Vol. 113, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 31, 1983 Pages 87-95

Effect of Aphidicolin on the Elongation Step of Adenovirus DNA Replication in vitro

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Received April 8, 1983

Adenovirus DNA synthesis carried out in vitro was inhibited by the aphidicolin. However, 30% of the DNA synthesis was resistant to aphidicolin even at a concentration of 200 μg/ml. When the distribution patterns of the radioactivity of the products synthesized in the presence of 50 μg/ml of the drug was examined after HindIII digestion of the product DNA, the radioactivity appeared preferentially in the fragments mapping nearest to the ends of the molecule. Pulse-chase experiment showed that the terminal fragments were synthesized with or without aphidicolin but that in the presence of aphidicolin the rate of elongation rapidly slowed down beyond this region, suggesting that a DNA polymerase sensitive to aphidicolin may participate in the synthesis of the internal region of adenovirus DNA.

The molecular mechanism of adenovirus (Ad) DNA replication has been extensively examined (1). Recent in vitro replication studies have shown that initiation of replication is primed at the ends of Ad molecules by a virus-coded protein with a mass of about 80K that is subsequently cleaved to the 55K terminal protein seen on DNA isolated from mature virus (2-5). Another virus-coded protein identified as the 72K single-stranded DNA binding protein (DBP) is required for elongation, but not for initiation, of Ad DNA replication (4-7). Of the three known cellular DNA polymerases, α, β, and γ, both α and γ have been implicated as forming part of the replication complex (8). The evidence for the involvement of DNA polymerase α in

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Abbreviations: Ad, adenovirus; DBP, DNA binding protein; DNA-pro, DNA-protein complex; NE, nuclear extract; CE, cytoplasmic extract; $NaDodSO_4$, sodium dodecyl sulfate; TCA, trichloroacetic acid; EDTA, ethylendiamine tetraacetic acid.

0006-291X/83 $1.50

Ad DNA replication is based mainly on the sensitivity of viral replication, both in vivo and in vitro, to the aphidicolin (9-13). However, very high levels of aphidicolin are needed to inhibit Ad DNA replication as compared to SV40 or cellular DNA synthesis (10). Recently, in vitro experiments have shown that initiation is carried out by the virus coded 140K DNA polymerase and is resistant to high levels of aphidicolin (4,14,15). The size of the product synthesized by the 140K DNA polymerase is small, less than full size (16).

The experiments reported here investigate the effect of aphidicolin on in vitro Ad DNA replication and suggest that DNA polymerase α is involved in elongation, based on the finding that aphidicolin preferentially inhibited the labeling of internal portions of the genome.

MATERIALS AND METHODS

Preparation of Components of the DNA Synthesis Reaction.

Ad DNA-pro was purified from Ad5 that had been banded twice in CsCl gradients (17). The Ad DNA-pro from guanidine-lysed virion was sedimented on sucrose gradients containing 4M guanidine hydrochloride as described (18). Ad5 nuclei and Ad5 cytoplasm were prepared, at 21 hr after infection, from 3.5ℓ of infected HeLa cells (40 p.f.u/cell) to which 3 mM hydroxyurea had been added at 2 hr after infection (7). The procedures for making Ad5 nuclear extract (Ad5NE) and cytoplasmic extract (Ad5cyto) were described previously in detail (19).

Reaction Conditions for in vitro Ad DNA Synthesis.

Reaction mixture (100 µl) contained 25 mM Hepes (pH 7.5), 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.05 mM each dATP, dGTP, and dTTP, 1.5 µM [α-^{32}P]dCTP (410 Ci/mmole, 5000-15000 cpm/pmol), 3.75 mM ATP, 20 µl of Ad5 infected nuclear extract (Ad5 NE)(protein concentration, 2.5 mg/ml), 5 µl of Ad5 cytoplasm (protein concentration, 25 mg/ml), and 0.1 µg of Ad5 DNA-pro. After incubation of the reaction mixture for the various time at 37°, and subsequent digestion with pronase (10 µg) and $NaDodSO_4$ (0.2%) for 20 min at 37°, the DNA was extracted with phenol saturated with 10 mM Tris (pH 8.1) and 1 mM EDTA and precipitated with ethanol. The precipitated DNA was redissolved in water, digested with HindIII for 1.5 hr at 37°, and electrophoresed on 1% agarose gel containing 40 mM Tris (pH 7.8), 1 mM EDTA, and 5 mM sodium acetate. The gels were dried and autoradiographed on Kodak Xray film XO mat.

