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Adenovirus DNA synthesized in the presence of aphidicolin

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ABSTRACT

Adenovirus types 2 and 5 DNA synthesized in vivo and in vitro in the presence of aphidicolin were studied. Inhibition of adenoviral DNA synthesis by aphidicolin was only 70% even at a concentration of 30 μg/ml of aphidicolin, at which the cellular DNA synthesis was completely inhibited. When initiation of the viral DNA synthesis was synchronized with hydroxyurea and labeled with [^{3}H]thymidine for 60 min, the viral DNA synthesized in the presence of 30 μg/ml of aphidicolin was not of full length (35 kb) but small (approximately 12 kb) by analysis of alkaline sucrose density gradient centrifugation. When initiation of the viral DNA synthesis was not synchronized, the viral DNAs ranging from full size to 12 kb were synthesized in the presence of aphidicolin, indicating that the nascent DNAs longer than about 12 kb can continue to elongate in the presence of aphidicolin. This 12 kb DNA was not derived from the degradation products of newly synthesized full size adenoviral DNA. The viral DNA synthesis was restored and the full size of adenoviral DNA was attained within 15 min following removal of aphidicolin. About 20% of the entire viral genome length from the 5'-end was not inhibited by aphidicolin, while the synthesis of interior fragments of the adenoviral DNA was markedly inhibited by aphidicolin, judging from the electrophoretic pattern on neutral agarose gel after digestion of DNA with Hind III. These results indicate that aphidicolin inhibits adenoviral DNA replication at the internal region located approximately 20-30% from both terminals.

INTRODUCTION

Replication of adenovirus (Ad) DNA (35 kilobase pairs, double stranded linear DNA with a terminal protein at 5'-ends) occurs in the nuclei of infected cells (1). When Ad DNA synthesis takes place, cellular DNA synthesis is suppressed. The first step of viral DNA synthesis is performed by strand displacement mechanism and the second, by complementary strand synthesis (1). Ad viral DNA replication initiates at the 5'-end of both strands using a precursor of the terminal protein as a primer. Chain growth of the new strand of viral DNA may occur continuously since no Okazaki pieces have been detected by analysis of pulse-labeled Ad DNA synthesized in infected-human cells (1).

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Although the nature of DNA polymerases involved in Ad DNA replication has not been clarified as yet, cellular DNA polymerases were considered responsible for viral DNA replication since no viral-coded DNA polymerase had been detected in Ad infected cells (2). Recently, however, the exsistence of adenovirus-coded DNA polymerase (140 K DNA polymerase) has been reported from a sequence analysis of a DNA region mapped by H5ts149, defective in viral DNA synthesis at a nonpermissive temparature (3) and from experiments of *in vitro* complementation of H5ts149 DNA replication (4). Among cellular DNA polymerases classified as DNA polymerase-α, β and γ (5), it is strongly suggested that DNA polymerase-β is not involved in Ad DNA replication since DNA polymerase-β activity has not been found in a DNA replication complex from Ad infected cells (6-9). Although it is still unclear whether DNA polymerase-α, γ or other enzymes are involved in Ad DNA replication, it has been suggested that DNA polymerase-γ participates primarily in Ad DNA replication (6, 10, 11) since ddTTP, which inhibits DNA polymerase-β and γ but not α, strongly inhibits Ad DNA replication in isolated nuclei. Enomoto *et al*. have demonstrated the involvement of a viral coded DNA polymerase that has the properties of both DNA polymerase-α and γ (12).

On the other hand, aphidicolin inhibits DNA synthesis *in vivo* by specifically inhibiting DNA polymerase-α activity, not β and γ (8, 13-16). Higher concentrations of aphidicolin are required for inhibition of Ad DNA synthesis than for cellular DNA synthesis (8, 9, 12, 14, 15, 17). Though the elongation of Ad DNA replication was inhibited by aphidicolin, the initiation and subsequent elongation up to 26 nucleotides from the 5'-end were not inhibited even in the presence of a high concentration of aphidicolin (18, 19). Foster *et al*. suggest that both DNA polymerase-α and the 72 K dalton single-stranded DNA binding protein play an important role in elongation, based on sensitivity toward aphidicolin of the Ad DNA replication of H5ts125, a conditional lethal mutant in the gene of 72 K DNA binding protein (20). In *in vitro* reconstitution system of adenovirus DNA synthesis, aphidicolin inhibits chain elongation but not initiation (21).

