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Transcription and Replication of Viral Deoxyribonucleic acid in Cells Coinfected with Adenovirus Types 2 and 12

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The yield of infectious virus was determined for KB cells infected with both adenovirus types 2 (ad 2) and 12 (ad 12). It was found that the yield of the former was greatly reduced, whereas that of the latter was not affected significantly. The reduction in virus yield was accompanied by an inhibition of ad 2 virus-specific ribonucleic acid (RNA) and viral deoxyribonucleic acid (DNA) synthesis at various times after infection. On the other hand, the rate of synthesis of ad 12 virus-specific RNA and viral DNA was not inhibited, but advanced in time. The total amount of ad 12 viral DNA synthesized was not affected by coinfection with ad 2. These results suggest that ad 2 infection hastens the maturation of ad 12.

Within the group of human adenoviruses, some are highly oncogenic in newborn rodents, such as type 12 (ad 12), and others are nononcogenic, such as type 2 (ad 2) (8, 14, 19). These two types also differ in their plaque morphology and ratio of particles to plaque-forming units (PFU) (12), and ad 12 contains a much higher proportion of particles capable of killing the host cells but unable to form a plaque than ad 2 (Rainbow and Mak, *unpublished data*). Furthermore, with KB cells, the yield of ad 12 is very low compared to ad 2, and it matures much later (13). It is of interest to examine whether ad 2 enhances the yield of ad 12 or advances its maturation when coinfecting a common host. The deoxyribonucleic acid (DNA) molecules of ad 2 and ad 12 are distantly related, sharing only about 20% of their nucleotide sequence (15); this system may allow one to examine the synthesis of virus-specific ribonucleic acid (RNA) and viral DNA in the coinfected cultures in addition to the virus yields. In this report, it is shown that ad 12 interferes with the normal development of ad 2, and that ad 2 advances the synthesis of ad 12 virus-specific RNA and viral DNA.

MATERIALS AND METHODS

Cells, medium, and virus. A clonal subline of human KB cells (7) was obtained from M. Green, St. Louis University Medical School, St. Louis, Mo. The cells were kept in exponential growth in suspension cultures using Joklik modified minimal essential medium (MEM; Grand Island Biologicals, Grand Island, N.Y.) supplemented with 5% horse serum. Virus stocks of both ad 2 and ad 12 (Huie) were obtained also from M. Green. These were propagated in KB cells in suspension and purified by the procedure of Green and Piña (10). The virus collected after the second equilibrium density gradient centrifugation using CsCl was diluted at least 10-fold with sterile tris(hydroxymethyl)aminomethane (Tris)-buffered saline [TBS, (22)] plus 20% glycerol and was stored at −45 C until use.

Infection and labeling of cells. Exponentially growing KB cells were centrifuged and resuspended to 10^7 cells per ml of MEM containing 1% fetal calf serum (FCS). Ad 12 was added to 0.3 PFU/cell and adsorbed for 1 hr at 37 C with continuous stirring. Then, a portion of the infected cells was diluted 35-fold with warm MEM plus 5% horse serum. To the remainder of the cells, ad 2 was added to 10 to 20 PFU/cell; it was adsorbed for 1 hr more and then diluted. A separate portion of cells at 10^7 per ml was stirred for 1 hr and then infected with ad 2 at 10 to 20 PFU/cell, allowing 1 hr for adsorption. Under these conditions, more than 99% of the cells in the three separate cultures were infected as judged by cloning (9). At different times after infection, portions of cells from each infected culture were labeled either with ^{3}H-uridine (1 μc/ml, 20 c/mM) for 30 min or with ^{3}H-thymidine (1 μc/ml, 15 c/mM) normally for 60 min at 37 C. The cells were then collected by centrifugation at 500 × *g* for 15 min at 0 C, washed with cold phosphate buffered saline [PBS, (4)], and stored at −20 C.