Materials.

Aphidicolin was kindly provided by Dr. T. Enomoto (Tokyo University). [α-^{32}P]dCTP was from Amersham.

RESULTS AND DISCUSSION

Effect of Aphidicolin on Viral DNA Synthesis *in vitro*

Aphidicolin had previously been shown to inhibit the incorporation of [^{3}H] thymidine into TCA precipitable material in both uninfected and infected HeLa cells (10,11,13). In all cases examined, the drug was added to the infected cells at the steady state when DNA replication was already under way, and these experiments left unanswered the question of which stage is affected by the aphidicolin. To clarify these points, we synchronized the initiation of Ad DNA replication by treating the infected cells with hydroxylurea or by the use of the temperature-sensitive mutant H5ts125 *in vivo* (Oguro, Yamashita, Ariga, and Nagano, submitted). Synchronization of the initiation of Ad DNA replication was also achieved *in vitro* system(3), where the initation occurs from the either end of Ad DNA in the reaction mixture on the exogenously added Ad DNA-pro. Fig. 1 shows the dose response curve of aphidicolin on the inhibition of Ad5 DNA synthesis *in vitro*. It is clear that in this case, as in the *in vivo* system reported elsewhere (Oguro *et al.*, submitted), the inhibition curve was biphasic and that the approximately 30%

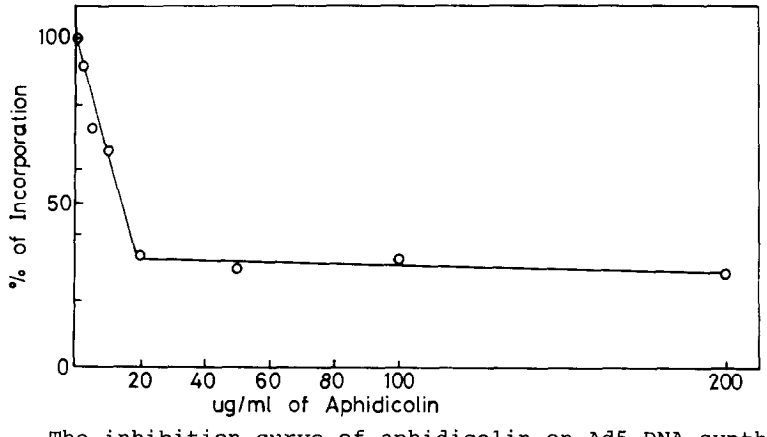

Fig. 1. The inhibition curve of aphidicolin on Ad5 DNA synthesis. The reaction was carried out with various concentrations of aphidicolin. After 60 min at 37°C, the acid insoluble radioactivity was counted. The radioactivity in the product formed without aphidicolin was 2.5 pmol.

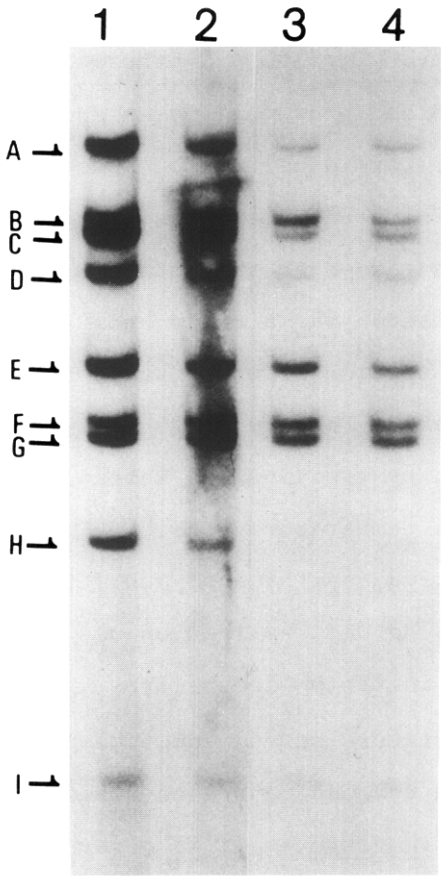

Fig. 2. Distribution patterns of the radioactivity of DNA synthesized in the presence of various concentrations of aphidicolin. After 60 min at 37°C, the DNA was extracted, digested with HindIII, and run on an agarose gel as in MATERIALS AND METHODS. The DNA bands were visualized by the autoradiography. The concentration of aphidicolin was as follows; lane 1: 0 μg/ml, lane 2: 10 μg/ml, lane 3: 50 μg/ml, lane 4: 100 μg/ml.

