Volume 9 Number 19 1981 Nucleic Acids Research

Characterization of the effect of aphidicolin on adenovirus DNA replication: evidence in support of a protein primer model of initiation

Steven Pincus, Warren Robertson and David Rekosh*

Department of Biochemistry, State University of New York at Buffalo, Buffalo, NY 14214, USA

Received 14 July 1981

ABSTRACT

Adenovirus DNA replication is inhibited by aphidicolin but the inhibition clearly has different parameters than the inhibition of purified DNA polymerase α. In adenovirus infected Hela cells, 10 μg/ml of aphidicolin reduced viral DNA synthesis by 80%. Cellular DNA synthesis was inhibited by 97% at 0.1 μg/ml. 10 μg/ml of drug had no effect on virus yield or late protein synthesis though higher concentrations of drug (50 μg/ml) caused an abrupt cessation of late protein synthesis and 100 μg/ml reduced virus yield by 3 logs. Concentrations of the drug from 0.5 μg/ml to 10 μg/ml were found to dramatically slow the rate of DNA chain elongation in vitro but not stop it completely, so that over a long period of time net incorporation was reduced only slightly compared to the control. 50 μg/ml or 100 μg/ml of drug completely inhibited incorporation in vitro.

Initiation of viral DNA replication - covalent attachment of dCMP to the preterminal protein - occurs in vitro. This reaction was found to be insensitive to inhibition by aphidicolin. We thus conclude that aphidicolin exerts its effect on adenovirus DNA chain elongation, but not on the primary initiation event of protein priming.

INTRODUCTION

Adenovirus DNA replication occurs in the nucleus of infected permissive cells and requires only a limited number of proteins encoded by its own genome plus others provided by the host cell (1). To date only 2 viral encoded gene products have been shown to be involved in the replication of viral DNA - a 72,000 dalton single-stranded DNA binding protein (DBP) (2-6) and an 87,000 dalton protein (preterminal protein) which is found covalently attached to replicating DNA (7). A processed form of the preterminal protein (M.W. = 55,000 daltons) is found at the 5' ends of the mature viral DNA molecule present in virions (8). Conversion of the preterminal protein to terminal protein happens at a late stage in virion assembly (7) and is mediated by a virus specified gene product (9, 10). The protein is linked to the DNA via a phosphodiester linkage from a serine residue to the 5' carbon of the deoxyribose of cytidine, the first nucleotide of the adenovirus DNA chain (11).

It has been proposed that initiaton of DNA replication involves the use of the preterminal protein as a primer as well as the DBP in some unspecified role (12). According to a model, first proposed by Rekosh et al. (8) and modified by Challberg and Kelley (13), an early step in the initiation process would involve covalent attachment of dCMP to the preterminal protein. This protein nucleotide complex could then prime DNA synthesis with the addition of nucleotides to the deoxycytidine already attached to the protein. Consistent with this notion, replication has been shown to initiate at the ends of the DNA molecule (14, 15) and the preterminal protein has been found covalently attached to short nascent DNA molecules both in vivo (16) and in vitro (17-19).

Despite several attempts, it has not been possible to detect any new DNA polymerase activity in cells infected with adenovirus (20, 21). These negative findings have led to the conclusion that a host cell polymerase is involved in the replication of viral DNA. Attempts to define the specific polymerase involved, however, have produced conflicting results with reports implicating only polymerase γ (22, 23), both polymerases α and γ together (24-26) as well as both polymerases β and α together (27). The role that each specific polymerase would play in the replicative process has not yet been defined.

Evidence suggesting that DNA polymerase α is involved in adenovirus DNA replication has come primarily from studies utilizing the drug aphidicolin (24-26, 28). This drug is thought to be a specific inhibitor of DNA polymerase α in uninfected cells (24, 28-30). However, although adenovirus DNA replication has been shown to be sensitive to the drug, a close look at the existing data reveals that important differences exist in the mode of inhibition of DNA polymerase α and adenovirus DNA replication, and thus the conclusion that DNA polymerase α is involved must be regarded with caution.

For example, several groups have shown that 50-300 times more aphidicolin is required to inhibit viral DNA replication in vivo to the same extent as cellular DNA replication (23, 24, 28) and it also has been shown that purified DNA polymerase α is inhibited in vitro by aphidicolin in a manner that is competitive for dCTP (26, 31) while adenovirus DNA synthesis in soluble replication complexes is inhibited by aphidicolin in a manner that is competitive with dTTP (26). In addition, a very elegant study with SV40 and SA7 (a simian adenovirus) in coinfected African Green Monkey Kidney cells has clearly shown that a much lower level of aphidicolin is required to inhibit SV40 DNA replication than SA7 replication (23). Since SV40

DNA replication is known to utilize DNA polymerase α (24) the results imply that SA7 replication does not utilize DNA polymerase α unless it is sequestered, modified, or used in some unusual way.

With these anomalies in mind it became of interest to try to better define the mechanism by which aphidicolin inhibits adenovirus DNA replication. We have reexamined the effect of aphidicolin *in vivo* in infected Hela cells and show that at concentrations of drug which inhibit cellular DNA replication by greater than 97%, adenovirus DNA replication is uneffected and normal yields of virus are produced. The advent of an *in vitro* system capable of initiation as well as elongation (17, 18) has allowed us to examine the specific phase of viral replication inhibited by aphidicolin. We conclude that aphidicolin acts by slowing the overall rate of elongation of viral DNA chains. In addition we show that the postulated initiation event - covalent attachment of dCMP to the 87K preterminal protein - does in fact occur *in vitro*, and is not sensitive to aphidicolin inhibition.

As this manuscript was being prepared we became aware of similar *in vitro* studies which have been published elsewhere (32).

MATERIALS AND METHODS

Cells, Virus, DNA, DNA Protein Complex: Hela cells were obtained from Ed Ziff (Rockefeller University) and grown in suspension in Joklik-modified Eagle spinner medium containing 5% horse serum. Monolayers of Hela cells obtained from Frank Graham (McMaster University) were grown in Eagles minimal essential medium supplemented with 5% fetal calf serum. Adenovirus type 5 (Ad5) was grown, purified, and titered as previously described (8). Ad5 DNA and DNA-protein complex was also prepared as previously described (33).

