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Degradation of Intracellular DNA in KB Cells Infected with *cyt* Mutants of Human Adenovirus Type 12

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A group of mutants (*cyt* mutants) with much reduced oncogenicity was isolated from the highly oncogenic human adenovirus type 12 (Takemori et al., Virology **36**:575-586, 1968). These mutants induce extensive cellular destruction during lytic infection of human cells and produce low yields of virions. We report here that human KB cells infected with *cyt* mutants synthesized a reduced amount of viral DNA as compared with cells infected with the parental virus. Furthermore, the newly synthesized viral and cellular DNAs were extensively degraded in mutant-infected cells. Viral DNA was first synthesized as complete genome size, and most of it was degraded to subgenomic size within 6 h after synthesis. This virus-induced DNA degradation function, as well as the low yield of virions, was prevented by co-infection with the parental virus.

Takemori et al. (16) isolated a class of mutants (*cyt* mutants) from the highly oncogenic human adenovirus type 12 (Ad12). Some mutants were isolated after UV irradiation, whereas others were spontaneous mutants. The *cyt* mutants generally have low oncogenicity in newborn hamsters and produce large, clear plaques on human embryonic kidney cells compared with the small, fuzzy-edged plaques produced by the parental virus. Cytopathic effects produced by these mutants are characterized by extensive cellular destruction. All the *cyt* mutants studied belong to a single complementation group (17). However, recombinants could be obtained from cells infected with two different *cyt* mutants (15). Revertants have been isolated from some spontaneous *cyt* mutants (17). These revertants regained the high tumorigenicity, the cytopathic effects, and the plaque morphology of the parental virus. Takemori et al. (16) have suggested that a single gene can affect both tumorigenicity and cellular destruction. However, no revertants have been isolated from the UV-induced *cyt* mutants. It is possible that these mutants have multiple mutations or deletions.

In an earlier report, we showed that one of the spontaneous *cyt* mutants (H12*cyt*70) produced a low yield of virions compared with the parental Ad12 (4). In this paper we report our finding that KB cells infected with H12*cyt*70 accumulated less viral DNA than those infected with the parental virus. Furthermore, a high fraction of the newly synthesized DNAs (both viral and cellular) were extensively degraded. Evidence is presented that DNA degradation is a characteristic of the *cyt* mutants.

MATERIALS AND METHODS

Virus and cells. Human Ad12 (strain 1131) (13) and several *cyt* mutants were used to infect human KB cells. *cyt* mutants used here H12*cyt*70, H12*cyt*52, H12*cyt*62, and H12*cyt*61. The former two mutants are spontaneous mutants, whereas the latter two mutants are UV-induced mutants. These mutants were previously designated as *cyt*129, *cyt*7, *cyt*133, and *cyt*135, respectively (4, 16) and were isolated from strain 1131 of Ad12 (16). A revertant isolated from H12*cyt*70 was also used. Crude virus stocks were obtained from N. Takemori and were used to infect human embryonic kidney cells. Lysates from these infections were used to infect KB cells grown as monolayer cultures. Virions purified by CsCl density gradient centrifugation were used throughout this study. Methods for cell culture, virus growth, and purification have already been described (5). For experiments, KB cells were infected in suspension with a multiplicity of infection of 200 to 1,000 virions per cell. After 90 min of adsorption, the cells were diluted with α-medium (GIBCO Diagnostics, Madison, Wis.) or with minimal essential medium enriched with 2% amino acid mixture and 2% vitamins (GIBCO), plus 10% fetal calf serum. Further incubation of infected cells was either in plastic flasks (Falcon Plastics, Oxnard, Calif.) or in 32-oz. (ca. 960-ml) prescription bottles (Brockway).

Detection and quantitation of viral DNA. Infected cells were labeled with [^{3}H]thymidine at 10 μCi/ml at different times after infection. To ensure the availability of [^{3}H]thymidine during long periods of labeling, nonradioactive thymidine was also added to the medium at a final concentration of 2 μg/ml. Total infected cell DNA was extracted as previously described (7). Radioactively labeled viral DNA was detected and quantitated by DNA-DNA hybridization (7, 19).