of the synthesis was still resistant to the aphidicolin even at very high concentrations. Fig. 2 shows an autoradiogram of samples treated with a range of aphidicolin from 0 to 100 μg/ml. After the incubation of the mixture at 37° for 60 min, the DNA was extracted and digested with HindIII. The HindIII map of Ad5 and Ad2 DNA is shown in Fig. 3. In the samples treated with 50 or 100 μg/ml of drug, radioactivity appeared preferentially in restriction fragments mapping nearest to the ends of the molecule (lane 3,4). The most internal fragments A, D, and H were hardly labeled, while the external

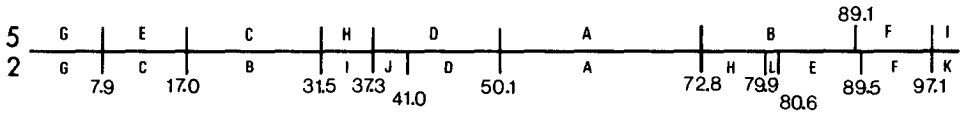

Fig. 3. The HindIII restriction map of Ad5 and Ad2 DNA. The map was taken from the review by Tooze (1).

fragments, G, E, F, and I were labeled extensively. To a lesser extent this was observed even at 10 μg/ml of drug (lane 2). The same results were also observed in Ad2 infected HeLa cells in vivo (Oguro et al., submitted). In this case, the terminal fragments, G, C, F, and K (see Fig. 3) were strongly labeld, while internal fragments, A and D were not.

Time Course Experiments in Ad5 DNA Synthesis in vitro.

Time course experiments were carried out (Fig. 4) in which one pair of samples was taken at various times after the start of incubation. At each time point, one of the reaction was stopped by the addition of EDTA to a final concentration of 25 mM (lane, 1,3,5, and 7), and at the same time 50 μg/ml of aphidicolin was added to the other reaction followed the further incubation (total 60 min)(lane 2, 4, and 6). In a first 5 min reaction (lane 3), the faint band of G was observed. In the 10 min reaction sample, the near terminal fragments, E, F, G, and I were labeled (lane 5), which clearly shows that the replication starts at the each end of the molecule. On the other hand, the sample treated with aphidicolin after the initial incubation without the drug showed different patterns (lane 2, 4, and 6). In the sample treated with aphidicolin after the first 5 min of reaction, DNA synthesis was observed only in the fragments nearest to the ends of the molecules (lane 4). This was also observed in the sample treated with the drug after the first 10 min reaction (lane 6). These results suggest that, in the presence of aphidicolin, the initiated molecules can elongate up to some restricted point and that cycles of reinitiation occur, followed by elongation