^{3}H Thymidine Pulse Labeling: Hela cells at a density of 2 x 10^5/ml were infected at a multiplicity of 10 PFU/cell. Varying amounts of aphidicolin were added and the infection was allowed to proceed for 24 hrs. at 37°C. Cells were pulse labeled with 20 uCi/ml of ^{3}H thymidine (sp. act.=50-80 Ci/mmol) for 30 minutes at 37°. Cells were then placed on ice, and non-radioactive thymidine was added to a concentration of 10 mM. The cells were pelleted and resuspended in water. DNA was precipitated by adding 5 volumes of cold 10% trichloro acetic acid (TCA) containing 10 mM sodium pyrophosphate. The precipitate was collected on GF/C glass fiber filters (2.5 cm). Each filter was washed with 10 ml of 10% TCA, 10 mM sodium pyrophosphate and 3 ml 95% Ethanol. The filters were dried and counted in Liquiscint (National

Diagnostics). Uninfected cells were treated in the same manner.

Alkaline Sucrose Gradient Analysis: Cells were treated with aphidicolin, and virus, and pulse labeled for 1 1/2 hrs with ^{3}H thymidine as described above. The samples were then chased with 20 μM nonradioactive thymidine for 1/2 hr. The cells were spun out and resuspended in 0.9M NaCl before being layered onto 10-30% sucrose gradients made up in 0.1M NaOH, 0.9M NaCl, 0.01M EDTA. The gradients were centrifuged for 16 hrs. at 23,000 rpm in an SW41 rotor at 4°C. Fractions from each gradient were collected by pumping from the bottom, TCA precipitated and collected on filters. The pellet was counted directly in Liquiscint.

^{35}S Methionine Labeling of Cells: Hela cells at a density of 3 x 10^{5} cells/ml were centrifuged and resuspended in medium containing 20% of the normal methionine. One set of cells was infected with Ad5 at a multiplicity of 10. A second set was left uninfected. After one hour, varying amounts of aphidicolin were added to the cultures and the cultures were maintained at 37° for 16 hrs. 2.5 uCi/ml of ^{35}S methionine (sp. act.=>400 Ci/mmol) was then added to each culture and incubation was continued at 37° for another 32 hrs. The cells were harvested and after several cycles of freeze-thawing in SDS gel sample buffer, the samples were boiled for 5 mins. and run on a 15.5% SDS polyacrylamide gel (8).

In Vitro DNA Replication: Hela cells at a density of 4-5 x 10^{5}/ml were concentrated to 1/10 their volume and infected with Ad5 at a multiplicity of 100 PFU/cell. After a 1 hr. absorption at 37° the cells were diluted back to their previous volume. Three hours post infection 10 mM hydroxyurea was added and the infection was allowed to proceed for another 15-18 hrs. Nuclei were prepared as previously described (17) and frozen in 1/2 ml aliquots. The extraction of thawed nuclei was as previously described (17).

The standard reaction mixture for in vitro DNA replication was slightly modified from that previously described (18, 19). A final reaction volume of 0.1 ml contained 180 ng adenovirus DNA protein complex, 35 ul of extract, 50 mM HEPES (N-2-hydroxyethyl piperazine-N-2-ethane-sulfonic acid)-NaOH (pH 7.5), 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 2 mM ATP, 10 mM creatine phosphate, 6 μg of creatine phosphokinase, 50 uM each of dCTP, dTTP, dGTP, 10 uMTTP, and 10 uCi ^{32}P-TTP (sp. act. = 400-1000 Ci/mmol in Tricine). Incubation was carried out at 37° for various times.

Analysis of the In Vitro Product: The reaction was stopped by adding 20 mM EDTA and 1% SDS. In some instances, a known amount ^{3}H Ad5 DNA was added to each sample to quantitate and normalize recovery. The samples were heated

at 65° for 20 mins., allowed to cool and treated with pronase at 1 mg/ml for 2 hrs. at 37°. They were then adjusted to 0.2M NaCl and extracted once with phenol, saturated with 0.1M Tris (pH 8.0), and once with chloroform-isoamyl alcohol (24:1). Two volumes of absolute ethanol (-20°C) was added and the samples were frozen in liquid nitrogen. After allowing the samples to thaw they were centrifuged in an Eppendorf centrifuge for 20 mins. The ethanol was removed and the pellet vacuum dried. The samples then were resuspended in 20 µl 10 mM Tris, 1 mM EDTA (pH 7.5) and 20 µl 2x restriction buffer was added. Ten units of restriction endonuclease was added and the samples were allowed to incubate at 37° for 4 hrs. at which time an additional 10 units of enzyme was added. Incubation was continued for an additional 16 hrs. 10 µl of restriction stop (20 mM EDTA 25% Glycerol 0.025% Bromphenol Blue) was added and the samples were subjected to electrophoresis on a 0.7% agarose gel containing .04M Tris, .005M acetate, .001M EDTA at 50V for 18 hrs.

Labeling of 87K Terminal Protein: In vitro DNA synthesis conditions were used with α ^{32}P dCTP, α ^{32}P dGTP, α ^{32}P dTTP, or α ^{32}P dATP at a concentration of 10 uCi/0.1 ml reaction mix (spec. act.=400-1000 Ci/mmol). Reactions contained either DNA protein complex, DNA, or no template and either 0, 10 µg/ml or 50 µg/ml aphidicolin. Reactions were incubated at 37° for 1 hr. and terminated by the addition of SDS gel sample buffer containing 1 mM phenyl methyl sulfonyl flouride (pmsf). The samples were then boiled and applied directly to the 15.5% polyacrylamide gel. The gel was washed extensively in destain solution (40% methanol 10% acetic acid) before drying. In some instances samples were treated with pronase or piperidine before loading (34).

Materials: Aphidicolin was obtained from Dr. Hesp, ICN, England. α ^{32}P dCTP, α ^{32}P dTTP, α ^{32}P dGTP, α ^{32}P dATP, ^{35}S methionine, and ^{3}H thymidine were purchased from New England Nuclear. Restriction enzymes were from Bethesda Research. Creatine phosphokinase was from Sigma.