In this study, it is reported that in the presence of aphidicolin approximately 12 kb DNA fragments are produced in Ad infected KB cells in which viral DNA synthesis is synchronized with hydroxyurea, and in *in vitro* Ad DNA replication system. This suggests that DNA polymerase-α is involved in elongation at a certain region of viral DNA located approximately 12 kb from each terminal and that the other enzyme(s) insensitive toward aphidicolin is involved in the initiation and elongation of the other region of viral DNA.

MATERIALS AND METHODS

Tritiated thymidine (47 Ci/mmole) and [α-^{32}P]dCTP (48 Ci/mmole) were purchased from Radiochemical Center, Amersham, England. Aphidicolin was kindly supplied from Dr. H. Todd, Imperial Chemical Industries. Hind III was from Takara-Shuzo Corp.

Cell culture, virus infection and determination of DNA synthesis in infected cells

A KB cell culture and Ad2 virus infection were carried out as described previously (22). Ad2 viral DNA synthesis was synchronized with 20 mM hydroxyurea as described previously (22). After hydroxyurea was removed 18 hr following infection, aphidicolin was added to the culture at a concentration of 30 μg/ml, and then [^{3}H]thymidine was administered to the infected cells (5 x 10^{5} cells) at a final concentration of 5 μCi/ml or 10 μCi/ml. This was followed by incubation for 60 min in the presence of aphidicolin. The radioactivity incorporated into the DNA was determined as described (19).

Alkaline sucrose gradient centrifugation

Analysis of labeled DNA was carried out by alkaline sucrose gradient centrifugation as described previously with slight modification (23). The infected cells pulse-labeled with [^{3}H]thymidine in the presence of 30 μg/ml of aphidicolin were washed with cold phosphate buffered saline (PBS), and suspended in 0.5 ml of PBS. An equal volume of solution containing 0.6 M NaOH, 1.0 M NaCl, and 20 mM EDTA was added to the suspension. The samples were mixed with [^{14}C] thymidine labeled Ad DNA as an internal marker, loaded onto 5-20% alkaline sucrose gradients in 0.3N NaOH, 0.5M NaCl and 10 mM EDTA and centrifuged in a SW 41 rotor at 35,000 rpm for 3.5 hr at 15°C. The radioactivity of each fraction was determined as described previously (23).

Electrophoretic analysis of restriction fragments of Ad DNA synthesized in vitro

For analysis of the Ad DNA synthesized *in vitro*, the Ad5 DNA-protein complex, nuclear extract, and cytoplasmic fraction prepared from Ad5 infected HeLa cells were incubated with [^{32}P]dCTP at 37°C for 60 min in the presence of 100 μg/ml of aphidicolin as described previously (24). The labeled DNA was digested with Hind III at 37°C for 90 min and electrophoresed on 1.0% agarose gels (24). The gels were dried and autradiographed on Kodak X ray film XRS.

RESULTS

Effects of aphidicolin on Ad2 DNA and cellular DNA synthesis in vivo

To investigate the effects of aphidicolin on Ad DNA synthesis, the

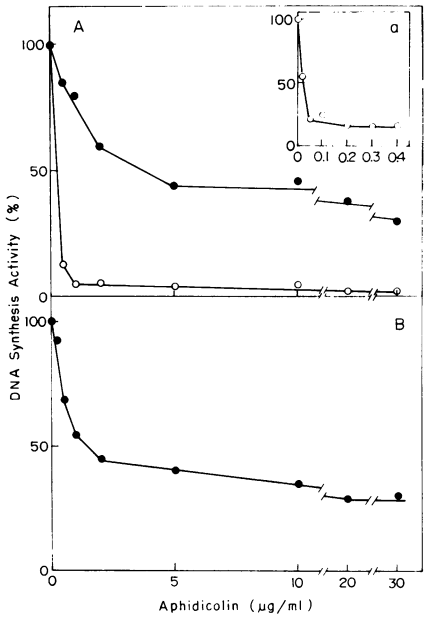


Fig. 1. Effects of aphidicolin on adenovirus and cellular DNA synthesis in vivo. The Ad2-infected and mock-infected cells were labeled with [^{3}H]-thymidine for 1 hr in both the presence and absence of aphidicolin and the radioactivities incorporated were counted by the method described in "Materials and Methods". The radioactivity incorporated in DNA in the absence of aphidicolin was 14,947 cpm and 31,595 cpm for adenovirus infected and mock -infected cells, respectively. A: ●, adenovirus infected cells not treated with hydroxyurea; o, mock-infected cells not treated with hydroxyurea. The inserted figure (o) is an expansion of the abscissa of Fig. 1. B: ●, adenovirus infected cells treated with hydroxyurea.

