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On the Fate of Adenovirus DNA in KB Cells

Electron microscopic and biochemical studies on the process of infection by adenoviruses have shown that after penetration into the cell the virions lose the pentons and are then gradually uncoated (*1*, *2*). Later adenovirus DNA is found in the cell nucleus, where DNA replication takes place. The mechanism of replication of linear adenovirus DNA molecules is still unknown. This report presents the first results of a study on the mechanism of adenovirus DNA replication and concerns especially the fate of the parental DNA molecules. To investigate whether tumorigenic and nontumorigenic adenovirus types differ in this aspect of the infection process, experiments were performed with adenovirus type 5 (Ad5) and type 12 (Ad12). As only parental molecules in the cell nucleus are important for replication, nuclei were prepared from infected cells and the parental viral DNA associated with them was examined.

KB cells were grown in monolayer cultures in Earle's balanced salt medium (Flow, Irvine, Scotland) containing 0.5% lactalbumin, 5% calf serum (Flow, Irvine, Scotland), 100 U/ml of penicillin G and 100 μg/ml of streptomycin sulfate (Mycofarm, Delft, Holland). Suspension cultures were obtained by trypsinization of monolayers and addition of the cells to the above medium. Purified virus preparations of adenovirus type 5 (ATCC) and type 12 (Huie) were obtained according to Green and Pina (*3*). For the isolation of viruses containing labeled DNA, 100 μCi thymidine-6-^{3}H were added to 3×10^7 infected cells. Suspension cultures in stoppered flasks were inoculated with labeled or nonlabeled virus pools (2×10^6 cells and $3–4 \times 10^{10}$ virions per milliliter). At several times p.i. cells were sedimented and washed after which the infected nuclei were isolated according to Penman (*4*), without detergent treatment. The associated viral DNA was isolated (*5*) and analyzed by centrifugation as described in the legend of Fig. 1. Fractions were counted in a liquid scintillation counter. The formation of newly synthesized viral DNA was studied with nonradioactive virus preparations under the conditions mentioned above with the addition of 5 μCi/ml thymidine-6-^{3}H (10 Ci/mmole) and 4 μg/ml mitomycin C (Sigma, St. Louis, Mo).

Double-diffusion experiments were performed with rabbit antiserum prepared against Ad5 and Ad12 (Grand Island Biological Co., Grand Island, NY) in 0.01 *M* veronal buffer, pH 8.4. Purified radioactive Ad5 and Ad12 DNA was prepared according to van der Eb, van Kesteren, and van Bruggen (*6*) with pronase and used as a marker in the centrifugation experiments. The purified DNA preparations of Ad5 and Ad12 contained only 31 S and 30 S molecules, respectively.

KB cells infected with radioactive Ad5 were isolated at different times p.i. Purified cell nuclei were prepared and the viral DNA in the nuclei was analyzed. Radioactivity measurements showed that under our conditions the amount of parental viral DNA in the nucleus did not increase after 5 hours of incubation. Calculations revealed that about 5000 molecules/nucleus were present. Analysis of Ad5 DNA showed that until 18 hours p.i. the parental molecules had the same sedimentation coefficient as DNA isolated from purified virions by phenol extraction (Fig. 1). No measurable amount of faster or slower sedimenting molecules could be detected.

Similar experiments with type 12 showed that 5 hours p.i. normal linear molecules (30 S) were found in the nucleus (Fig. 2). However, 9 hours p.i. a 20 S component could be detected and 18 hours p.i. an extensive degradation of the parental molecules had taken place. In some experiments 13 S fragments were also observed. The 18-hour samples of Ad5 and Ad12 DNA were analyzed by CsCl equilibrium density gradient centrifugation in the presence of eth-

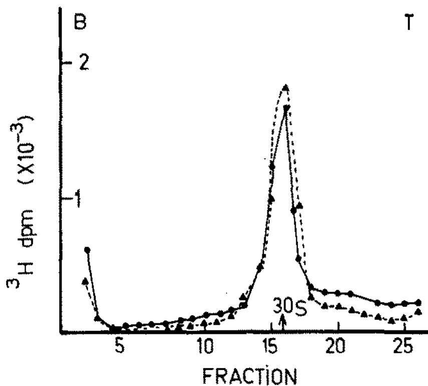

FIG. 1. Sucrose gradient centrifugation in 5–20% sucrose, 0.01 M Tris, pH 8.1, 0.001 M EDTA, and 0.1% sodium dodecylsulfate of viral DNA, isolated from nuclei of KB cells infected with Ad5 virions containing ^{3}H-DNA. Centrifugation was performed for 6 hours at 24,000 rpm at 15° in the Spinco SW25 rotor. Viral DNA was isolated 5 hours (▲) and 18 hours (●) p.i. The position of ^{14}C-labeled Ad12 DNA (30S) is marked by an arrow.