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Size of DNA molecules. The size of single-stranded DNA molecules was assayed by alkaline sucrose density gradient centrifugation (8). Infected cells were labeled with [^{3}H]thymidine for an appropriate period of time and then washed once with phosphate-buffered saline. The cells, together with Ad2 virions containing [^{14}C]DNA as marker, were lysed in 0.5 N NaOH containing 10 mM EDTA, 0.1 M NaCl, and 1% sodium dodecyl sulfate on top of a 5 to 20% sucrose gradient. The sucrose gradient was made up in 0.3 N NaOH, 2 mM EDTA, 0.1 M NaCl, and 0.1% sodium dodecyl sulfate. The gradient was centrifuged at 24,000 rpm for 13 h at 20°C with a Beckman SW27.1 rotor. About 40 fractions were collected from the bottom of the tube, and the radioactivity was determined by scintillation counting after trichloroacetic acid precipitation and collection onto nitrocellulose membrane filters.

The size of double-stranded DNA was similarly analyzed by a 5 to 20% sucrose gradient containing 0.1 M Tris (pH 7.1), 0.15 M NaCl, 2 mM EDTA, and 0.1% sodium dodecyl sulfate. Centrifugation was at 23,000 rpm for 12.5 h at 20°C with a Beckman SW27.1 rotor. Before centrifugation, the infected cells were lysed in 0.15 M NaCl, 0.15 M sodium citrate, 5 mM EDTA, and 0.5% sodium dodecyl sulfate, and then treated with 200 μg of pronase per ml (predigested for 2 h at 37°C) for several hours.

Virus yield determinations. [^{3}H]thymidine was added to infected cultures at 14 h after infection. At 40 h the cells were harvested and treated with deoxycholate and Freon 113 as described previously (4). The treated lysate was analyzed for virion content by rate zonal centrifugation. Radioactivity sedimented in the position of a marker virion ([^{14}C]Ad12 virions) was taken as an estimate of virus yield (4, 8).

RESULTS

Amount of viral DNA synthesized in cells infected with *cyt* mutant or parental virus. In a previous communication, we reported that the *cyt* mutant (H12*cyt*70)-infected cells produced fewer virions compared with the parental virus-infected cells (4). There may be a correspondingly smaller amount of viral DNA synthesized in cells infected with the *cyt* mutant. The total amount of Ad12 DNA synthesized in infected KB cells was determined by a previously described method (7). It was shown that the amount of radioactively labeled viral DNA hybridized, at saturation, to unlabeled viral DNA immobilized on filters is equal to the amount of unlabeled DNA on these filters (7). Infected cells were labeled with [^{3}H]thymidine between 30 and 40 h after infection (10 μCi/ml). Ad12 DNA (0.2 μg) immobilized on nitrocellulose filters was hybridized to saturation with excess amounts of [^{3}H]DNA from the infected cells. The specific activity of the viral DNA could thus be determined. The amount of radioactivity incorporated into viral DNA was also determined by multiplying the total amount of radioactivity incorporated by the fraction of radioactivity hybridized to excess amounts of Ad12 DNA. Amounts of viral DNA accumulated in infected cells from two different experiments are shown in Table 1. The cells infected by the mutant virus accumulated only about 40% of that accumulated in cells infected with the parental virus. Data shown in Table 1 suggest that infection of KB cells by the *cyt* mutant failed to shut off host DNA synthesis. However, in other experiments, we observed that more than 85% of the DNA synthesized late after infection of KB cells by H12*cyt*70 could hybridize to Ad12 DNA. It is not clear that factors contribute to this variation.

Molecular size of the intracellular viral DNA. The reduced amount of viral DNA present in *cyt* mutant-infected cells could account, in part, for the reduced yield of the *cyt* mutant virions. However, it is possible that some of the viral DNA molecules may not be of full genome

TABLE 1. *Total amount of viral DNA synthesized at 40 h after infection of KB cells by Ad12 parental strain and cyt mutant (H12cyt70)*

Infection with:	(A) Total radioactivity in 10^6 cells (cpm)	(B) % Radioactivity hybridized to Ad12 DNA[a]	(C) Radioactivity hybridized at saturation to 0.2 μg of Ad12 DNA (cpm)	(D) Sp act of viral DNA (cpm/μg)	(E) Total amt of viral DNA[b] (μg/10^6 cells)
Expt I					
Parental strain	37,620	100	3,300	16,510	2.28
H12*cyt*70	34,930	43	3,600	18,000	0.83
Expt II					
Parental strain	46,210	96	3,500	17,510	2.53
H12*cyt*70	53,510	42	4,700	23,500	0.96

[a] [^{3}H]DNA from infected cells was hybridized to 2 μg of Ad12 DNA. The unhybridized [^{3}H]DNA was then denatured and rehybridized to another 2 μg of Ad12 DNA. The figures shown in the table represent the sum of percent hybridized for the two hybridizations.