initiation of Ad DNA synthesis was synchronized by hydroxyurea treatment. Type 1 Ad DNA synthesis primarily undergoes stress under these particular conditions (1). Fig. 1 shows the inhibitory effects of aphidicolin on DNA synthesis in Ad2 infected and uninfected KB cells. Cellular DNA synthesis was highly sensitive to aphidicolin (Fig. 1a and 1A). However, a much higher concentration of aphidicolin was required for the inhibition of Ad DNA synthesis whose initiation was or was not synchronized with hydroxyurea (Fig. 1A and 1B). From the results in Figs. 1A and 1B, it is apparent that the inhibition pattern is biphasic and that about 30% of the Ad DNA synthesis is still resistant toward very high concentrations of the drug, suggesting that the initiation of Ad DNA synthesis occurs in the presence of aphidicolin since initiation of Ad DNA synthesis occurred synchronously under the conditions

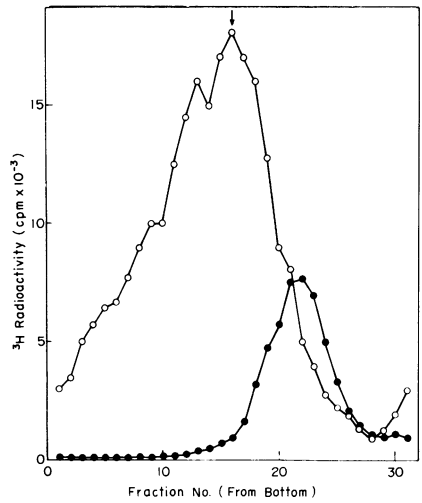


Fig. 2. The pattern of alkaline sucrose density gradient centrifugation of Ad2 DNA synthesized in vivo in the presence (●) and absence (o) of 30 μg/ml of aphidicolin. The infected KB cells were treated with hydroxyurea and labeled with [^{3}H]thymidine (5 μCi/ml) for 1 hr. Thereafter, the cells were loaded onto a 5 to 20% alkaline sucrose density gradient and centrifuged as described in "Materials and Methods". The arrow indicates the Ad DNA as an internal marker (34 S).

shown in Fig. 1B. The level of inhibition by aphidicolin, however, varied for each experiment.

Size of Ad2 DNA synthesized in the presence of aphidicolin

The initiation of Ad2 DNA synthesis in infected KB cells was synchronized by treatment of a culture with hydroxyurea. The DNA synthesized in either the presence or absence of aphidicolin was analyzed by alkaline sucrose density gradient centrifugation (Fig. 2). Nearly all the DNA labeled with [^{3}H]thymidine in the absence of aphidicolin sedimented at the same position as the marker Ad DNA. Aphidicolin produced homogenous and small size DNA; the size was calculated to be about 12 kb. This indicates that aphidicolin inhibits Ad DNA synthesis in a limited region located about 12 kb from both 5'-termini.

A sedimentation pattern of Ad DNA synthesized in infected KB cells not treated with hydroxyurea is shown in Fig. 3. In the case of non-synchronized Ad DNA synthesis, DNA labeled with [^{3}H]thymidine in the presence of aphidicolin was heterogeneous; the 12 kb DNA and nearly full size DNA along with intermediates were detected under these conditions, indicating that the

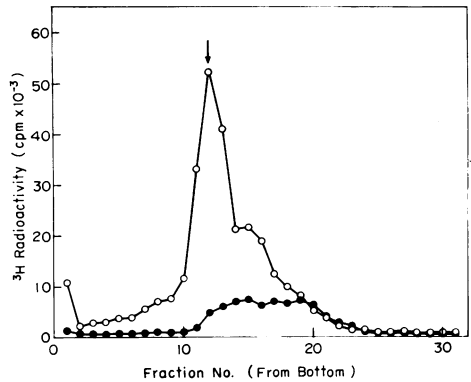


Fig. 3. The pattern of alkaline sucrose density gradient centrifugation of viral DNA synthesized in adenovirus type 2-infected cells not treated with hydroxyurea. Adenovirus-infected cells were labeled with [^{3}H]thymidine (5 μCi/ml) for 30 min in the presence (●) and absence (o) of aphidicolin, and then centrifuged as described in "Materials and Methods". The arrow indicates Ad DNA as an internal marker.