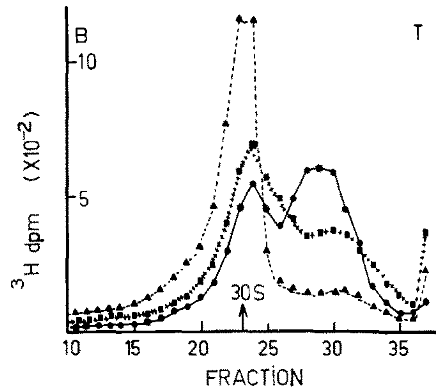

FIG. 2. Sucrose gradient centrifugation in 5–20% sucrose, 0.01 M Tris pH 8.1, 0.001 M EDTA, and 0.1% sodium dodecylsulfate of viral DNA isolated from nuclei of KB cells infected with Ad12 virions containing ^{3}H-DNA. Centrifugation was performed for 6 hours at 24,000 rpm at 15° in the Spinco SW25 rotor. Viral DNA was isolated 5 (▲), 9 (■), and 18 hours (●) p.i. The position of ^{14}C-labeled Ad12 DNA (30S) is marked by an arrow.

idium bromide (120 μg/ml) for 50 hours at 38,000 rpm in the Spinco R50 rotor. Molecules with densities of covalently closed circular molecules could not be observed.

The cause of the degradation of Ad12 parental DNA might be the endonuclease activity found in the pentons of purified Ad12 virions (7). Experiments were performed to identify an endonuclease activity in virions of both types. Ad5 and Ad12 virus preparations in Tris 0.01 M, pH 8.0, and NaCl 0.15 M were stored in the presence of purified ^{3}H-labeled Ad5 and Ad12 DNA, respectively. During storage viral capsomers were released and DNA degradation could occur.

After 6 days of incubation almost no intact virions were found by CsCl density centrifugation and an extensive breakdown of the virions was detected by double-diffusion precipitation. The incubation samples were analyzed by sucrose gradient centrifugation. Control experiments were performed in absence of viral protein.

Analysis of labeled DNA showed that under these circumstances Ad12 DNA was almost completely converted into 20 S fragments while in the Ad5 system no degradation could be detected.

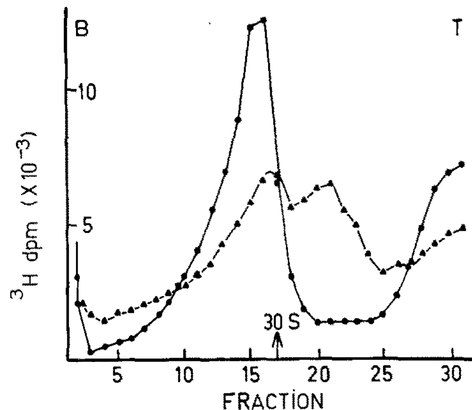

FIG. 3. Sucrose gradient centrifugation in 5–20% sucrose, 0.01 M Tris, pH 8.1, 0.001 M EDTA, and 0.1% sodium dodecylsulfate of new viral DNA, synthesized 15–18 hours p.i. in nuclei of infected KB cells. Centrifugation was performed for 6 hours at 24,000 rpm at 15° in the Spinco SW25 rotor. Cells were infected with nonradioactive virus preparations of Ad5 (●) or Ad12 (▲). The position of ^{14}C-labeled Ad12 DNA (30S) is marked by an arrow.

These observations make it very likely that breakdown of Ad12 DNA is performed by the endonuclease activity in Ad12 virions. The absence of such an activity in Ad5

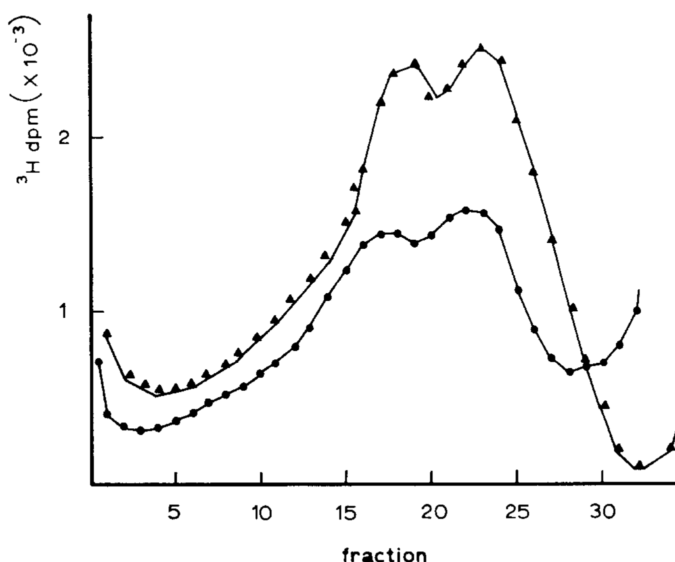

FIG. 4. Sucrose gradient centrifugation in 5–20% sucrose, 0.01 *M* Tris, pH 8.1, 0.001 *M* EDTA, and 0.1% sodium dodecylsulfate of new viral DNA, synthesized 15–18 hours p.i. in nuclei of Ad12-infected KB cells. DNA was isolated 18 hours p.i. from 0.6 × 10^5 cells (●) and after a chase with unlabeled thymidine until 23 hours p.i. from 10^6 cells (▲). Centrifugation was performed for 6 hours at 24,000 rpm at 15° in the Spinco SW25 rotor.

virions might explain the absence of degradation of Ad5 DNA in the cell nucleus. The meaning of this difference between the two types is unclear. As suggested by Doerfler (*7*) the fragmented state of adenovirus DNA in abortively infected cells may be related to the endonuclease activity in the virions (*8*).