[b] Total amount of viral DNA: column (A) × column (B)/column (D) × 100.

size. Thus, the size of the viral DNA synthesized was examined by alkaline sucrose gradients after the cells were labeled with [^{3}H]thymidine between 13 and 40 h after infection. Figure 1 shows the sedimentation profiles of intracellular DNA from infected cells. In the parental virus-infected

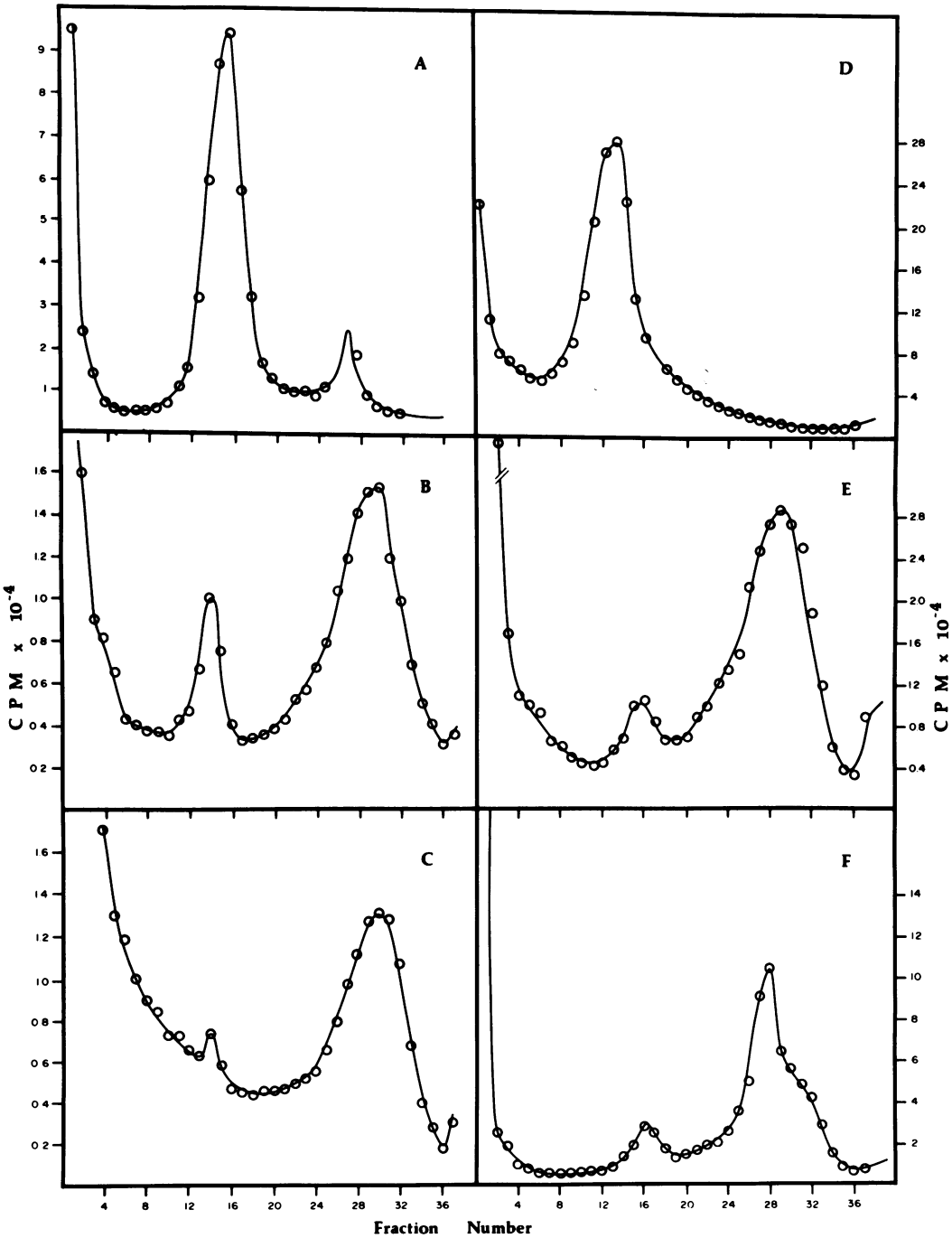

FIG. 1. *Sedimentation profiles of intracellular DNA in KB cells infected with Ad12 parental virus (strain 1131) and various cyt mutants in a 5 to 20% alkaline sucrose gradient. Sedimentation is from right to left. Cells were infected with parental virus (A), H12cyt70 (B), H12cyt52 (C), a revertant of H12cyt70 (D), H12cyt62 (E), and H12cyt61 (F). Complete viral genome sediments at positions corresponding to fractions 14 to 16.*

cells, about 50% of the radioactivity cosedimented with the marker viral DNA ([^{14}C]Ad2) (Fig. 1A). In contrast, *cyt* mutant (H12*cyt*70)-infected cells synthesized substantial amounts of DNA molecules of smaller size (Fig. 1B). Similar differences in sedimentation profiles were observed when the intracellular DNA was analyzed by neutral sucrose gradients (Fig. 2). Thus, it appears that the small DNA molecules are double stranded.

DNA degradation by other *cyt* mutants. To establish whether *cyt* mutation in general induces DNA degradation in infected KB cells, three additional *cyt* mutants (H12*cyt*62, H12*cyt*52, H12*cyt*61) and a revertant isolated from H12*cyt*70 were examined for their effects on DNA degradation. Infected cells were labeled with [^{3}H]thymidine between 13 and 40 h after infection, and the [^{3}H]DNA was analyzed by alkaline sucrose gradients. These data are shown in