Preparation of cellular RNA and DNA. Whole cell RNA was extracted from a frozen pellet of infected cells by the hot phenol-sodium dodecyl-sulfate (SDS) procedure (21) and dialyzed against 0.1 × SSC (0.15 M NaCl; 0.015 M Na citrate). Recovery was normally between 80 to 100%. Whole cell DNA was extracted by resuspending the frozen pellet with 1 × SSC, 0.1 M

Tris (*p*H 8.0), 5×10^{-3} M ethylenediaminetetraacetate (EDTA), 0.3 M trichloroacetate, and 0.5% SDS; the cells were allowed to lyse at room temperature for 15 min. The lysate was incubated with Pronase B (Calbiochem, Los Angeles) at 0.8 mg/ml (preincubated for 2 hr at 37 C) for 3 hr at 37 C. The lysate was extracted three times with equal volume of redistilled phenol at room temperature. Nucleic acids were precipitated with twice the volume of ethyl alcohol at 4 C overnight. RNA was removed by ribonuclease (50 μg/ml) incubated at 37 C for 30 min (17) followed by three phenol extractions at room temperature; the aqueous phase was treated three times with ether, bubbled with compressed air, and dialyzed extensively against 0.1 × SSC. Recovery is usually 80 to 100% with this procedure.

Virus yield. At 60 hr after infection, 100-ml samples of the infected cultures were centrifuged at 500 × *g* for 15 min, resuspended in 5 ml of TBS + 20% glycerol, and stored at −45 C. After thawing, the cells were disrupted at 0 C by sonication for 2 min with a Biosonik probe. Cell debris was removed by centrifugation (500 × *g*, 10 min) and the infectious virus was determined by plaque assay (12). To determine the yield of ad 12 in the coinfected cultures, the virus preparation was treated with goat anti-ad 2 serum (a generous gift from G. D. Dressman, Saint Louis University, Institute for Molecular Virology) for 15 min at 37 C before plaque assay.

Preparation of adenovirus DNA molecules. DNA molecules from purified ad 2 and ad 12 viruses were prepared by the method of Green and Piña (11). ^{14}C-labeled ad 2 DNA was obtained from purified ad 2 virus from cells grown in the presence of ^{14}C-thymidine (0.04 μc/ml, 60 mc/mM) added at 6 hr after infection.

RNA and DNA synthesis. After the infected cells had been labeled with either ^{3}H-uridine or ^{3}H-thymidine, a known sample was treated with cold 0.3 M trichloroacetic acid, collected on membrane filters, and washed three times with cold trichloroacetic acid. After drying, the radioactivity was counted in a Beckman scintillation counter in 5 ml of counting fluid which consists of 4 mg of 2,5-diphenyloxazole (PPO) and 0.3 μg of 1,4-bis-2-(5 phenyloxazolal)-benzene (POPOP) dissolved in each ml of toluene.

Detection of virus-specific RNA and viral DNA. Determination of virus-specific RNA was done by DNA-RNA hybridization on nitrocellulose membrane filters (sartonius filters; British Drug Houses, Canada) by the method of Gillespie and Spiegelman with slight modifications (5, 16). ^{3}H-RNA bound to DNA-containing filters after drying was counted in a Beckman scintillation counter.

Viral DNA was detected by DNA-DNA hybridization on nitrocellulose membrane filters (16, 20). Sonicated and heat-denatured radioactive DNA extracted from either purified virus or infected cells was incubated in 2 × SSC, 10^{-2} M Tris (*p*H 7.0) and 0.1% SDS at 64 C for 24 hr with viral DNA-containing membrane filters. Then the filters were washed exhaustively with 3×10^{-3} M Tris in 0.1 × SSC (*p*H 9.4) by filtration. The amount of radioactivity bound was counted in a Beckman scintillation counter after drying.

RESULTS

Virus yield in coinfected cultures. The intracellular virus yield of ad 2 was reduced to about 3% of normal yield when coinfected with ad 12 (Table 1). The number of infectious virus from coinfected cultures after anti-ad 2 serum treatment was found to be only two times that infected by ad 12 alone (Table 1). This indicates that coinfection with ad 2 does not help much the intracellular yield of ad 12.