RESULTS

Effect of Aphidicolin on Viral DNA Synthesis In Vivo: Aphidicolin previously has been shown to inhibit the incorporation of ^{3}H thymidine into TCA precipitable material in both uninfected and infected Hela cells (23, 24, 28). In all cases examined, incorporation in infected cells was found to be more resistant, requiring 50-300 times the amount of drug to reach the same level of inhibition as uninfected cells. In these experiments, infection was allowed to proceed for a given length of time, aphidicolin was added and

after a brief period ^{3}H thymidine incorporation was measured. Because this particular protocol was used, the experiments do not address the possibility that aphidicolin might effect events at the very beginning of the viral DNA replication process. They only examine the effect of the drug at steady state when DNA replication is already ongoing. Accordingly we performed an experiment where aphidicolin, together with virus was added to cells at time zero and 24 hrs. later ^{3}H thymidine incorporation was measured. Uninfected cells were also treated with drug for 24 hrs. before labeling. Figure 1 shows the results of such an experiment. From the data it is clear that in this case, as in those previously reported, DNA synthesis in the infected cell culture is much less sensitive to aphidicolin than DNA synthesis in the uninfected cell culture. It should also be noted that the curve representing the data from the infected cell culture appears to be biphasic. A significant percentage of DNA synthesis in the infected culture has the same sensitivity as synthesis in the uninfected cell culture and a significant percentage is more resistant. One possible explanation of these data is that some cellular DNA synthesis continues in the infected cell culture.

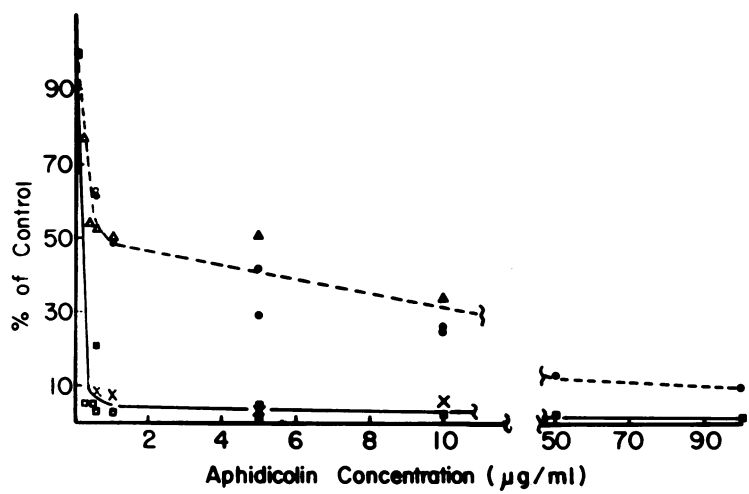

Figure 1: The Effect of Aphidicolin on ^{3}H Thymidine Incorporation into DNA in Infected and Uninfected Hela Cells.

Cells were treated with different concentrations aphidicolin at the time of infection or mock infection and 24 hrs. later they were pulse labeled for 30 mins. with ^{3}H thymidine as described in Materials and Methods. The data shown represents 3 separate experiments. •, ○, Δ Infected Cells; ■, □, x Uninfected Cells.

Accordingly we carried out an analysis of the product synthesized under these conditions of infection and drug treatment to determine if a significant amount of cellular DNA synthesis was in fact continuing in the infected cell culture and if a differential inhibition of viral and cellular DNA synthesis by aphidicolin could be demonstrated. To do this pulse labeled cells were pelleted, resuspended in buffer, and layered onto alkaline sucrose gradients, prepared and centrifuged as described in Materials and Methods. Under these conditions cellular DNA pellets, while viral DNA sediments in a peak at 31S. The results are shown in Figure 2.

Figure 2 panels A and B show the profile of DNA obtained from the uninfected cell culture treated with or without 1 μg/ml of aphidicolin. As expected, the only significant amount of radioactivity found in both gradients is in the pellet of gradient A, indicating, as already shown in Figure 1, that cellular DNA synthesis is inhibited by at least 97% under these conditions. Figure 2 panels C and D show the profile of DNA from the infected cell culture in the absence (Figure 2C) or presence (Figure 2D) of 1 μg/ml aphidicolin. Several points should be noted. Even at 24 hrs. after infection in the untreated sample (Figure 2C) there is significant radioactivity in the cell pellet, indicating that cellular DNA replication is not completely switched off, consistent with results from other laboratories, infecting at similar multiplicities (35, 36). In the case of infected cells treated with 1 μg/ml of aphidicolin however (Figure 2D) this residual cellular DNA replication is reduced to 10%, of control. (Compare pellets of Figure 2C to Figure 2D). The viral DNA peak is unaltered. Figures 2E and 2F show that even when cells are treated with concentrations of aphidicolin as high as 5 and 10 μg/ml, adenovirus DNA replication still continues at a significant rate (20-25% of the control). Thus we are able to conclude that 1 μg/ml of aphidicolin effectively inhibits cellular DNA synthesis without effecting viral DNA synthesis.

As these cultures were infected with a moi of 10 pfu/cell, it appears likely that every cell in the culture was infected, and therefore it appears that aphidicolin effectively inhibits cellular DNA synthesis without effecting viral DNA synthesis in the same cell. It should be noted however, that we have not actually demonstrated that every cell in the culture was infected. There may be cells in the culture refractile to infection by the virus and although the differential effect of the drug on cellular versus viral synthesis is clear, the conclusion that the

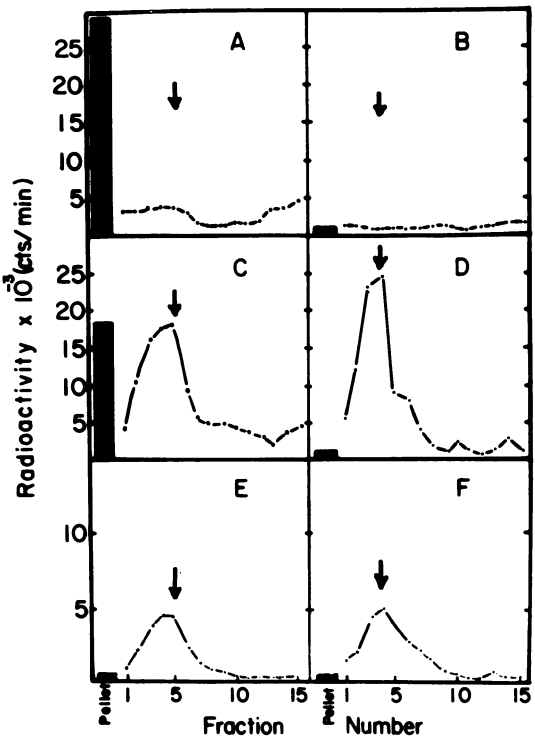

Figure 2: Alkaline Sucrose Gradient Analysis of DNA Synthesized in the Presence or Absence of Aphidicolin in Infected or Uninfected Hela Cells.