Fig. 1. Cells infected with all *cyt* mutants showed extensive DNA degradation, whereas those infected with the revertant virus and the parental virus did not. For some *cyt* mutants, such as H12*cyt*52 and H12*cyt*62, a high multiplicity of infection (~1,000 virions/cell) was necessary to induce DNA degradation. However, infection of KB cells by parental virus at 1,000 virions/cell showed only very little DNA degradation (data not shown). It should be noted that cells infected with *cyt* mutants synthesized only a small amount of DNA, which cosedimented with the viral DNA marker.

Size distribution of viral DNA in Ad12-infected KB cells. To determine whether the small molecules were of viral origin, infected cells were labeled with [^{3}H]thymidine, and the [^{3}H]DNA was analyzed by alkaline sucrose gradients as before. The DNA from different regions of the gradients were pooled, neutralized by HCl, precipitated by ethanol, and hybridized to Ad12 DNA immobilized on filters. Table 2 shows the distribution of viral DNA in various size classes. Seventy percent of the parental viral DNA was of complete genome size; in contrast, less than 18% of the *cyt* mutant viral DNA was of genome size. It should be noted that only about 30% of the small DNA fragments hybridized to Ad12 DNA. Thus, cellular DNA was also degraded.

Origin of the small viral DNA fragments. To investigate whether the smaller viral DNA molecules were due to incomplete synthesis or degradation of complete viral DNA molecules, pulse-chase experiments were performed. At 23 h after infection, the infected cells were labeled with [^{3}H]thymidine for 1 h and chased for 3 and 6 h in the presence of excess thymidine. The intracellular [^{3}H]DNA was analyzed by alkaline