Rate of synthesis of RNA and DNA after infection. The amounts of ^{3}H-uridine and ^{3}H-thymidine incorporated into cultures infected with ad 2 and ad 12 only, and coinfected with both viruses are shown in Fig. 1A and 1B. The pattern of RNA synthesis in all the infected cultures was similar, showing a much decreased rate of incorporation at late times after infection. For DNA synthesis, the ad 2-infected culture showed a much higher rate than the other cultures at all times tested. However, a decreased rate occurred at late times after infection for all of them.

Synthesis of ad 2 and ad 12 virus-specific RNA. Virus-specific RNA was detected by annealing 1 μg of either ad 2 viral DNA or ad 12 viral DNA immobilized on membrane filters with ^{3}H-RNA extracted from infected cells. Virus-specific RNA from ad 2-infected cells reached a maximum at 18 hr after infection, whereas ad 12-infected ones reached it at 24 hr. ^{3}H-RNA from ad 2-infected cells did not hybridize significantly with ad 12 DNA at all times examined and vice versa. Thus, ad 2 and ad 12 virus-specific RNA from coinfected cultures can be detected by hybridization with ad 2 and ad 12 DNA, respectively. The results are shown in Fig. 2 for cultures infected with either ad 2 or ad 12 singly, and coinfected with both viruses. It can be seen that in the singly infected cultures, ad 12-specific RNA synthesis showed an 8-hr delay as compared to the synthesis of ad 2-specific RNA. In the coinfected culture, ad 12-specific RNA synthesis was advanced in time

TABLE 1. *Virus yield in adenovirus-infected KB cells*

Infection	Virus yield[a]	Virus yield after anti-ad 2 serum treatment[b]	
		PFU/10^6 cells	Surviving fraction
Ad 2 only	4×10^9	8×10^5	2×10^{-4}
Ad 2 and ad 12	1.2×10^8	4×10^6	3.3×10^{-2}
Ad 12 only	2×10^6	2×10^6	1.00

[a] Virus yield was determined at 60 hr after infection by plaque assay; values expressed as plaque-forming units (PFU) per 10^6 cells.

[b] 15 min with anti-ad 2 at 37C.

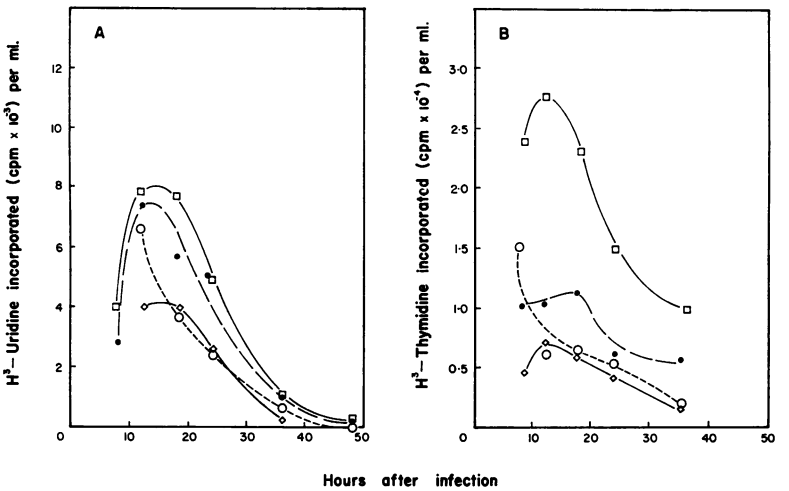

FIG. 1. *Incorporation of precursors into nucleic acids of KB cells at various times after infection with adenoviruses. (A) ³H-uridine incorporated into acid-insoluble fraction, 30-min pulse. (B) ³H-thymidine incorporation into acid insoluble fraction, 60-min pulse. Symbols: (□) ad 2-infected (20 PFU/cell), (●) ad 2- and ad 12-co-infected, (○) ad 12 at 0.3 PFU/cell, (◇) ad 12 at 1.5 PFU/cell.*