Cells were infected or mock infected and treated with different concentrations of aphidicolin as described in Materials and Methods. 24 hrs. later the cells were pulse labeled with ^{3}H thymidine, harvested and spun on alkaline sucrose gradients as described. Fraction 1 corresponds to the bottom of the gradient. A: Uninfected Cells, without aphidicolin; B: Uninfected Cells, with 1 µg/ml aphidicolin; C: Infected Cells, without aphidicolin; D: Infected Cells, with 1 µg/ml aphidicolin; E: Infected Cells, with 5 µg/ml aphidicolin; F: Infected Cells, with 10 µg/ml aphidicolin. The arrow indicates the 31S viral DNA peak.

differential effect can be seen, in the same cell, must be regarded with caution.

Effect of Aphidicolin on Viral Protein Synthesis and Virus Yield: The effect of differing concentrations of aphidicolin on uninfected and infected cell protein synthesis was next examined. Aphidicolin previously has been shown to have little effect on uninfected cell protein synthesis (28). However, since late protein synthesis in infected cells requires viral DNA replication and transcription from replicated genomes (37) it

seemed likely that total late protein synthesis would be reduced at concentrations of drug which reduced viral DNA synthesis significantly.

To examine this, cells were infected as described in Materials and Methods and treated with differing amounts of aphidicolin from the start of infection. ^{35}S methionine was added to the cultures at 16 hrs. and labeling was allowed to proceed until 48 hrs. post infection when the cells were harvested and the proteins were analyzed on an SDS gel. Under these conditions uptake of label remained linear throughout the labeling period. A long label was carried out in order to measure total accumulated late proteins, rather than a rate of protein synthesis as would be measured by a short pulse label. Uninfected cell cultures were treated similarly. Figure 3 shows the results of this experiment. Suprisingly, it is clear

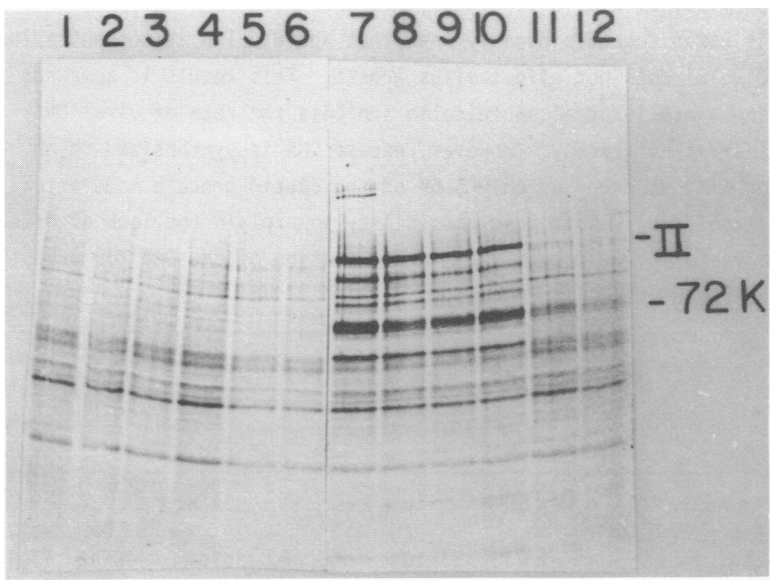

Figure 3: The Effect of Aphidicolin on Protein Synthesis in Uninfected and Infected Cells.

Cells were infected or mock infected and treated with different concentrations of aphidicolin as described in Materials and Methods. 16 hrs. later ^{35}S methionine was added as described and incubation was continued for 32 hrs. An autoradiogram of an SDS polyacrylimide gel of the labeled proteins is shown. Slots 1-6 are from uninfected cells; Slots 7-12 are from uninfected cells. Slots 1 and 7 had .5 μg/ml aphidicolin; Slots 2 and 8 had 1 μg/ml aphidicolin; Slots 3 and 9 had 5 μg/ml aphidicolin; Slots 4 and 10 had 10μg/ml aphidicolin; Slots 5 and 11 had 50 μg/ml aphidicolin; Slots 6 and 12 had 100 μg/ml aphidicolin. Samples containing no aphidicolin appeared identical to Slots 1 and 7 (not shown).

from the data that little effect can be seen on viral protein synthesis, at concentrations of drug up to and including 10 μg/ml, a concentration which reduced the rate of DNA replication by 80%. At concentrations greater than 10 μg/ml, however, late proteins are clearly inhibited but synthesis of the 72K DBP, an early protein, is still visible though somewhat reduced. This indicates that the effect of the drug is not on viral protein synthesis in general but probably is a consequence of its effects on DNA replication. Little effect on uninfected cell protein synthesis is seen.

To further confirm that full yields of late proteins are produced at these concentrations of drug, we measured virus yield, by harvesting the cells and performing a plaque assay. Table 1 shows that little effect on yield is seen at concentrations up to 10 μg/ml, though 100 μg/ml reduced the yield by 2-3 logs. These data and those for late protein synthesis taken together clearly show that aphidicolin at concentrations up to 10 μg/ml does not effect virus growth. This result is somewhat surprising since 10 μg/ml aphidicolin inhibits the rate of viral DNA replication significantly. However, excess DNA is synthesized in an infection without drug and only 5-6% of the capsid protein made actually gets packaged (38). Thus, one possibility to explain the lack of effect on virus yield may be more efficient utilization of the DNA for transcription, translation, and assembly.

TABLE 1

THE EFFECT OF APHIDICOLIN ON VIRUS YIELD

Aphidicolin Concentration (μg/ml)	Exp. 1 PFU/Cell 24 hr. Yield	Exp. 1 PFU/Cell 48 hr. Yield	Exp. 2 PFU/Cell 48 hr. Yield
0	535	910	----
0.1	650	1055	----
0.5	595	1085	----
1.0	---	----	1250
5.0	565	915	----
10.0	325	775	833
100.0	---	----	1.9

Hela cells in suspension were infected with adenovirus type 5 at a moi of 10 as described in Materials and Methods. Aphidicolin was added simultaneously with virus. Cells were harvested and virus yields were titered at times indicated.

Interestingly, the shut off of late protein synthesis between 10 µg/ml and 50 µg/ml of drug is rather abrupt. This abrupt transition provides a convenient method to titer the concentration of aphidicolin required to inhibit viral DNA replication completely. We have analysed the concentration of aphidicolin required to inhibit Ad12, Ad3, and Ad7 replication in this manner and find that in the same cell type concentrations of aphidicolin equivalent to that required to inhibit Ad5 replication are required to cause inhibition of these serotypes, though the end point is somewhat different in different cell lines (data not shown).