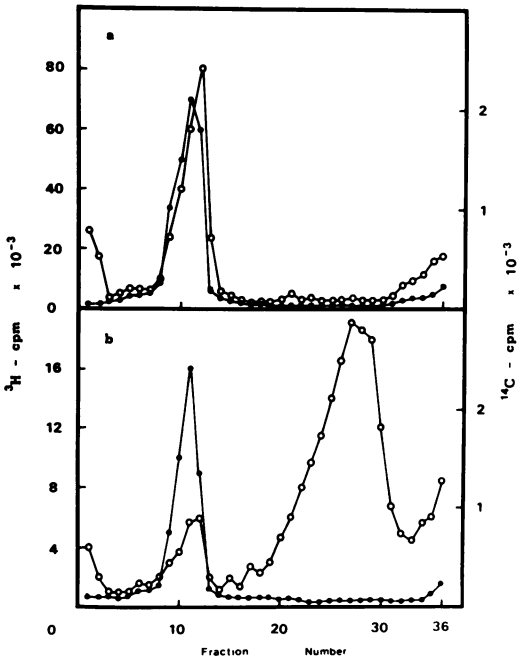

FIG. 2. *Sedimentation profile of intracellular DNA in KB cells infected with Ad12 parental strain and the cyt mutant in a 5 to 20% neutral sucrose gradient. Sedimentation is from right to left. (a) Parental virus-infected cells* (○); *[^{14}C]Ad2 marker DNA* (●). *(b) cyt mutant (H12cyt70)-infected cells* (○); *[^{14}C]Ad2 marker DNA* (●).

sucrose gradients as described. The results of this experiment are shown in Fig. 3. It can be seen that in the cells infected with parental virus, most of the radioactivity could be chased into complete genome size. There was no difference in the radioactivity profile between a 3-h and a 6-h chase. On the other hand, cells infected with the *cyt* mutant showed a very different profile depending on the duration of the chase. After the pulse, the profile was the same as that from cells infected with the parental virus. With a 3-h chase, about 46% of the radioactivity sedimented more slowly than the complete genome. With a 6-h chase, this fraction had increased to greater than 60%. By this time, only 20% of the radioactivity sedimented at the position as the complete genome compared with about 74% in the cells infected with parental virus.

It was noted that very often, more than 50% of the DNA synthesized at 23 h after infection with *cyt* mutant virus was of cellular origin (data not shown). It is possible that the results observed in Fig. 3 relate only to cellular DNA. The small viral DNA molecules observed may have arisen by a different mechanism. To test this possibility, cells were infected with the *cyt* mu-

TABLE 2. *Size and distribution of intracellular viral DNA after infection of KB cells with Ad12 parental strain and cyt mutant (H12cyt70)*

Fraction	(I) Molecular size[a]	(II) Total cpm	(III) % Radioactivity hybridized to Ad12 DNA[b]	(IV) Total viral DNA (cpm)[c]	(V) Amt of viral DNA[d]
Parental					
A	>1.37	223,000	14.6	32,600	7.4
B	1.26–0.73	355,000	88.9	315,600	71.5
C	0.66–0.26	66,000	81.8	53,900	12.2
D	0.22–0.04	25,000	46.3	11,600	2.6
E	<0.02	74,000	37.8	28,000	6.3
cyt mutant					
F	>1.37	286,000	3.3	9,400	8.0
G	1.26–0.73	56,000	37.8	21,200	18.0
H	0.66–0.26	65,000	27.2	17,700	15.0
I	0.22–0.04	143,000	32.6	46,600	39.6
J	<0.02	72,000	31.7	22,800	19.4

[a] As a fraction of the complete viral genome.
[b] [^{3}H]DNA recovered from each fraction in the gradient was hybridized with 1 μg of Ad12 DNA.
[c] Total viral DNA: column (II) × column (III)/100.
[d] Amount of viral DNA in each fraction is expressed as percentage of total viral DNA in the gradient.

tant, pulse-labeled with [^{3}H]thymidine, and chased for 6 h, and the cell lysate was centrifuged in alkaline sucrose gradients. The region of the gradient equivalent to fractions 9 to 12, as shown in Fig. 3, was collected, neutralized, and ethanol precipitated (class I). The region equivalent to fractions 20 to 28 was similarly treated (class II). The amount of [^{3}H]labeled viral DNA in these classes was determined by DNA-DNA hybridization, and the results are shown in Table 3. It can be seen that after a 6-h chase, two thirds of the viral [^{3}H]DNA was degraded to subgenomic size. These results suggest that viral DNA was made as complete genome and then degraded.