TABLE 2. *Hybridization of ad 2 or ad 12 DNA with ³H-RNA from adenovirus-infected KB cells at different times after infection*[a]

Infection	Hr after infection	Ad 2 DNA		Ad 12 DNA	
		³H-RNA input	Per cent bound	³H-RNA input	Per cent bound
		counts/min	*counts/min*	*counts/min*	*counts/min*
Ad 2 (20 PFU/cell)	8	2,310	0.9	11,660	<0.1
	12	2,810	5.0	14,480	0.1
	18	2,250	18.7	9,890	0.4
	24	2,830	19.3	9,890	0.5
	36	2,300	10.4	12,650	0.1
	48	2,560	3.0	8,058	0.1
Ad 12 (0.3 PFU/cell)	12	10,820	0	2,480	0.5
	18	11,850	0.14	2,550	5.5
	24	10,750	0.4	2,820	20.2
	36	10,950	0.2	2,380	13.4
	48			2,220	5.0

[a] Samples of infected cells were pulse-labeled with ³H-uridine (1 μc/ml) for 30 min. ³H-RNA was annealed with 1 μg of either ad 2 or ad 12 DNA.

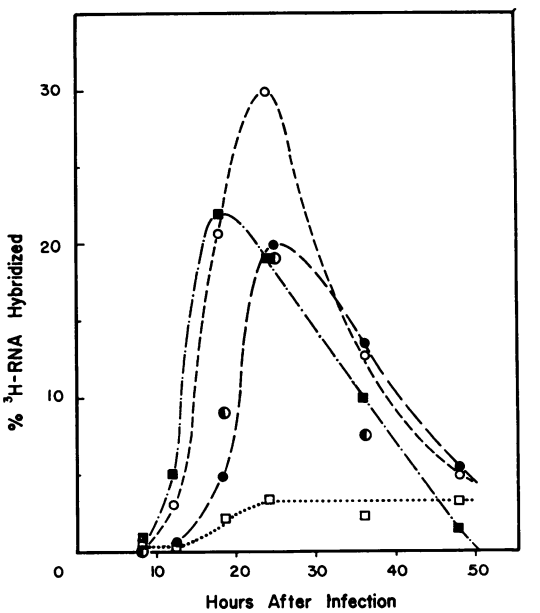

FIG. 2. *Synthesis of virus-specific RNA at different times after infection with adenoviruses. Percentage of ³H-RNA (30-min pulse) hybridized with 1 μg of viral DNA is plotted. Symbols: (■) ad 2-infected (20 PFU/cell), (○) ad 2- and ad 12-coinfected and then hybridized with ad 12 DNA, (□) ad 2- and ad 12-coinfected and then hybridized with ad 2 DNA, (●) ad 12-infected (0.3 PFU/cell), (◐) ad 12-infected (1.5 PFU/cell).*

to the pattern of ad 2-infected cultures, but it reached the maximal value at the same time as ad 12-infected cultures. At the same time, ad 2-specific RNA synthesis was greatly suppressed. Since in the coinfected cultures the total number of input virions per cell was higher than that in ad 12-infected alone, it is possible that this advancement in time was due to the effects of virions per cell rather than ad 2 effect. This possibility was ruled out since cells infected with additional ad 12 virions equivalent to about 10 to 20 PFU of ad 2

(12) per cell did not show an advance in ad 12-specific RNA synthesis (Fig. 2).