Analysis of the Effect of Aphidicolin In Vitro: Recently, Challberg and Kelley have described an in vitro system capable of initiation of adenovirus DNA replication de novo (17, 18). The system requires DNA containing terminal protein as substrate. We have utilized this system to further characterize the effects of aphidicolin on viral DNA replication. However, preliminary characterizations of the in vitro system were first carried out to assure that initiation and replication were occuring properly. In our hands initiation in this system is extremely sensitive to varying extract concentrations. Low concentrations of extract (5λ per 100λ reaction mix) promoted a repair type of reaction rather than the replication reaction, while higher concentrations (35λ per 100λ reaction mix) promoted replication (data not shown). The optimal concentration of extract varied somewhat from prep to prep and consequently was always titered. The simplest and best criteria for initiation and correct replication was found to be analysis of the distribution of label in restriction cut product with time. Since initiation occurs at the ends of the molecules terminal fragments should label first followed by more internal ones. Figure 4 shows a typical time course analysis of DNA synthesis in the extract used in subsequent experiments. In this particular time course the product was cut with restriction enzyme KPN_I. The KPN_I map of Ad5 DNA is shown at the bottom of Figure 4. Slots A, B, C, and D show the product formed after 5, 10, 30, and 60 minutes of incubation at 37°C with either DNA (1) or DNA protein complex (2) as template. From the data it is clear that only the DNA protein complex functions properly as a template in a replication type reaction when DNA protein complex is used. Slot A2 shows that at 5 mins. only fragments G and H are labeled; Slot B2 shows that at 10 mins. fragments H, A. D, and G are primarily labeled; Slot C2 and Slot D2 show that at 30 and 60 mins. the

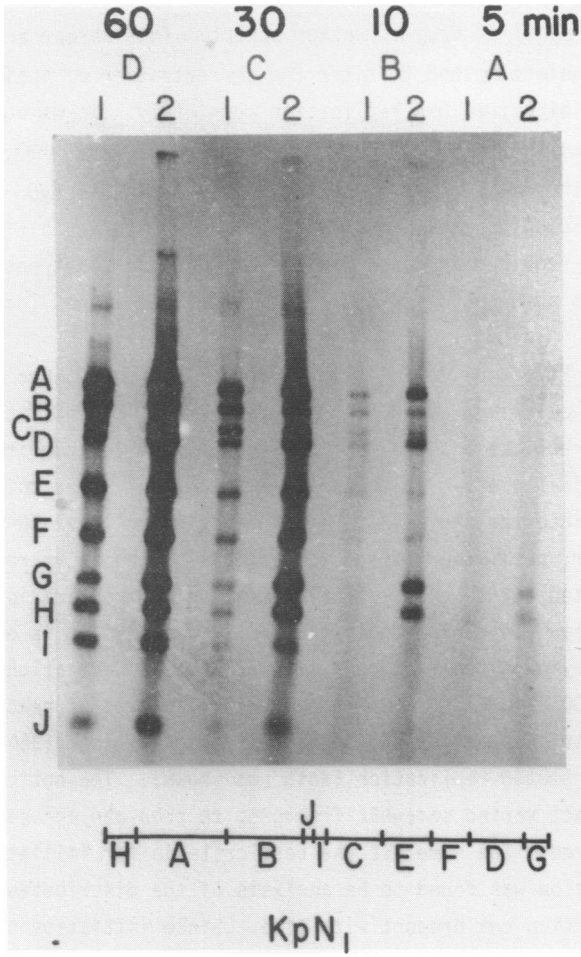

Figure 4: Time Course Analysis of KPN$_I$ Digested In Vitro DNA Product.
An in vitro reaction mix containing pronase treated DNA (Slot 1) or Ad5 DNA protein complex (Slot 2) was prepared and incubated with α ^{32}P dTTP as described in Materials and Methods for A: 5 mins.; B: 10 mins.; C: 30 mins.; D: 60 mins. The reaction was stopped, the DNA extracted and cut with KPN$_I$. The samples were then analyzed on a 0.7% agarose gel. A fluorogram of the gel is shown.

label becomes uniform. Pronase treated DNA on the other hand functions less efficiently and directs the synthesis of a more uniformly labeled product predominantly after long incubations.

To analyze the effect of aphidicolin on replication, an incubation mix

was prepared and preincubated in the absence of template for 15 mins on ice, with differing amounts of aphidicolin. DNA protein complex was then added and the reactions were placed at 37°. Samples were taken at different times after the start of incubation and at each time point the reaction was stopped by pipetting the sample into 20 mM EDTA and 1% SDS. The product was preciptated and treated as described before digestion with restriction enzyme Hind III. The digested DNA was then analyzed on agarose slab gels.

Figure 5A shows an autoradiogram of samples treated with a range of aphidicolin from 0 to 100 µg/ml and incubated for 60 mins. From these data it is apparent that in going from 10 µg/ml to 50 µg/ml of drug there is an abrupt shut off of incorporation (compare Slots 6 to Slots 7 and 8). This is remeniscent of the abrupt shut off seen in vivo in this concentration range. In the samples treated with 5 or 10 ug/ml of drug, radioactivity appears preferentially in restriction fragments mapping nearest to the ends of the molecule. The most internal fragments A, D, and H are hardly labeled at all, while the most external fragments G, E, F, and I are labeled in amounts roughly equivalent to the control.

To investigate this phenomena further a detailed time course, was undertaken. This is shown in Figure 5B. Slots 1-3, 4-6, 7-9, and 10-12 show the product formed after 30, 45, 60, or 90 mins. of incubation with either 0, .5, or 5 µg/ml of drug. A comparison of the restriction patterns at the various time points shows clearly that it takes a longer time for radioactivity to appear in the internal fragments when aphidicolin is present than when it is not. Even at .5 µg/ml an effect can be seen at the early time points, though by 90 mins. the pattern appears similar to the control, though slightly diminished in intensity. At 5 µg/ml the most internal fragments, D and H, are still reduced in intensity after 90 mins. of incubation. From these data we conclude that aphidicolin exerts its effect, at concentrations up to 10 µg/ml, by slowing the overall rate of DNA chain elongation. Initiation appears to be uneffected since the terminal restriction fragments label almost as well as in the control. At concentrations greater than 10 µg/ml an unexplained more complete inhibition is apparent.