DNA degradation and virion production in cells doubly infected with H12*cyt*70 and parental strain viruses. It is clear from these experiments that low virion yield and DNA degradation are the properties of *cyt* mutants. Since parental cytopathic effects are dominant over those of the *cyt* mutant (17), we therefore investigated the effects of double infection of cells by parental and H12*cyt*70 virus. KB cells were first infected with the mutant virus (100 virions/cell) and then immediately superinfected with the parental virus (100 virions/cell). The infected cells were labeled with [^{3}H]thymidine between 14 and 40 h after infection, and the intracellular [^{3}H]DNA was analyzed by alkaline sucrose gradients. Results shown in Fig. 4 indicate that cells double infected had a sedimentated profile similar to that of the parental virus-infected cells. The relative number of virions produced by cells singly infected or doubly infected is shown in Table 4. Cells infected with parental virus or both viruses produced the same amount of virions, whereas the *cyt* mutant-infected cells produced 13-fold less.

DISCUSSION

The *cyt* mutants of Ad12 isolated by Takemori show a number of biological properties different from those of the parental strain (16, 17). These include low oncogenicity in hamsters, the inability to induce cellular transformation in hamster cells, and extensive cellular destruction of infected HEK cells. Virion productions in KB cells infected with the same four *cyt* mutants studied here are low compared with that of cells infected by the parental strain (4; I. Mak, unpublished data).

We have shown in this report that infection of KB cells by *cyt* mutants induced both viral and cellular DNA degradation. The function of inducing DNA degradation is a property of the *cyt* mutants since infection of KB cells by a revertant isolated from H12*cyt*70 produced very little DNA degradation (Fig. 1). Furthermore, this virus-induced DNA degradation is a recessive function since it can be complemented by coinfection with parental virus. DNA degradation readily explains the low yields of virions produced by cells infected with *cyt* mutants. Since cellular DNA was also degraded by *cyt* mutant infection, this may also provide an explanation for the extensive cellular destruction at late times after infection.

Results from pulse and chase experiments indicated that Ad12 DNA was degraded after a complete genome was synthesized. Thus, the *cyt* mutation does not affect the process of initiation,

