Adenovirus Type 2 DNA Replicated in Arginine-Starved KB Cells Is Infectious

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Adenovirus type 2 DNA from extracts of KB cells rendered nonpermissive by arginine starvation is infectious.

Productive infection by adenovirus is aborted when arginine (Arg) is omitted from the culture medium in which infected cells are maintained (11, 12); assembly of virions does not occur (8). Furthermore, Arg is unique among the amino acids nutritionally required by cultured cells because virus is made in the absence of any others. Most double-stranded DNA animal viruses appear to have a similar Arg requirement (7, 10). During infection of KB cells with adenovirus type 2 (Ad2) in the absence of Arg, viral DNA and structural polypeptides are made, and the DNA can be packaged into virions which are produced after Arg restoration. Viral polypeptides accumulate but are not used (13).

Whether processing of the viral DNA or of structural polypeptides is the target of the Arg-dependent factor is not known. If the Ad DNA made in Arg-starved cells is infectious, i.e., able to transfect fresh cells, it must contain all of the genetic information and be a product of complete (possibly normal) replication. A difference in the efficiency of transfection from that obtained with viral DNA from normal productive infection would suggest an aberrant and perhaps unpackageable nucleoprotein intermediate.

Ad2 and a subline of KB cells were used. The medium, essentially Eagle basal essential medium with Arg (Arg⁺) or without Arg (Arg⁻) and supplemented with 2% dialyzed calf serum (bobby calf serum; GIBCO Laboratories, Grand Island, N.Y.), was previously described in detail, as were the procedures for infecting cells and [³H]thymidine labeling (7). Infectivity was titrated by a fluorescence focus method (2).

Viral DNA was labeled preferentially by [³H]thymidine incorporation from 14 to 20 h postinfection (p.i.), at which time cellular DNA synthesis is inhibited (4). Greater than 90% of the DNA, thus labeled in both Arg⁺ and Arg⁻ samples, was of viral length on alkaline sucrose gradients, confirming this fact. Total macromo-

lecular incorporation of [³H]thymidine in Arg⁻ infections at 20 h p.i. was 20% that of Arg⁺ infections (data not shown).

For transfection, viral DNA and associated polypeptides were harvested at 20 h p.i. as follows. First, the viral nucleoprotein was extracted from infected cell nuclei with ammonium sulfate essentially by the method of Wilhelm et al. (15), except (NH₄)₂SO₄ was increased to 0.3 M and incubation took place at 37°C for 6 min. These changes enhanced recovery and gave better separation of material in subsequent gradients. After the first extraction, the nuclear remnants were lysed with 0.1% sodium lauryl sarcosinate (Sarkosyl; Chemical Additives Co., Farmingville, N.Y.) on ice for 20 min. Relevant fractions in both extracts were separated on velocity gradients (10 to 60% sucrose-2 mM EDTA-10 mM Tris adjusted to pH 8.0 at 5°C and 100 mM (NH₄)₂SO₄ [1]).

The concentrations of viral DNA in the (NH₄)₂SO₄ extracts could be estimated directly by absorbance at 260 nm because this procedure has been shown to extract viral nucleoproteins free of contamination by cellular chromatin (15). These results were verified in this laboratory; prelabeled cellular DNA could not be extracted under these conditions. Direct measurement of viral DNA in the Sarkosyl fraction was prevented by the presence of cellular DNA. Therefore, the quantities of viral DNA in Sarkosyl fractions were calculated from the values in the (NH₄)₂SO₄ fractions and the proportion of precipitable [³H]thymidine-labeled viral DNA recovered from each procedure, assuming uniform specific activity. The partitioning of viral DNA between the two extracts was somewhat variable (Table 1).

The transfection method detailed by Graham and Van der Eb was used (5). Briefly, nucleoproteins were dialyzed and diluted in isotonic buffer at pH 7.0 to 7.1. Samples of 0.5 ml were

TABLE 1. Specific infectivity of Ad2 DNA replicated in Arg⁺ or Arg⁻ KB cells^a

Expt ^b	FFU $\times 10^{-4}$ per μ g of DNA $\pm$ SD		[³ H], sample/total $\times 100$	
	Arg ⁺	Arg ⁻	Arg ⁺	Arg ⁻
A				
(NH ₄) ₂ SO ₄ extract	6.0 $\pm$ 2.9	0.89 $\pm$ 0.53	0.70	0.35
Sarkosyl extract	3.3 $\pm$ 1.4	1.6 $\pm$ 0.6	0.30	0.65
B				
(NH ₄) ₂ SO ₄ extract	4.0 $\pm$ 1.8	1.3 $\pm$ 0.6	0.37	0.35
Sarkosyl extract	5.6 $\pm$ 2.0	6.8 $\pm$ 5.1	0.63	0.65

^a Ad nucleoproteins were extracted at 20 h p.i. as described in the text from nuclei of infected KB cells grown with or without Arg. Extracts were inoculated on KB monolayers, and infectivity was determined by a fluorescence focus assay 48 h later. Specific infectivity (focus-forming units [FFU] per microgram of DNA) was determined. Calculation of the total specific infectivity was based on the partitioning of viral DNA between the two extracts (columns 3 and 4).

