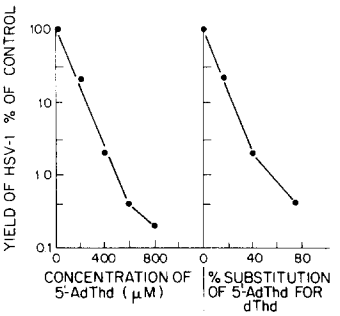


Fig. 3. Relationship between the degree of substitution, the antiviral activity and the concentration of 5'-AdThd. Reduction in the yield of HSV-1 is plotted as a function of the drug concentration (A) and the percent substitution of drug for thymidine in HSV-1 DNA (B). HSV-1-infected Vero cells were exposed to different concentrations of 5'-AdThd or 5'-[^{14}C]AdThd and DNA was harvested as described in Fig. 1. The degree of substitution was determined using a specific activity of 1.4 Ci/mol for 5'-[^{14}C]AdThd.

Effects of the incorporation of 5'-AdThd on HSV-1 DNA integrity.

Previous studies with a related analog, 5-iodo-5'-amino-2',5'-dideoxyuridine (AIdUrd), have shown that a concentration-dependent incorporation of the analog into HSV-1 DNA resulted in single- and double-stranded breaks in the DNA [6]. Since similar effects with 5'-AdThd might explain the correlation between incorporation and antiviral activity, HSV-1 DNA synthesized in infected Vero cells exposed to increasing concentrations of 5'-AdThd was examined with respect to its physical integrity by alkaline and neutral sucrose gradients (Fig. 4). Three concentrations of 5'-AdThd were chosen, i.e., 200, 400, and 800 μM, which inhibit the yield of HSV-1 by 1.5, 2.0 and 2.5 log respectively. There appeared to be a significant increase in the single-stranded breaks only with 800 μM and no detectable increase in the double-stranded breaks at any of the concentrations tested, suggesting that 5'-AdThd in the DNA does not exert a major antiviral effect by causing fragmentation of the DNA.

Effect of 5'-AdThd on HSV-1 RNA synthesis

Since 5'-AdThd in the DNA might exert its antiviral activity at the level of transcription, the levels of HSV-1-specific RNAs in infected cells exposed to 5'-AdThd were examined. It was found that the overall level of [^{3}H]uridine-labeled HSV-1-specific RNA as determined by hybridization to HSV-1 DNA bound to nitrocellulose filters was not significantly changed by exposure to 5'-AdThd. There was, however, a marked increase in HSV-1-specific poly(A^-) RNA and a concomitant decrease in HSV-1-specific poly(A^+) RNA (Fig. 5). The degree of change in the distribution of HSV-1-specific RNAs increased with the degree of substitution of 5'-AdThd for thymidine in the herpes DNA.

Discussion

5'-AdThd has been shown to have a number of metabolic effects in HSV-1-infected or in uninfected mammalian cells. It can be phosphorylated by the herpes-specific