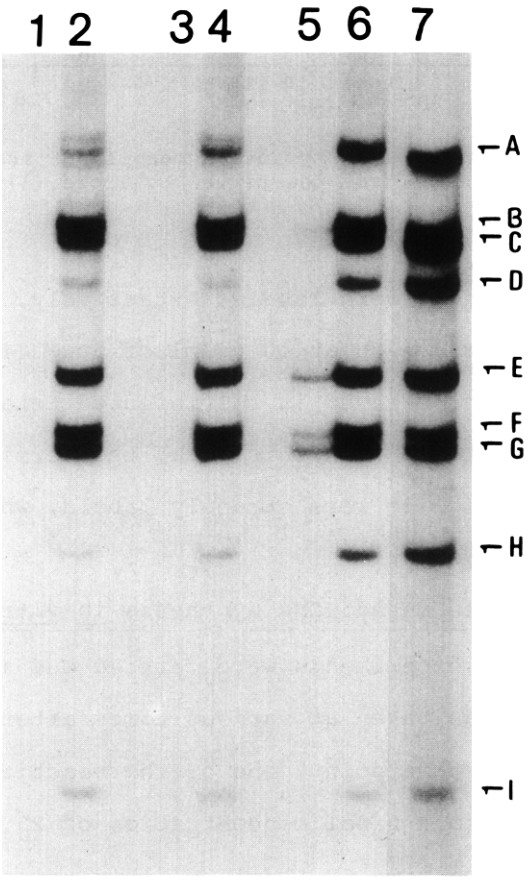

Fig. 4. Time course of Ad5 DNA synthesized in vitro. Two reactions were carried out at 37°C as described in MATERIALS AND METHODS. At the various time after the start of the reaction in the absence of aphidicolin, one reaction was stopped by the addition of EDTA to a final concentration of 25 mM and the other reaction was continued up to 60 min in the presence of 50 μg/ml of aphidicolin. The DNA products were processed as in Fig. 2. Lanes 1 and 2: 0 min, lane 3 and 4: 5 min, lane 5 and 6: 10 min, lane 7: 60 min.

to a restricted point, which might be reflected by the different intensity of the radioactivity of the fragment (compare E, G, F in land 5 and 6), or that the rate of elongation slows down rapidly, especially in the internal region of molecule.

Pulse-chase Experiment of Ad5 DNA Synthesis in vitro.

To further examine the effect of the aphidicolin, pulse-chase experiments were carried out (Fig. 5). The sample in lane 1 was taken after the first 10 min incubation, without further incubation. The near terminal fragments, E, F, and G were labeled.

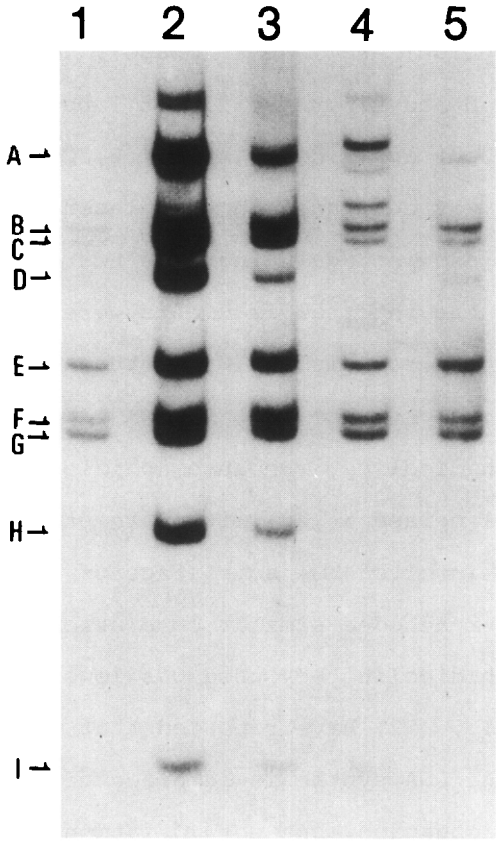

Fig. 5. Pulse-chase experiments of the Ad5 DNA synthesis reaction. The reaction was carried out at 37°C as described in MATERIALS AND METHODS. Lane 1: the reaction without aphidicolin was stopped at 10 min after the start of the incubation by the addition of EDTA. Lane 2: the reaction was carried out for 60 min without aphidicolin. Lane 3: at 10 min after the start of the reaction, 50 μg/ml of aphidicolin was added to the mixture and the reaction was continued further up to the total 60 min as in Fig. 4. Lane 4: at 10 min after the start of the reaction, 50 μg/ml of aphidicolin and 200 fold concentration of cold dCTP (300 μM) were added to the mixture and the reaction was continued up to 60 min. Lane 5: after the reaction for 10 min with 1.5 μM cold dCTP in place of [^{32}P]dCTP, 1.5 μM of [^{32}P]dCTP and 50 μg/ml of aphidicolin were added to the mixture and the reaction was continued up to 60 min. The DNA products were processed as in Fig. 2.

The sample in lane 2 was a control sample after 60 min of incubation in the absence of drug. In the sample receiving aphidicolin after the first 10 min reaction without the drug, followed the further incubation (total 60 min), the same result shown in Fig. 4 was obtained (lane 3). In the sample shown in lane 4, the reaction procedure was as for the sample in lane 3 except that aphidicolin was added at 10 min after the start of the reaction together with

a two-hundred fold excess of cold dCTP. From a comparison of the samples in lane 1 (pulse) and lane 4 (chase), it is clear that the internal fragments, D and H were still not labeled. The reaction was carried out without aphidicolin and [^{32}P]dCTP for the first 10 min, after which it was continued in the presence of aphidicolin and [^{32}P]dCTP (lane 5), showing again that the near terminal fragments were labeled, while the internal fragments were little labeled.