Labeling of the 87K Preterminal Protein with α ^{32}P dCTP: The model which we have previously proposed for initiation of adenovirus DNA replication (8), as modified by Challberg and Kelley (13), involves the covalent attachment of the preterminal protein to the first nucleotide (dCMP) of the adenovirus DNA chain, prior to chain elongation. Since 10 µg/ml of aphidicolin substantially slows chain elongation, we reasoned that if the protein primer

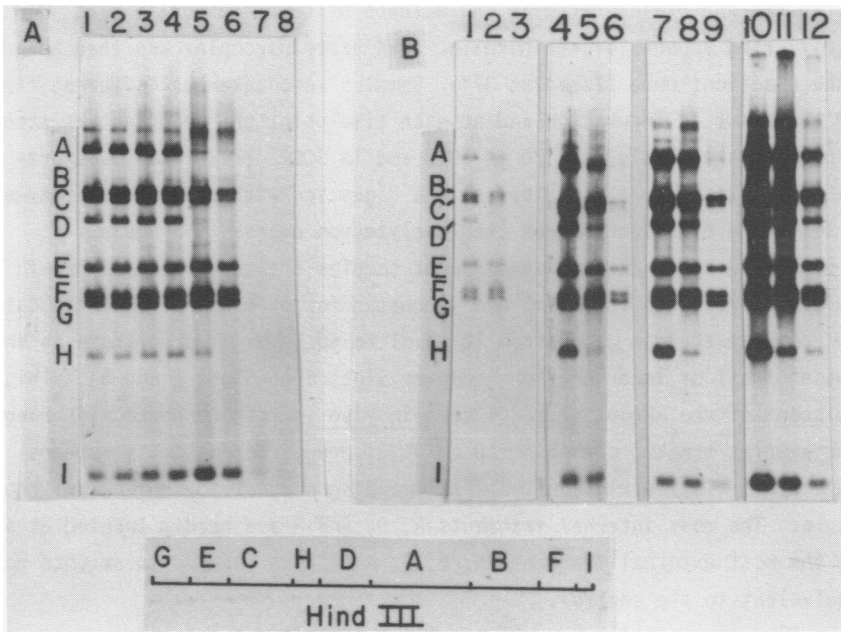

Figure 5: The Effect of Aphidicolin of Adenovirus DNA Replication In Vitro.
A: An in vitro reaction mix containing Ad5 DNA protein complex and α^{32}P dTTP was prepared and incubated with aphidicolin for 60 mins. as described in Material and Methods. The reaction was stopped, the DNA extracted and cut with Hind III as described. ^{3}H Ad5 DNA was added to the samples prior to extraction to normalize the recoveries. The samples were analyzed on a 0.7% agarose gel. A fluorogram of the gel is shown. Slot 1: no aphidicolin; Slot 2: 0.1 μg/ml aphidicolin; Slot 3: 0.5 μg/ml; Slot 4: 1 μg/ml; Slot 5: 5 μg/ml; Slot 6: 10 μg/ml; Slot 7: 50 μg/ml; Slot 8: 100 μg/ml.
B: Three in vitro reaction mixes containing Ad5 DNA protein complex, α^{32}P dTTP and aphidicolin at 0 μg/ml, 0.5 μg/ml or 5 μg/ml were incubated as described. DNA was extracted and cut with Hind III. ^{3}H Ad5 DNA was added prior to extraction to normalize recoveries. The samples were analyzed on a 0.7% agarose gel. Slots 1, 4, 7, and 10 had 0 μg/ml aphidicolin; Slots 2, 5, 8, and 11 had 0.5 μg/ml aphidicolin; Slots 3, 6, 9, and 12 had 5 μg/ml aphidicolin. Sample in Slots 1-3 were incubated for 30 mins.; Slots 4-6 for 45 mins.; Slots 7-9 for 60 mins.; Slots 10-12 for 90 mins.

model were correct, an initiation complex containing the preterminal protein and dCMP might form and accumulate in vitro in the presence of aphidicolin. We also speculated that the more complete inhibition of replication seen at 50 μg/ml of aphidicolin might be due to a second effect of the drug on initiation as well as on elongation. If this were the case, the presumptive complex which we hoped to find when 10 μg/ml of aphidicolin was used might not form when 50 μg/ml of aphidicolin was used.

To test these possibilities an in vitro DNA synthesis reaction mix was incubated with or without Ad5 DNA protein complex in the presence or absence of 50 μg/ml aphidicolin using α ^{32}P dCTP, α ^{32}P dATP, α ^{32}P dTTP, or α ^{32}P dGTP as label. After an hour of incubation at 37° the reaction was terminated by the addition of SDS gel sample buffer and boiling. The samples were then loaded directly onto an SDS gel slab together with an ^{125}I labeled preterminal protein marker from tsl-39° virions (7, 10) (Slot M). An autoradiogram of the gel is shown in Figure 6A. Slots 1-8 show the proteins labeled in the reactions which contained 50 μg/ml aphidicolin while Slots 9-16 show the proteins labeled without aphidicolin. Essentially the same results are seen whether 10 μg/ml or 50 μg/ml of aphidicolin is used (data not shown). Analysis of the data reveals that only the samples from the reaction mix containing both DNA protein complex and α ^{32}P dCTP contain a labeled band which comigrates with authentic 87K preterminal protein (Slots 1 and 9). Slot 1, which contained 50 μg/ml aphidicolin contained less background and less material at the top of gel than Slot 9, presumably because labeled DNA was not synthesized in this sample due to the high concentration of drug. Both samples clearly have roughly equivalent amounts of the 87K protein band. Other bands are also seen on the gel. In particular, a band is seen at about 30K which labels with all four nucleotides and another smaller molecular weight band which labels only with α ^{32}P dGTP is also seen. These bands were not investigated further.

In another experiment the nature of the 87K band was partially characterized. The 87K band present in Slot 1, Figure 6B, was shown to be sensitive to pronase digestion, (Figure 6B, Slot 4) and to alkaline hydrolysis by piperidine (Figure 6B, Slot 7). Alkaline sensitivity is a property of the authentic preterminal protein nucleotide linkage (13, 34). The material removed from the 87K band by alkali was further examined and shown to comigrate with dCMP by chromotography on PEI plates (data not shown). The 87K band does not appear when pronase treated DNA (Figure 6B, Slot 2) or no DNA (Figure 6B, Slot 3) is used as template. Thus from these data we conclude that the postulated initiation reaction involving the addition of dCMP to the preterminal protein occurs in vitro, in the presence of DNA protein complex. Contrary to our original speculations the reaction does not appear to be enhanced by 10 μg/ml aphidicolin or inhibited by 50 μg/ml aphidicolin, although aphidicolin does reduce the "background" and makes the band more visible.