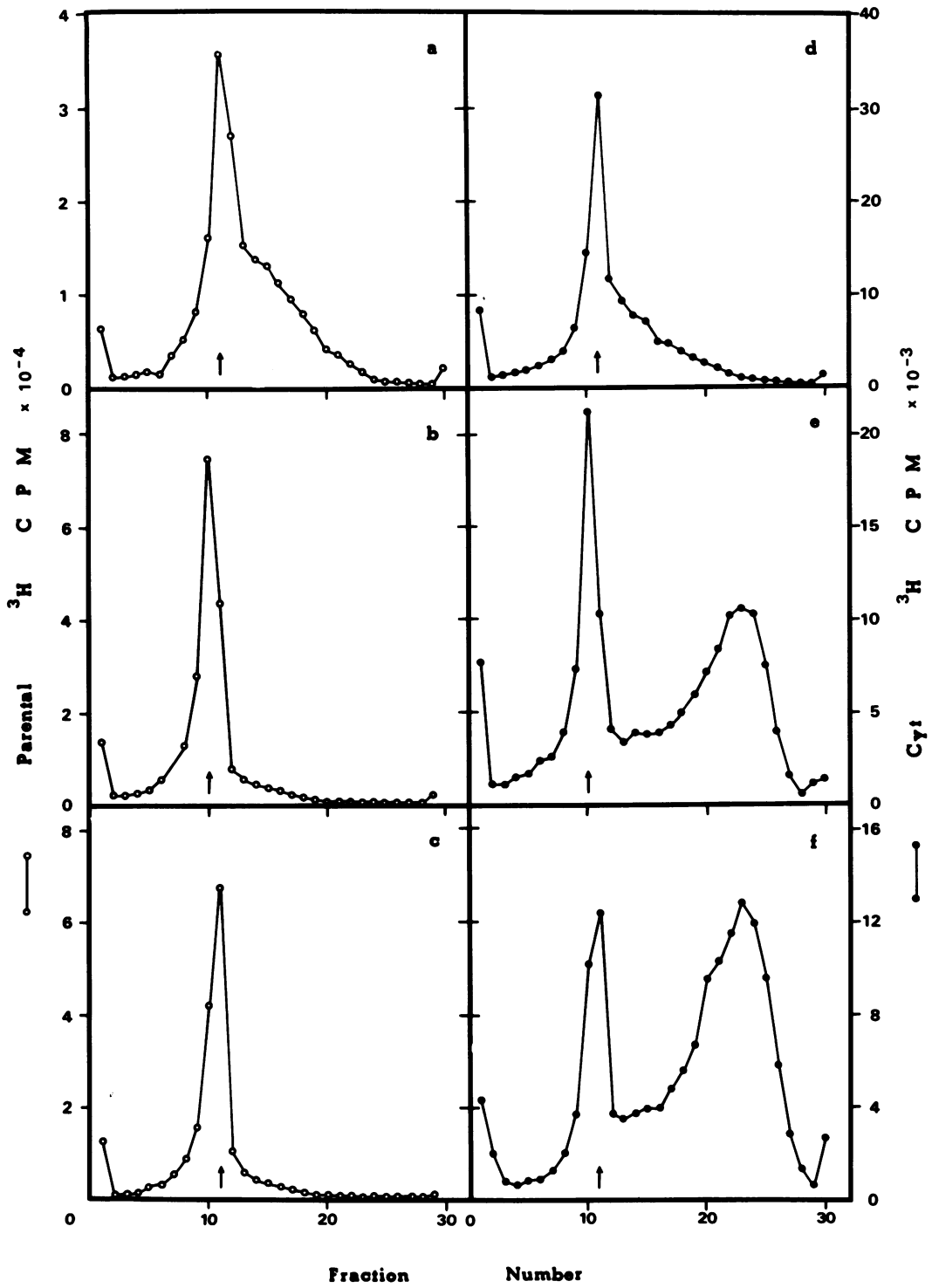

FIG. 3. *Sedimentation profile of pulse-labeled intracellular DNA in KB cells infected with Ad12 parental strain and cyt mutant (H12cyt70) in a 5 to 20% alkaline sucrose gradient. Sedimentation is from right to left. (a) Pulse-labeled for 1 h at 23 h after infection with parental strain; (b) pulsed-labeled and subjected to a 3-h chase; (c) pulse-labeled and subjected to a 6-h chase; (d) pulse-labeled for 1 h at 23 h after infection with the cyt mutant; (e) pulse-labeled and subjected to a 3-h chase; (f) pulse-labeled and subjected to a 6-h chase. Arrows indicate the position of [^{14}C]Ad2 marker DNA from virions.*