^b In experiment A, residual infectivity of input viral DNA was determined from extracts at 8 h p.i. and subtracted to obtain the values shown in the table. These were $4.3 \times 10^2 \pm 1.1 \times 10^2$ and $5.5 \times 10^2 \pm 2.5 \times 10^2$ for Arg⁺ and Arg⁻ cultures, respectively, in (NH₄)₂SO₄ extracts. Sarkosyl fractions had average background values of $8.0 \times 10^3 \pm 1.9 \times 10^3$, and $2.5 \times 10^3 \pm 1.3 \times 10^3$ for Arg⁺ and Arg⁻ cultures, respectively. Alternatively, in experiment B, background infectivity was determined from extracts at 20 h p.i. in which cytosine arabinofuranoside (50 μ g/ml) was present throughout infection. These values were $1.7 \times 10^3 \pm 0.9 \times 10^3$ and $1.8 \times 10^3 \pm 1.0 \times 10^3$ for Arg⁺ and Arg⁻ cultures, respectively, in (NH₄)₂SO₄ extracts. The Sarkosyl extracts had average background infectivity values of $2.0 \times 10^4 \pm 1.6 \times 10^4$ and $8.4 \times 10^3 \pm 4.6 \times 10^3$ for Arg⁺ and Arg⁻ cultures, respectively. The calculated total focus-forming units per culture were 5.1×10^4 and 1.4×10^4 for Arg⁺ and Arg⁻ cultures, respectively, in experiment A and 5.0×10^4 and 4.7×10^4 for Arg⁺ and Arg⁻ cultures, respectively, in experiment B. In experiment B, resistance in each sample DNase was monitored by treating aliquots with DNase I for 30 min. The percentage of DNase-resistant infectivity was 9.5 and 6.7% for Arg⁺ and Arg⁻ cultures, respectively, in (NH₄)₂SO₄ extracts and 6.6 and 7.9% for Arg⁺ and Arg⁻ cultures, respectively, in Sarkosyl extracts.

added to washed KB cell monolayers. After 4 h of adsorption at 37°C, cells were washed with phosphate-buffered saline, fed with complete medium containing 10% booby calf serum, and incubated at 37°C. Enumeration of fluorescing infected cells was made after 48 h. Because CaCl₂ precipitation of the extracted DNA did not enhance transfectivity, values for both precipitated and untreated samples were averaged in the calculations. Others have also found that the uptake of Ad2 DNA by KB cells is not enhanced by calcium treatment (6).

The relationship between the numbers of infected cells and the amounts of DNA is shown in Fig. 1. With increasing DNA within the range assayed, infectivity, as indicated by fluorescing cells, increased linearly for both Arg⁺ and Arg⁻ preparations, reaching a maximum of at least 1.0 log₁₀ over background (8 h p.i.) samples. The fact that these fluorescent foci contained complete virions was confirmed by demonstrating infectivity on subculture.

Specific infectivity (focus-forming units per microgram of DNA) of newly replicated DNA was calculated from three concentrations of DNA after correcting for infectivity attributable to input DNA. This background DNA was determined by two methods and subtracted to obtain values given in the table. In experiment A, background infectivity was demonstrated at 8 h p.i., a time before the replication of viral DNA.

Alternatively, in experiment B, residual infectivity at 20 h p.i. in samples containing the DNA synthesis inhibitor cytosine arabinofuranoside (Sigma Chemical Co., St. Louis, Mo.) at 50 μ g/ml throughout the infection was assayed. Less than 10% of the infectivity remained DNase resistant, as determined after treating extracts for 30 min at 37°C with DNase I (Worthington Biochemicals Corp., Freehold, N.J.).

The calculated total infectivity attributable to replicated viral DNA, adjusted in both nucleoprotein extractions, was within fourfold in all samples.

Using transfection, we showed the Ad nucleoproteins produced in infected KB cells deprived of Arg to be infectious, even though mature virions are not made in such cells. This DNA must have been entirely replicated and polymerized without errors to be able to specify new virus. Therefore, faithful replication of the DNA is not the target of the Arg-dependent factor(s). In the absence of virions, the infectivity of genomes before assembly has been shown.