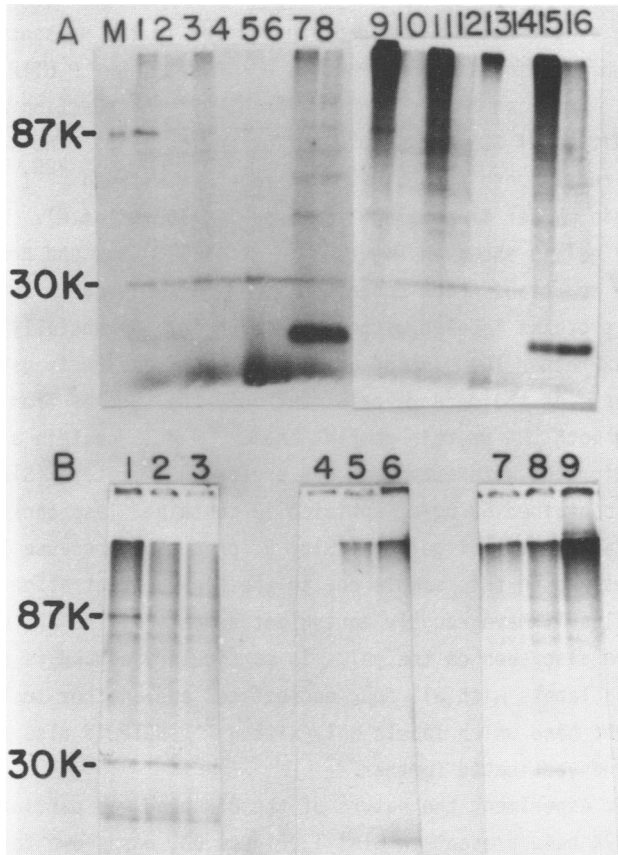

Figure 6: Labeling of the 87K Preterminal Protein with α ^{32}P dCTP.

A: An in vitro reaction mix was prepared and incubated in the presence (Slots 1-8) or absence (Slots 9-16) of 50 μg/ml aphidicolin. Samples in ODD numbered slots contained DNA protein complex; samples in EVEN numbered slots contained no added template; Slots:1, 2, 9, 10 contained α ^{32}P dCTP; Slots 3, 4, 11, 12 contained α ^{32}P dATP; Slots 5, 6, 13, 14 contained α ^{32}P dTTP; Slots 7, 8, 15, 16 contained α ^{32}P dGTP. Samples were incubated for 60 mins. at 37°C as described. The incubation was terminated by the addition of SDS gel sample buffer and boiling. They were then loaded directly onto an SDS polyacrylamide gel and subjected to electrophoresis. A fluorogram of the gel is shown.

B: An in vitro reaction mix was prepared and incubated in the presence of 50 μg/ml aphidicolin for 60 mins. The mix contained α ^{32}P dCTP and Ad5 DNA protein complex (Slots 1, 4, 7); or pronase treated Ad5 DNA (Slots 2, 5, 8); or no added template (Slots 3, 6, 9). Samples in Slots 1, 2, and 3 received no additional treatment after incubation; Samples in Slots 4, 5, and 6 were incubated at 37°C with 1 mg/ml pronase for 60 mins. prior to loading on the gel; Samples in Slots 7, 8, and 9 were treated with 1M piperidine for 60 mins. at 37°C prior to loading on the gel. Samples were processed as in part A. A fluorogram of the resulting gel is shown.

DISCUSSION

Our results, consistent with those previously published (23, 24, 26, 28), clearly demonstrate that adenovirus DNA replication and growth is sensitive to inhibition by aphidicolin, although substantially higher concentrations are required for complete inhibition than is normally required for the complete inhibition of cellular DNA replication. This differential effect is not merely an apparent difference, caused by some feature of infection which creates a metabolic alteration of the cells (e.g. altered nucleotide pools), since the effect is also seen in vitro, and cellular DNA replication can be inhibited by the drug in the same cells, at the same time, that virus can be shown to grow normally. Because aphidicolin is a specific inhibitor of DNA polymerase α in the uninfected cell, it is tempting to speculate that DNA polymerase α is involved in some stage of adenovirus DNA replication. However, the different parameters for inhibition of polymerase α and viral DNA replication cannot be overlooked and must be addressed in any model that implicates polymerase α in the viral replicative process. It is equally likely that an interaction with some virus specified factor is involved in the action of aphidicolin on viral DNA replication. Both Herpes virus (24, 39) and Vaccinia virus (37) are known to specify viral encoded polymerases which are sensitive to aphidicolin at different concentrations then DNA polymerase α. Although there is no indication that adenovirus encodes a polymerase (20, 21) we cannot rule out the notion that it encodes a polymerase subunit or some other protein involved in DNA replication which is sensitive to the drug. Almost any of the viral early proteins would be a possible candidate.

Attempts to demonstrate a differential sensitivity to aphidicolin between adenovirus subgroups proved unsuccessful (data not shown). DNA synthesis directed by all of the different adenovirus serotypes examined is equally resistant to inhibition. Thus, if a viral specified function does mediate sensitivity to aphidicolin, it must be a highly conserved one. The fact that SA7, a simian adenovirus, shows a sensitivity to the drug that is roughly equivalent to the sensitivity of human adenoviruses supports this idea (23).

Although a definition of the precise target of aphidicolin action is not yet forthcoming from the data available, we have sought to better define the phase of viral DNA replication effected by the drug. Analysis of DNA replication in the in vitro system which we are using, has allowed us to define two distinct phases of replication - an initiation phase represented by the covalent attachment of dCMP to the preterminal protein and an elongation

phase leading to the synthesis of full length adenovirus DNA.

The initiation phase has been studied in some detail in vitro. In our system, dCMP is added directly to an 87K protein which comigrates with the 87K preterminal protein from tsl-virions. The addition of dCMP to this protein is totally dependent on the presence of added DNA protein complex suggesting that an initiation complex must form between the soluble 87K protein and the DNA protein complex, before nucleotide can be added. Of the 4 nucleotides tested, only dCMP is added to the protein. This is the first nucleotide of the Ad5 DNA chain (39, 40). We have further shown that the linkage of dCMP to the soluble 87K protein is sensitive to cleavage with piperidine, as is the linkage between the DNA bound protein and DNA, suggesting that both proteins may indeed be the same. Definitive proof that they are awaits peptide mapping. Similar results and conclusions have recently been reported by others (32). The fact that the initiation reaction still occurs in the presence of 50 µg/ml aphidicolin definitively rules out DNA polymerase α as a catalyst in the formation of the protein-nucleotide bond. Further experiments are needed to determine if the nucleotide addition reaction is autocatalytic or mediated by an additional enzyme.