Synthesis of ad 2 and ad 12 viral DNA. If coinfection of cells with ad 2 and ad 12 advanced the development of ad 12 as indicated in the synthesis of virus-specific RNA, the synthesis of ad 12 viral DNA should also be advanced in time. To detect viral DNA synthesis, ^{3}H-DNA extracted from infected cells was annealed with 2 μg of either ad 2 or ad 12 DNA immobilized on membrane filters. Since ^{3}H-DNA extracted from ad 2-infected cells cross-hybridize with ad 12 viral DNA about 25% and vice versa (Table 3), the amount of ^{3}H-DNA from coinfected cultures bound to each of the viral DNA must be corrected by the amount due to cross-hybridization. The corrected per cent of ^{3}H-DNA hybridized with either ad 2 or ad 12 DNA is shown in Fig. 3 for different times after infection. As can be seen, the synthesis of ad 12 DNA was greatly accelerated, beginning about 15 hr after infection; this is later than that for ad 2, when cells were singly infected with either ad 2 or 12. However, in the coinfected culture, the sharp increase in the synthesis of ad 12 DNA was advanced in time by about 8 hr, whereas ad 2 viral DNA synthesis was inhibited. The decline of per cent hybridized at late times after infection is probably due to the fact that some ^{3}H-viral DNA renatured in solution, thus unable to be retained on the filters, and was not due to reinitiation of host DNA synthesis (13). The pattern of advanced synthesis of ad 12 DNA by coinfection with ad 2 is similar to the virus-specific RNA synthesis in these cultures.

TABLE 3. *Hybridization of ad 2 or ad 12 viral DNA with ^{3}H-DNA from adenovirus-infected KB cells at different times after infection*[a]

Infection	Hr after infection	^{3}H-DNA input (counts/min)	Percentage bound		Percentage of cross-hybridized[b]
			Ad 2 DNA (2 μg/filter)	Ad 12 DNA (2 μg/filter)	
ad 2	12	1,330	45	15	33
	18	2,460	90	27	30
	36	6,160	62	18	29
ad 12	12	2,020	1.2	6.4	18
	18	2,130	8.6	39.0	22
	24	1,000	23	81	28
None		20,800	0.2		

[a] Samples of cells were labeled with ^{3}H-thymidine (1 μc/ml) for 60 min. ^{3}H-DNA extracted from infected cells was annealed with ad 2 or ad 12 viral DNA.

[b] Percentage of cross-hybridized is a ratio of the per cent bound to heterologous DNA to the per cent bound to homologous DNA.

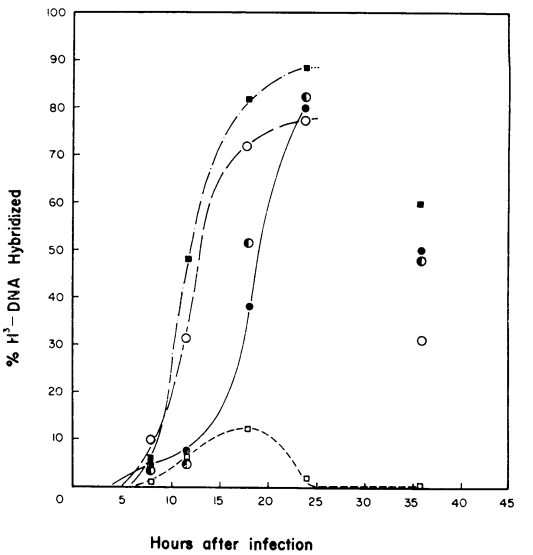

FIG. 3. *Viral DNA synthesis at different times after infection with adenoviruses. Percentage of ^{3}H-DNA (60-min pulse) bound to 2 μg of viral DNA on nitrocellulose membranes is plotted. Symbols:* (■) *ad 2-infected (20 PFU/cell),* (○) *ad 2- and ad 12-coinfected and then hybridized with ad 12 DNA,* (□) *ad 2- and ad 12 coinfected and then hybridized with ad 2 DNA,* (●) *ad 12-infected (0.3 PFU/cell),* (◐) *ad 12-infected (1.5 PFU/cell).*