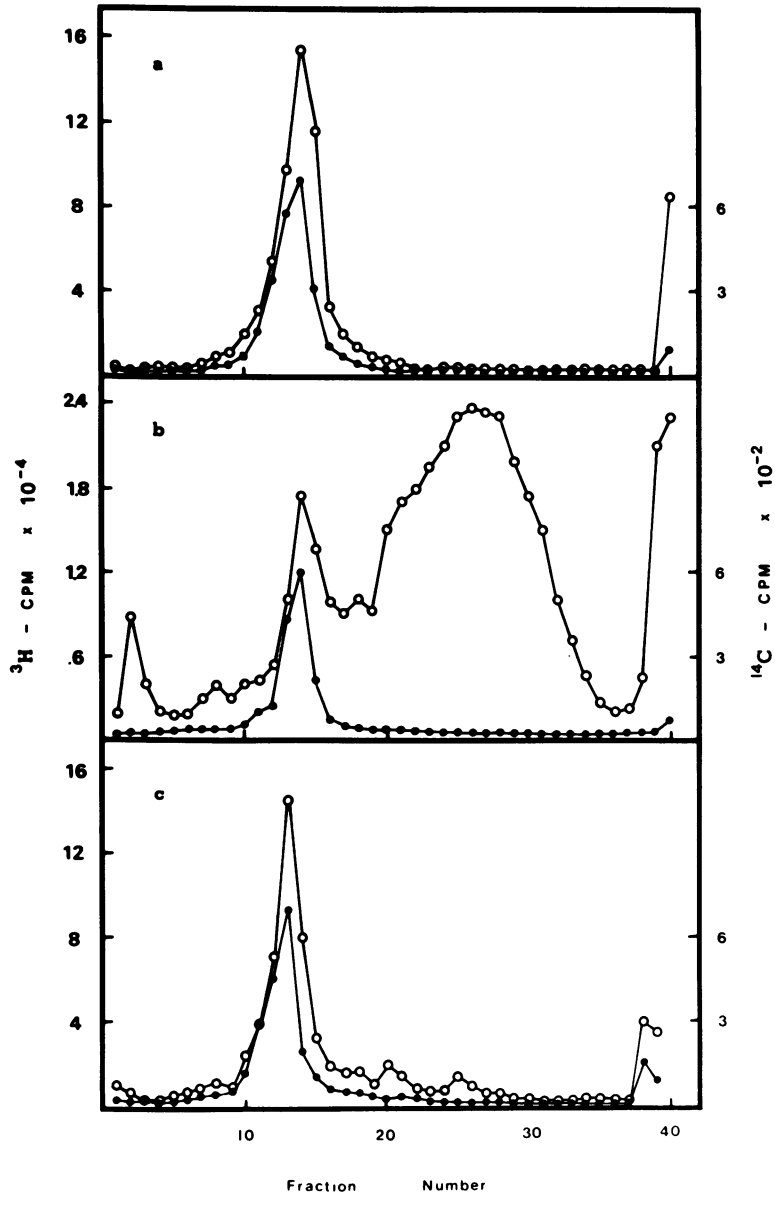

FIG. 4. *Sedimentation profile of intracellular DNA in KB cells infected with Ad12 parental strain, the cyt mutant (H12cyt70), and a mixture of the parental strain and the cyt mutant in a 5 to 20% alkaline sucrose gradient. Sedimentation is from right to left. (a) Cells infected with the parental strain* (○); *[^{14}C]Ad2 marker DNA* (●). *(b) Cells infected with the cyt mutant* (○); *[^{14}C]Ad2 marker DNA* (●). *(c) Cells co-infected with the parental strain and the cyt mutant* (○); *[^{14}C]Ad2 marker DNA* (●).

elongation, or termination of viral DNA synthesis.

Recently, D'Halluin et al. (3) showed that a temperature-sensitive mutant of Ad2 (H2*ts*111) degrades DNA at the nonpermissive temperature. We have found that infection of KB cells by Ad5 dl313, which has a deletion between 3.5 and 10.5 map units (6), caused extensive degradation of newly synthesized DNA (R. B. Lai Fatt, unpublished data). Thus, it is possible that DNA degradation function may reside in the early region 1B of Ad5. Since the organization of the Ad12 genome and its pattern of transcription are similar to those of Ad5 (11, 14), the

TABLE 3. *Amount of viral DNA degraded in cyt mutant (H12cyt70)-infected KB cells*[a]

Expt	Amt of viral DNA[b] (% of total radioactivity)	
	Full genome size	Smaller than genome size
Pulse of [^{3}H]thymidine (1 h)	43	0
Chase (6 h)	12	24

[a] Infected cells were labeled for 1 h at 23 h after infection and chased for 6 h. See text for method of analysis.

[b] Fraction of radioactivity hybridized to Ad12 DNA multiplied by the fraction of radioactivity in the respective peaks. (See text.)

TABLE 4. *Amount of virions produced by KB cells after infection*

Infection with:	Total cpm in virion band[a] (× 10^{-3})
Parental strain	64.6
H12*cyt*70	4.8
Parental strain plus H12*cyt*70	66.0

[a] Radioactivity in the virion band which cosedimented with [^{14}C]Ad2 marker virion by sucrose gradient sedimentation (4).

mutation responsible for DNA degradation in the *cyt* mutants may also be located in early region 1. It should be pointed out that Ad5 dl313 is DNA negative in KB cells and that Ad2*ts*111 is also DNA negative at the nonpermissive temperature, whereas *cyt* mutants are DNA positive. It is not clear whether similar gene products are involved in DNA degradation in all three serotypes.

Endonuclease has been found to be associated with virions, as well as in cells infected by different serotypes of human adenoviruses (1, 2, 9, 18). These nucleases induced double-stranded DNA breaks, giving a product of about 100 to 350 base pairs in size (12). Thus, a similar nuclease may be overproduced in *cyt* mutant-infected cells. On the other hand, the *cyt* mutants may fail to produce a factor which protects the DNA or a DNase inhibitor similar to that found in Ad5-infected cells (10).

The relationship between the ability to degrade DNA and low oncogenicity is not clear. These two functions may be closely linked but not identical since one of the *cyt* mutants reported here (H12*cyt*52) transforms rat kidney cells in culture as efficiently as the parental virus. The remaining three mutants have a much-reduced transforming capacity (I. Mak and S. Mak, manuscript in preparation).

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