Although little effect of aphidicolin can be detected on initiation we can detect a dramatic effect on elongation. Interestingly the effect appears to be a very gradual one causing a slowing of elongation with increasing concentration of drug until, at concentrations of greater than 10 µg/ml, an abrupt shut off is seen. This abrupt shut off suggests that either a second target is hit at these high concentrations or that a cooperative type of inhibition is occurring. It is likely that these in vitro results accurately reflect the in vivo situation since our results on inhibiton of late protein synthesis suggest the same type of phenomenon.

There are several possible ways that aphidicolin may slow elongation, at intermediate concentrations of drug, which our data do not allow us to discriminate between. For example we cannot distinguish between an effect on the instantaneous rate of chain elongation at all nucleotide linkages, at a limited number of specific nucleotide linkages or at a limited number of random nonspecific nucleotide linkages. Experiments are in progress to distinguish between these possibilities and to elucidate the target of the drug.

ACKNOWLEDGEMENTS

We thank Joel Huberman, Mary-Helen Binger and the DNA Club of Greater

Buffalo for encouragement and helpful discussions; Jacquie McDonough for excellent technical assistance; Angela Dudek for typing the manuscript; Edwin B. Rekosh for printing the figures; and Dr. B. Hesp of Imperial Chemical Industries, Ltd. for his generous gift of aphidicolin. This work was supported by Public Health Service grant number CA 25674 from the National Cancer Institute.

*To whom reprint requests should be addressed.

REFERENCES

1. Winnacker, E.L. (1978) Cell 14, 761-773.
2. Jeng, Y.H., Wold, W.S., Sugawara, K., Gilead, Z. and Green, M. (1977) J. Virol. 22, 402-411.
3. Levinson, A.D., Postel, E.M. and Levine, A.J. (1977) Virology 79, 144-159.
4. Linne, T., Jornvall, M. and Philipson, L. (1977) Eur. J. Biochem. 76, 481-490.
5. Sugawara, K., Gilead, Z. and Green, M. (1977) J. Virol. 21, 338-346.
6. Klein, M., Maltzman, W. and Levine, A.J. (1979) J. Biol. Chem. 254, 11051-11060.
7. Challberg, M.D. and Kelly, T.J., Jr. (1981) J. Virology 38, 272-277.
8. Rekosh, D.M.K., Russell, W.C., Bellett, A.J.D. and Robinson, A.J. (1977) Cell 11, 283-295.
9. Weber, J. (1976) J. Virol. 17, 462-471.
10. Stillman, B.W., Lewis, J.B., Chow, L.T., Matthews, M.B. and Smart, J.E. (1981) Cell 23, 497-508.
11. Desiderio, S.V. and Kelly, T.J., Jr. (1980) J. Mol. Biol. 145, 319-337.
12. vanderVliet, P.C. and Levine, A.J. (1973) Nature, New Biology 246, 170-173.
13. Challberg, M.D., Desiderio, S.V. and Kelly, T.J., Jr. (1980) Proc. Natl. Acad. Sci. USA 77, 5105-5109.
14. Weingartner, B., Winnacker, E.L., Tolun, A. and Pettersson, V. (1976) Cell 9, 259-268.
15. Sussenbach, J.S. and Kuijk, M.G. (1977) Virology 84, 509-517.
16. Robinson, A.J., Bodnar, J.W., Coombs, D.H. and Pearson, G. (1979) Virology 96, 143-158.
17. Challberg, M.D. and Kelly, T.J., Jr. (1979) Proc. Natl. Acad. Sci. USA 76, 655-659.
18. Challberg, M.D. and Kelly, T.J., Jr. (1979) J. Mol. Biol. 135, 999-1012.
19. Stillman, B.W. (1981) J. Virol. 37, 139-147.
20. Bolden, A., Aucker, J. and Weissbach, A. (1975) J. Virol. 16, 1584-1592.
21. deJong, A., vanderVliet, P.Ch. and Jansz, H.S. (1977) Biochem. Biophys. Acta 476, 156-165.
22. Shaw, C.H., Rekosh, D.M.K. and Russell, W.C. (1980) J. Gen. Virol. 48, 231-236.
23. Kwant, M.M. and vanderVliet, P.C. (1980) Nucleic Acid Res. 8, 3993-4007.
24. Krokan, H., Schaffer, P. and DePamphilis, M.L. (1979) Biochemistry 18, 4431-4443.
25. Van derWerf, S., Bourke, J.P., Mehali, M. and Girard, M. (1980) Virology 104, 56-72.
26. Habara, A., Kano, K., Nagano, H., Mano, Y., Ikegami, S., and Yamashita, T. (1980) Biochem. Biophys. Res. Comm. 92, 8-12.

27. Ikeda, J.E., Enomoto, T. and Hurwitz, J. (1981) Proc. Nat. Acad. Sci. USA 78, 884-888.
28. Lorgiaru, M., Ikeda, J.E., Jarkovsky, Z., Horwitz, S.B. and Horwitz, M.S. (1979) Nuc. Acid. Res. 6, 3369-3386.
29. Ikegami, S., Taguchi, T., Ohashi, M., Oguro, M., Nagano, H. and Mano, Y. (1978) Nature 275, 458-460.
30. Wist, E., and Prydz, H. (1979) Nucleic Acid Res. 6, 1583-1590.
31. Oguro, M., Shioda, M., Nagano, H., Namo, Y., Hanaoka, F., and Yamada, M. (1980) Biochem. Biophys. Res. Comm. 92, 13-19.
32. Lichy, J.H., Horwitz, M.S. and Hurwitz, J. (1981) Proc. Nat. Acad. Sci. USA 78, 2678-2682.
33. Sharp, P.A., Moore, C. and Haverty, J.L. (1976) Virology 75, 442-456.
34. Rekosh, D. (1981) J. Virol., in press.
35. Daniell, E. (1980) Virology 107, 514-519.
36. Longiaru, M. and Horwitz (1981) Mol. and Cell. Biol. 1, 208-215.
37. Thomas, G.P. and Matthews, M.B. (1980) Cell 22, 523-534.
38. Green, M. (1962) Cold Spring Harb. Symp. Quant. Biol. 27, 219-235.
39. Pedrali-Noy, B. and Spadari, S. (1980) J. Virol. 36, 457-464.
40. Steenbergh, P.H. and Sussenbach, J.S. (1979) Gene 6, 307-318.
41. Arrand, J.R. and Roberts, R.J. (1979) J. Mol. Biol. 128, 577-594.