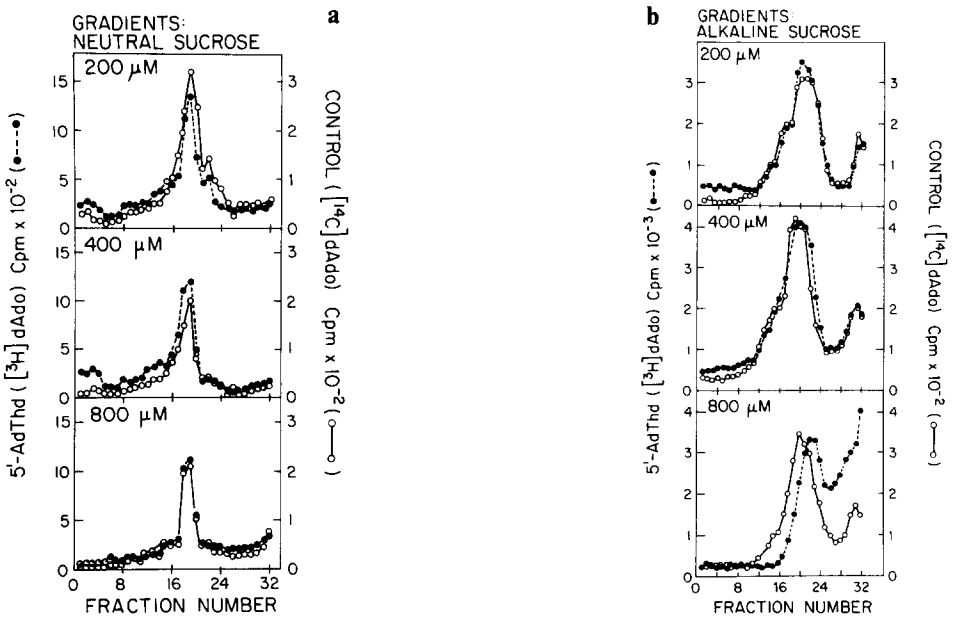


Fig. 4. (a) Absence of double-stranded breaks in 5'-AdThd-substituted HSV-1 virion DNA. Virions grown in the presence of various concentrations of 5'-AdThd were purified and their DNA was analyzed by neutral sucrose gradients to detect double-stranded breaks in the DNA. Purified virions grown in the presence of [^{3}H]dAdo and 5'-AdThd were mixed with virions grown in the presence of [^{14}C]dAdo and no drug. The virions were disrupted by 0.5% SDS and 2% sarkosyl in 20 mM Tris-HCl, pH 7.5, 1 M NaCl and 1 mM EDTA and layered onto a 15–40% neutral sucrose gradient. Centrifugation was carried out in a SW 50.1 rotor for 2.5 h at 35 000 rpm. Fractions were collected, trichloroacetic acid-precipitated and counted. ○, Control virion DNA; ●, 5'-AdThd-substituted DNA. (b) Presence of single-stranded breaks in 5'-AdThd-substituted HSV-1 virion DNA. Control virion DNA and drug-substituted DNA were prepared as in (a) except that disruption of the virions was performed on top of the 15–40% alkaline (pH 12.1) sucrose gradient. Centrifugation, trichloroacetic acid precipitation and counting were performed as in (a).

thymidine kinase but not by the cellular enzyme [3], and its antiviral effects depend on this phosphorylation since TK$^-$ mutants are resistant to this activity (Otto and Prusoff, unpublished observation). In addition, it has been found by Fischer and Baxter [7] that 5'-AdThd inhibits the feedback inhibition normally exerted by dTTP on thymidine kinase and thus acts to increase the level of dThd nucleotides, including analogs of dThd which can be phosphorylated by the cellular enzyme such as IdUrd.

In the present study we have demonstrated that 5'-AdThd is incorporated into HSV-1 DNA at antiviral concentrations of the compound. Under these conditions no detectable change is seen in the integrity of the DNA except for some increased single-stranded breaks at 800 μM which may indicate increased fragility of the highly substituted DNA at the phosphoramidate bond. The antiviral activity increases in a dose-dependent manner which parallels the degree of substitution of 5'-AdThd for dThd in the viral DNA. This kind of correlation has been shown for other nucleoside analogs, such as BVDU [5-(2-bromovinyl)-2'-deoxyuridine], IdUrd (5-iodo-2'-deoxyuridine) and AIdUrd (5-iodo-5'-amino-2',5'-dideoxyuridine) in HSV-1 infected