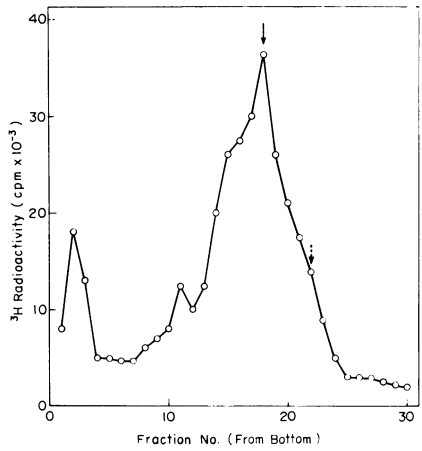


Fig. 4. Aphidicolin does not induce degradation of the already synthesized adenoviral DNA. Adenovirus type 2 DNA synthesis was synchronized with hydroxyurea. Ad infected KB cells were labeled with [^{3}H]thymidine (10 μCi/ml) for 1 hr at 18 hr postinfection, washed with Eagle's MEM and then incubated with 30 μg/ml of aphidicolin for 1 hr. Ad DNA was analyzed by alkaline sucrose gradient centrifugation as described in "Materials and Methods". The Ad DNA marker (34 S) is represented by the line-arrow. The dotted-arrow represents the sedimentation position of Ad DNA synthesized in the presence of aphidicolin (12 Kb).

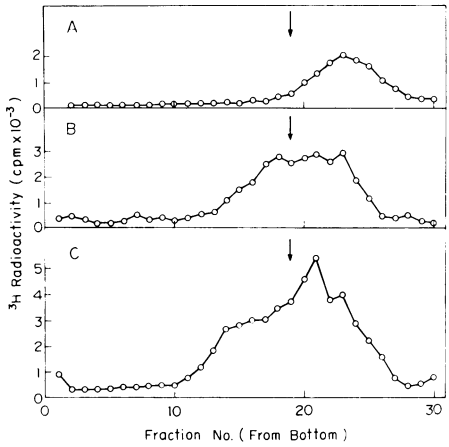


Fig. 5. Elongation of the 12 kb DNA following removal of aphidicolin. After removal of hydroxyurea followed by incubation with [^{3}H]thymidine and aphidicolin for 1 hr, the culture was washed with Eagle's MEM, administered a cold thymidine to a final concentration of 200 μM and incubated for 0 min (A), 15 min (B) or 30 min (C). The procedures for alkaline sucrose gradient centrifugation are described in "Materials and Methods". Arrows indicate the position of the adenoviral full length DNA.

nascent DNAs longer than 12 kb elongate in the presence of the drug and that production of the 12 kb DNA is not due to the hydroxyurea treatment but due to the aphidicolin treatment because the 12 kb fragment is observed in non-synchronized Ad DNA synthesis in the presence of aphidicolin.

Evidence against the degradation of Ad2 DNA synthesized in the presence of aphidicolin

An investigation was made as to whether or not the 12 kb DNA shown in Fig. 2 is produced as a result of the degradation of full size Ad DNA or cellular DNA. When Ad DNA was labeled with [^{3}H]thymidine for 1 hr without the drug and further incubated with 30 μg/ml of aphidicolin, the 12 kb DNA could not be found (Fig. 4). This indicates that the 12 kb DNA was newly synthesized in the presence of aphidicolin and that aphidicolin did not cause the Ad DNA to degradate to the 12 kb fragment.