Total viral DNA synthesized. The amount of viral DNA synthesized at 36 and 48 hr after infection was determined for singly infected and coinfected cultures. Since host DNA synthesis is shut off at these times (6, 13), the total amount of viral DNA can be determined as follows. Infected cells were labeled for 2 hr with ^{3}H-thymidine and the amount of radioactivity incorporated into the DNA was determined. The specific activity of the ^{3}H-viral DNA was found by determining the radioactivity bound to a known amount of immobilized viral DNA by using saturating amounts of ^{3}H-DNA in the DNA-DNA hybridization reaction. The total ^{3}H-thymidine (counts/min) incorporated, divided by the specific activity, would yield an estimate of the amount of viral DNA in the infected cells. To test the validity of this method for specific activity determination, two types of control experiments were carried out. (i) Increasing amounts of ad 2-^{14}C-DNA (specific activity, 5,200 counts/min per μg or 2,300 counts/min per μg), mixed with equal amount of ^{3}H-labeled noninfected KB cell DNA, were annealed with 0.2 μg of ad 2 DNA on filters. The maximum amount of ^{14}C-radioactivity bound was equivalent to 0.23 to 0.24 μg of viral DNA in both cases, whereas no ^{3}H-activity was bound to the filters (Fig. 4A). This indicates that the amount of DNA

bound is equal to that immobilized on the filters and, also, that the presence of cell DNA had no effect on the viral DNA-DNA hybridization. (ii) Increasing amounts of ^{3}H-DNA extracted from ad 2-infected cells (48 hr after infection) were hybridized with 0.2 ot 0.3 μg of ad 2 DNA on filters. From Fig. 4B, it can be seen that the ^{3}H-DNA bound reached maximum at 950 and 1,400 counts/min, respectively, giving specific activities of 4,750 and 4,650 counts/min per μg, respectively, supporting the validity of this method.

The amount of viral DNA molecules at 36 and 48 hr after infection for singly infected and coinfected cultures are shown in Table 4. In the determination of specific activity of ad 12 DNA in the coinfected culture, the effects of ad 2 DNA was neglected since its synthesis was shut off early (*see* Fig. 3). The total amount of viral DNA in the ad 12 singly infected cultures was less than that in ad 2 or coinfected cultures at 36 hr, but reached similar values at 48 hr after infection. These results suggest that the synthesis of ad 12 viral DNA in the coinfected cultures was not significantly increased above the singly infected cultures, but was advanced to that of ad 2-infected cultures, lending support to the data in Fig. 3. The amount of ad 2 in the coinfected cultures was not determined due to difficulties in determining the specific activity with the present method.

It is of interest to note that KB cells infected with either ad 2 or ad 12 synthesized about the