In order to investigate whether splitting of Ad12 parental molecules has an influence on the daughter molecules, newly synthesized viral DNA was investigated. Although cellular DNA synthesis is strongly inhibited late after infection, residual cellular synthesis was blocked with mitomycin C. KB cells were infected with nonlabeled Ad5 and Ad12 virus preparations in the presence of 4 μg/ml mitomycin C. After addition of this drug viral DNA synthesis proceeds and virus is formed, while cellular DNA synthesis is inhibited by 99% (*9*, *10*, and own results). Under our conditions Ad5 and Ad12 DNA synthesis started at about 14 hours p.i. Newly synthesized DNA was labeled by addition of ^{3}H-thymidine to the infection medium 15–18 hours p.i. and analyzed. More than 98% of the new DNA had the buoyant density of the parental molecules (1.714 g/cm^3 for Ad5 and 1.708 g/cm^3 for Ad12). This means that new DNA was neither of cellular origin nor in a single-stranded form.

After infection with Ad5, sucrose gradient centrifugation of new DNA showed only 31 S molecules. Infection with Ad12 resulted in the presence of 30 and 20 S molecules (Fig. 3). The distribution was almost identical to that obtained for parental molecules at 18 hours p.i.

The 20 S fraction of new Ad12 DNA might originate from replication of 20 S parental molecules or from the action of Ad12 endonuclease on new 30 S molecules. Since the observed rate of degradation of parental molecules was rather low and the time of exposure of new DNA to endonuclease activity was about four times shorter than that of parental DNA, the second possibility is rather unlikely. Moreover, the amount of new DNA was estimated to be about 3–10 times higher than the amount of parental molecules which also does not favour degradation.

To obtain more information on the origin of 20 S molecules a pulse-chase experiment was performed. KB cells were infected with Ad12 virions and newly synthesized DNA

was labeled with ^{3}H-thymidine 15–18 hours p.i. At 18 hours p.i. a sample was taken and the associated DNA extracted, while the remainder of the cells was washed and further incubated in fresh medium containing 10^{-5} *M* thymidine. At 23 hours p.i. DNA was extracted and analyzed by sucrose-gradient centrifugation together with the 18-hour sample (Fig. 4). During the chase the amount of labeled DNA did not increase, while the distribution of labeled 30 and 20 S molecules did not change.

From this experiment it may be concluded that 20 S molecules are probably not formed by degradation of 30 S molecules. The absence of degradation during the chase cannot be explained by a dilution effect caused by new unlabeled molecules resulting in a strongly decreased rate of degradation of labeled molecules since DNA formed between 20 and 23 hours p.i. is also mainly in the fragmented form (*11* and own observations). If these 23-hour fragments should originate by endonuclease action, one should expect that 30 S molecules formed 15–18 hours p.i. should be degraded as well. The possibility that 30 S molecules have a chance to be degraded only if attached to the replication site cannot be excluded but is not very likely. Although these experiments are not fully conclusive, they suggest that Ad12 fragments are able to replicate. This would make a mechanism of DNA replication involving circular replicating molecules unlikely. The absence of covalently closed circular molecules, as determined in ethidium bromide density centrifugation, is in accordance with this view. An extended study on replicating adenovirus DNA is in progress.

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REFERENCES

1. SUSSENBACH, J. S., *Virology* **33**, 567–574 (1967).
2. CHARDONNET, Y., and DALES, S., *Virology* **40**, 462–477 (1970).
3. GREEN, M., and PINA, M., *Virology* **20**, 199–207 (1963).
4. PENMAN, S., *J. Mol. Biol.* **17**, 117–130 (1966).
5. HIRT, B., *J. Mol. Biol.* **26**, 365–369 (1967).
6. VAN DER EB, A. J., VAN KESTEREN, L. W., and VAN BRUGGEN, E. F. J. *Biochim. Biophys. Acta* **182**, 530–541 (1969).
7. DOERFLER, W., *J. Virol.* **6**, 652–666 (1970).
8. BURLINGHAM, B. T., and DOERFLER, W., *Fed. Proc.* **28**, 434 (1969).
9. GINSBERG, H. S., *Cold Spring Harbor Symp. Quant. Biol.* **27**, 234 (1962).
10. MAGEE, W. E., and MILLER, O. V., *Biochim. Biophys. Acta* **55**, 818 (1962).
11. DOERFLER, W., *Virology* **38**, 587–606 (1969).

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