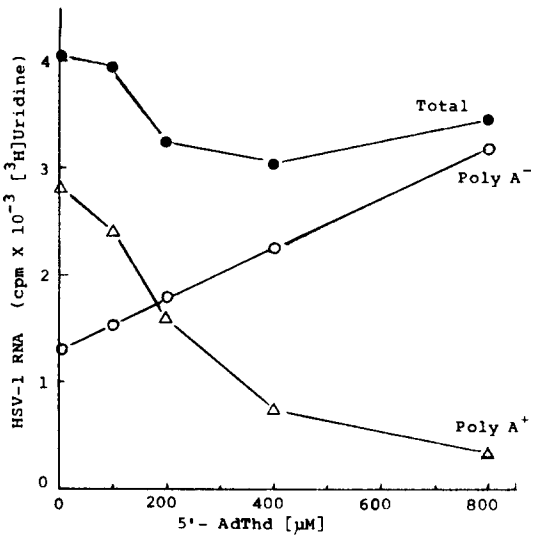


Fig. 5. Analysis of RNA synthesized in HSV-1-infected Vero cells at various concentrations of 5'-AdThd. Vero cells infected at 10 PFU/cell were incubated in the presence of [^{3}H]uridine and various concentrations of 5'-AdThd. After 18 h the cells were lyzed and the RNA extracted with phenol/chloroform/isoamylalcohol 25 : 25 : 1. The RNA was separated into poly(A^+) and poly(A^-) by column chromatography on oligo(dT) Sepharose. Aliquots were analyzed by hybridization to purified HSV-1 DNA bound to nitrocellulose filters and the HSV-1-specific radioactivity bound was determined.

cells [6,10]. It is interesting to note that a 20% substitution of either AIdUrd or BVDU for dThd in the viral DNA reduces the infectious virus yield approximately 96%, while 5'-AdThd requires approximately 38% substitution to achieve the same antiviral effect. AIdUrd and BVDU, however, produce a significant increase in the single- and double-stranded DNA breaks while 5'-AdThd produces no significant DNA breaks at the same antiviral level.

While much of the antiviral effect of the incorporation of AIdUrd or BVDU into viral DNA could be explained by the fragmentation of the DNA, the antiviral effect of the incorporation of 5'-AdThd is still a question. Previously we had shown that HSV-1-infected cells exposed to the analog exhibited altered protein patterns on SDS gels with reduction in the late beta and the gamma proteins [13]. It has been known for some time that herpes simplex mRNA is polyadenylated [2]. Here we demonstrate that with increasing concentrations of 5'-AdThd the ratio of poly(A^-) to poly(A^+) HSV-1-specific RNA increases. It should be noted that this effect is more pronounced late in infection (12–18 h) than at earlier times (4–6 h). These effects on late proteins and RNAs suggest that the effects are due to the incorporation of 5'-AdThd into the newly synthesized DNA and not to some other (early) effect.

Effects on transcriptional events might have been predicted following incorporation of 5'-AdThd into viral DNA in light of studies by Nottoli et al. [12]. It was found that the substitution of NH_2 for OH at C(5') of thymidine nucleotides results in an altered conformation for the protons at the C(5')–C(4') bond and the P–N(5')–C(5')

bonds as well as a change in the torsion angle at C(5')–N(5') as compared to C(5')–O(5'). It was suggested that the results of such changes would be alterations in the conformation of the sugar-phosphate backbone of the DNA. Thymidine-rich regions in the DNA might therefore be expected to exhibit abnormal conformations. It is known that eukaryotic as well as viral mRNAs possess a highly conserved sequence, AAUAAA, 10–30 nucleotides upstream from the poly(A) [11]. This site appears to be a required recognition site for accurate cleavage and polyadenylation of the mRNA [11]. The coding sequence in the DNA therefore would be TTATTT and the substitution by 5'-AdThd at this site could have profound effects on polyadenylation, i.e., a reduction in the amount of poly(A^+) containing RNA, and on accurate cleavage of the RNA, i.e., transcripts of increased length. The later effect would be detected as an apparent increase in the total amount of viral (hybridizable cpm's) RNA synthesized, specifically poly(A^-) RNA. Such an effect is detected at 800 μM 5'-AdThd where the degree of substitution of 5'-AdThd for dThd in the viral DNA approaches 85%.

Thus these data, taken together with the data presented previously [13], demonstrate that increasing concentrations of 5'-AdThd result in increased levels of substitution in the viral DNA, increased levels of poly(A^-) herpes-specific RNA, a marked decrease in the level of poly(A^+) herpes-specific RNA, decreased late protein synthesis and decreased yields of infectious virus.

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