Pulse-chase experiments were carried out in Ad infected KB cells synchronized with hydroxyurea. Ad infected KB cells were cultured for 18 hr in the presence of 20 mM hydroxyurea. After removal of hydroxyurea, aphidicolin and [^{3}H]thymidine were added to the culture, which was washed with Eagle's MEM 1 hr later. After chase for 15 min or 30 min by adding cold thymidine (final concentration of 200 μM), the pulse-labeled 12 kb DNA elongated to full size

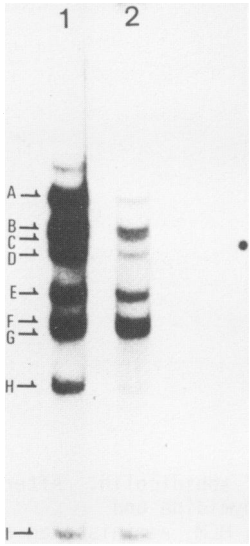


Fig. 6. Neutral agarose gel electrophoresis pattern of adenovirus type 5 DNA synthesized in in vitro system in the presence (lane 2) and absence (lane 1) of aphidicolin. Assay and gel electrophoresis were carried out as described in "Materials and Methods". Exposure time for the autoradiography was 16 hr.

DNA (35 Kb) (Figs. 5B and 5C). These results indicate that the 12 kb DNA is not simply an artifact.

Location of nascent Ad5 DNA arrested by aphidicolin

An examination was made to determine whether DNA chain elongation occurs from the 5'-ends of both strands in the presence of aphidicolin as it does in the absence of aphidicolin. In vitro Ad DNA synthesis system using the Ad5 DNA-protein complex, nuclear extract and cytoplasmic fraction were prepared from Ad5 infected HeLa cells. Initiation occurred from both 5'-ends synchronously using the Ad DNA-protein complex as a template. DNA synthesized in the presence of aphidicolin was digested with Hind III (Fig. 6, lane 2). Terminal fragments (G,E,F,I, see Fig. 7) of Ad5 DNA were strongly labeled but not internal fragments (H,D,A). Fragments B and C were weakly labeled compared with the control.

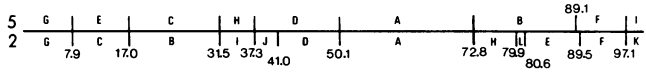


Fig. 7. The restriction map of adenovirus type 2 and 5 DNA by Hind III digestion. The map was taken from the review by Tooze (2).

Another system, the initiation of viral DNA replication was synchronized by up and down shifts of temperature in H5ts125 infected HeLa cells (25, 26). H5ts125 cannot initiate a new round of DNA replication but can elongate at a non-permissive temperature. H5ts125 infected HeLa cells were cultured for 16 hr at a permissive temperature (34°C). Then, culture temperature was shifted up to a non-permissive temperature (40°C) and maintained at 40°C for 2 hr. After shift down to 34°C, viral DNA were labeled with [^{3}H]thymidine for 1 hr in the presence of 50 μg/ml of aphidicolin. The newly synthesized DNA was extracted by the Hirt procedure (27). The patterns of labeled DNA fragment following Hind III digestion were similar to those obtained from Ad2 infected cells synchronized with hydroxyurea (data not shown). It is concluded that aphidicolin inhibits in vivo and in vitro Ad DNA synthesis in a limited region located about 30% from both terminals.

DISCUSSION

In this paper, it has been demonstrated that the initiation and subsequent elongation up to 30% of the chain length of the entire Ad DNA occur in the presence of high concentration of aphidicolin, a specific inhibitor of DNA polymerase-α, on the basis of alkaline sucrose density gradient centrifugation and Hind III-restriction fragment analysis in agarose gel electrophoresis. The 12 kb DNA synthesized in the presence of aphidicolin is not a degradation product of full size Ad DNA.

The enzymes involved in the Ad DNA synthesis have not been fully understood so far (1). The present paper indicates that initiation and subsequent elongation up to 30% of the entire length from the 5'-end occur by an enzyme insensitive to high concentrations of aphidicolin, that elongation at the limited point occurs by an enzyme sensitive to aphidicolin and that elongation exceeding the above limited point does by an enzyme insensitive toward the drug. Recently, however, a new DNA polymerase resistant to high concentrations of aphidicolin has been reported and may possibly be a virus coded protein (12, 4). This virus DNA polymerase (140 K) is capable of forming a complex with the precursor terminal protein (80 K) to prime the DNA synthesis, suggesting the involvement of Ad-coded DNA polymerase in the initiation and subsequent elongation up to a restricted region. The reason why DNA polymerase-α is involved in the replication of the limited region remains to be clarified. Switching of enzyme involved in viral DNA synthesis may possibly occur at a special configuration or base sequence of DNA in the region.

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