Previous transfection studies have used DNA or DNA-protein complexes from purified, mature virions. Protein-free DNA, purified from adenovirions, can transfect KB cells at low efficiency (5). The DNA-terminal protein complex from such virions is considerably more infectious (3, 14). Also, core structures when isolated from the noninfectious virions of mutant

FIG. 1. Relationship between infectivity and amount of Ad2 DNA in Arg⁺ and Arg⁻ cellular extracts. Viral nucleoprotein extracts from Ad2-infected KB nuclei at 20 h p.i. were used to inoculate KB monolayers, and infectivity was determined 48 h later by a fluorescence focus method. After a primary (NH₄)₂SO₄ extraction (○) and a subsequent Sarkosyl treatment (●) and fractionation on sucrose velocity gradients, the partially purified DNA-containing extracts were tested. DNA was estimated as described in text. Samples taken 8 h p.i., (NH₄)₂SO₄ extract (△), and Sarkosyl extract (▲) were used to determine infectivity attributable to residual inoculum. Six samples were assayed at each point and the average focus-forming units (FFU) ± 1 standard deviation per plate are shown.

Ad2 ts1 grown at 39°C are infectious (9). The infective nucleoproteins examined in these experiments presumably represent replicative or transcriptional or both forms before assembly into virions occurs.

The functional competence of a viral genome made during an abortive infection induced by Arg starvation was demonstrated. Testing nascent intracellular viral nucleoproteins during the infectious cycle by transfection was shown to be feasible.

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LITERATURE CITED

1. Brison, O., C. Kedinger, and P. Chambon. 1979. Adenovirus DNA template for late transcription is not a replicative intermediate. *J. Virol.* 32:91-97.
2. Champe, P. C., W. A. Strohl, and R. W. Schlesinger. 1972. Demonstration of an adenovirus-inhibitory factor in adenovirus-induced hamster tumor cells. *Virology* 50:482-494.
3. Chinnadurai, G., S. Chinnadurai, and M. Green. 1978. Enhanced infectivity of adenovirus type 2 DNA and a DNA-protein complex. *J. Virol.* 26:195-199.
4. Ginsberg, H. S., L. J. Bello, and A. J. Levine. 1967. Control of biosynthesis of host macromolecules in cells infected with adenoviruses, p. 547-572. *In* J. S. Colter and W. Paranchych (ed.), *The molecular biology of viruses*. Academic Press, Inc., New York.
5. Graham, F. L., and A. J. Van der Eb. 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* 52:456-467.
6. Groneberg, J., D. T. Brown, and W. Doerfler. 1975. Uptake and fate of the DNA of adenovirus type 2 in KB cells. *Virology* 64:115-131.
7. Heilman, C. A., and H. Rouse. 1980. Adenovirus type 2 polypeptide synthesis in arginine-depleted cells. *Virology* 105:159-170.
8. Marusyk, R., E. Norrby, and H. Marusyk. 1972. The relationship of adenovirus-induced paracrystalline structures to the virus core protein(s). *J. Gen. Virol.* 14:261-270.
9. Mirza, A., and J. Weber. 1980. Infectivity and uncoating of adenovirus cores. *Intervirology* 13:307-311.
10. Obert, G., F. Tripiet, H. Nonnenmacher, and A. Kirn. 1980. Evidence for two arginine-dependent steps in vaccinia virus multiplication: role of polyamines in the synthesis of late mRNA. *Ann. Virol. Inst. Pasteur* 131:13-24.
11. Rouse, H. C., V. H. Bonifas, and R. W. Schlesinger. 1963. Dependence of adenovirus replication on arginine and inhibition of plaque formation by pleuropneumonia-like organisms. *Virology* 20:357-365.
12. Rouse, H. C., and R. W. Schlesinger. 1967. An arginine-dependent step in the maturation of type 2 adenovirus. *Virology* 33:513-522.
13. Rouse, H. C., and R. W. Schlesinger. 1972. The effects of arginine starvation on macromolecular synthesis in infection with type 2 adenovirus. I. Synthesis and utilization of structural proteins. *Virology* 48:463-471.
14. Sharp, P. A., C. Moore, and J. L. Haverty. 1976. The infectivity of adenovirus 5 DNA-protein complex. *Virology* 75:442-456.
15. Wilhelm, J., O. Brison, C. Kedinger, and P. Chambon. 1976. Characterization of adenovirus type 2 transcriptional complexes isolated from infected HeLa cell nuclei. *J. Virol.* 19:61-81.