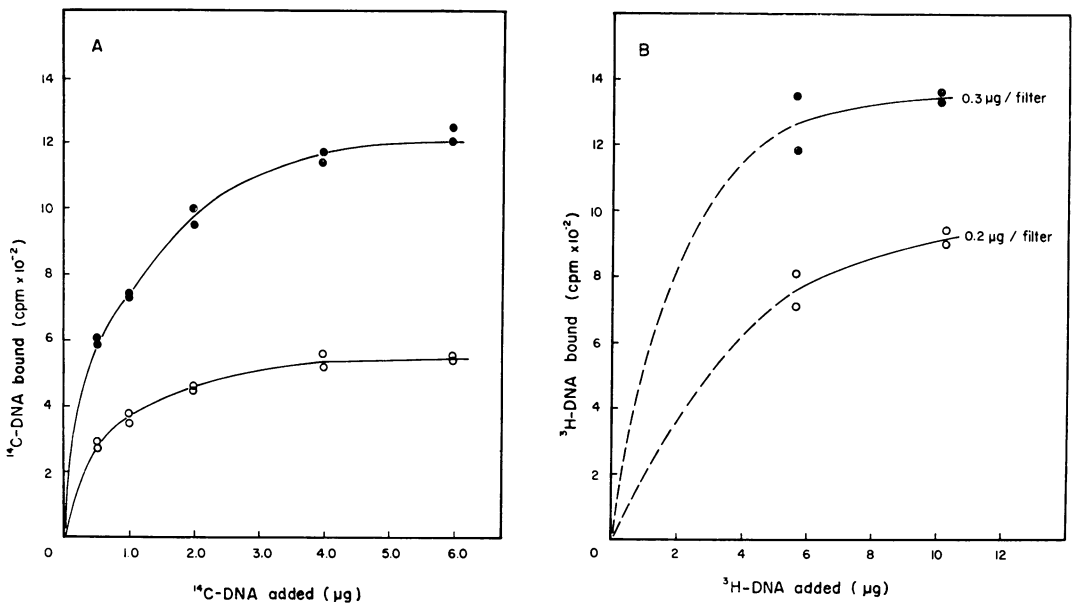

FIG. 4. *Saturation of immobilized DNA with ^{14}C-ad 2 DNA and ^{3}H-DNA from ad 2-infected cells. (A) 0.2 μg of immobilized ad 2 DNA was hybridized with increasing amounts of ^{14}C-ad 2 DNA, with specific activities of 5,200 counts/min per μg (●) and 2,300 counts/min per μg (○). (B) Immobilized ad 2 DNA [0.3 μg (●) or 0.2 μg (○)] was hybridized with ^{3}H-DNA (120 min of labeling with ^{3}H-thymidine) extracted from cells at 48 hr after infection.*

TABLE 4. *Total amount of ad 2 or ad 12 viral DNA synthesized at two different times after infection*

Hr after infection	Infection	^{3}H-DNA (counts/min per 10^6 cells)	Viral DNA determined	Specific activity of viral DNA[a] (counts/min per μg)	Total amount of viral DNA[b] (μg per 10^6 cells)
36	ad 2	9.5×10^4	ad 2	20,000	4.7
	ad 2 and ad 12	14.7×10^4	ad 12	25,000	6.0
	ad 12	4.5×10^4	ad 12	35,000	1.3
48	ad 2	9.3×10^4	ad 2	6,000	15
	ad 2 and ad 12	5.6×10^4	ad 12	4,000	14
	ad 12	5.5×10^4	ad 12	6,000	9

[a] For the method of determination of specific activity of viral DNA, *see* text.
[b] Total viral DNA = ^{3}H-DNA (column 3)/specific activity (column 5).

same amount of viral DNA. This suggests that the assembly of ad 12 viral DNA into complete virion is less efficient since the yield of virions per cell in ad 2-infected cells is greater than 10 times that of ad 12-infected cells (S. Mak, *unpublished data*).

DISCUSSION

The low yield of infectious adenovirus type 12 in KB cells was increased only slightly by coinfection with adenovirus type 2 which is a high yielder; the adenovirus type 2 yield was greatly inhibited by coinfection, suggesting an interference phenomenon. Similar results have been reported for cells doubly infected with unrelated animal viruses (3); in that report, the one with longer latent period was inhibited in contrast to the present study where the yield of the faster developing one is suppressed. It is unlikely that ad 2 was excluded by ad 12 infection since the development of ad 12 was advanced in the coinfected cultures. It is apparent that the level of interference is on the transcription and replication of viral DNA; however, it is not certain whether translation process is also affected.

Although ad 2 did not increase the yield of ad 12, the development of the latter was speeded up similar to that of ad 2 alone. This is evident from the time course of synthesis of ad 12 viral DNA and its specific RNA. It is not clear whether ad 2 infection of KB cells has supplied early new product(s) such as proteins which ad 12 can use, as suggested by Chany and Brailovsky (2), or merely by shutting off host metabolism, thus allowing ad 12 to develop sooner than normally.

It was also noted that the total amount of ad 12 viral DNA in the coinfected culture was similar to the singly infected cells, which is consistent with the finding that infectious virus yield was not increased greatly. This suggests that coinfection does not increase the efficiency of utilization of DNA for the infectious virions in contrast to the case where the assembly of human adenovirus DNA into infectious virus was greatly enhanced by coinfection with SV40 in African green monkey cells (1, 18).

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