These results, consistent with those previously published (10,11,13), clearly demonstrate that Ad DNA replication is sensitive to inhibition by aphidicolin. Because aphidicolin is a specific inhibitor of DNA polymerase α (20), it is tempting to speculate that DNA polymerase α is involved in some stage of Ad DNA replication. Pincus et al., (21) reached a similar conclusion, although the concentration of aphidicolin they used was lower than that used here. Foster et al., (22) have reported that the sensitivity of aphidicolin to the Ad DNA synthesis is affected by the 72K DBP which is needed for the elongation, but not initiation, of the Ad DNA replication (4-7). The size of the DNA molecules synthesized by the Ad coded 140K DNA polymerase which is resistant to aphidicolin, is only one quarter that of the total genome (16). Therefore, this suggested that Ad DNA polymerase takes part in the initial reaction of Ad DNA replication and DNA polymerase α continues the elongation process.

ACKNOWLEDGMENT

This work was supported by grants from the Ministry of Education, Science, and Culture, Japan. I am grateful to Miss M. Matsumoto for beautiful typing of this manuscript.

REFERENCES

1. Tooze, J. ed. (1980). Molecular Biology of Tumor Viruses, 2nd ed., part 2. Cold Spring Harbor Laboratory.
2. Challberg, M.D., Desiderio, S.V., and Kelly, T.J. (1980). Proc. Natl. Acad. Sci. USA. 77, 5105-5109.
3. Challberg, M.D. and Kelly, T.J. (1979). Proc. Natl. Acad. Sci. USA. 76, 655-659.
4. Enomoto, T., Lichy, J.H., Ikeda, J., and Hurwitz, J. (1981). Proc. Natl. Acad. Sci. USA. 78, 6779-6783.

5. Lichy, J.H., Horwitz, M.S., and Hurwitz, J. (1981). Proc. Natl. Acad. Sci. USA. 78, 2678-2682.
6. Horwitz, M.S. (1978). Proc. Natl. Acad. Sci. USA. 75, 4291-4295.
7. Kaplan, L.M., Ariga, H., Hurwitz, J., and Horwitz, M.S. (1979). Proc. Natl. Acad. Sci. USA. 76, 5534-5538.
8. Winnacker, E.L. (1978). Cell 14, 761-773.
9. Huberman, J.A. (1981). Cell 23, 647-648.
10. Krokan, H., Schaffer, P., and DePamphillis, M.L. (1979). Biochemistry 18, 4431-4443.
11. Longiaru, M., Ikeda, J., Jarkovsky, Z., Horwitz, S., and Horwitz, M.S. (1979). Nucleic Acids Res. 6, 3369-3386.
12. Ohashi, M., Taguchi, T., and Ikegami, S. (1978). Biochem. Biophys, Res. Comm. 82, 1034-1040.
13. van der Werf, S., Bouche, J.-P., Mechall, M., and Gerard, M. (1980). Virology 104, 56-72.
14. Lichy, J.H., Field, J., Horwitz, M.S., and Hurwitz, J. (1982). Proc. Natl. Acad. Sci. USA. 79, 5225-5229.
15. Stillman, B.W., Tamanoi, F., and Mathews, M.B. (1982). Cell 31, 613-623.
16. Ikeda, J., Enomoto, T., and Hurwitz, J. (1981). Proc. Natl. Acad. Sci. USA. 78, 884-888.
17. Maizel, J.V.Jr., White, D.O., and Schaff, M.D. (1968). Virology 36, 115-125.
18. Sharp, P.A., Moore, C., and Haverty, J.L. (1976). Virology 75, 442-456.
19. Horwitz, M.S. and Ariga, H. (1981). Proc. Natl. Acad. Sci. USA. 78, 1476-1480.
20. Ikegami, S., Taguchi, T., Ohashi, M., Oguro, M., Nagano, H., and Mano, Y. (1978). Nature 275, 458-460.
21. Pincus, S., Robertson, W., and Rekosh, D. (1981). Nucleic Acids Res. 9, 4919-4938.
22. Foster, D.A., Hantzopoulos, P., and Zubay, G. (1982). J. Virol. 43, 679-686.