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# Mapping of an Adenovirus Function Involved in the Inhibition of DNA Degradation

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A function involved in the inhibition of DNA degradation has been assigned through complementation tests to a product of region E1b of the adenovirus genome (between 4.5 and 10.5 map units). DNA degradation induced by the adenovirus type 12 (Ad12) *cyt* mutant H12*cyt*70 and the Ad5 early deletion mutant *dl*313 (with the deletion between 3.5 and 10.7 map units) was inhibited by coinfection with Ad5 region E1a (between 0 and 4.5 map units) mutants *dl*312 and *hr*1 and region E1b mutant *hr*6. The defect of inhibition of DNA degradation in Ad5 *dl*313 was also complemented in 293 cells. This DNase-inhibitory function does not appear to involve polypeptide IX or the 58,000-dalton polypeptide. Wild-type Ad12 induced DNA degradation in hamster embryo cells, suggesting that the DNase-inhibitory function is not expressed in these nonpermissive cells. Additional evidence suggests the involvement of a second viral product which positively influences the DNase activity and which appears to be an early function.

The cytocidal mutants (*cyt* mutants) of highly oncogenic adenovirus type 12 (Ad12) were shown to have a much reduced oncogenicity in newborn hamsters (41) and in general are transformation defective (41; I. Mak and S. Mak, unpublished data). These mutants were isolated on the basis that they produced large clear plaques on human embryonic kidney cells (41). We subsequently showed that infection of KB cells induced extensive degradation of both cellular and viral DNA, leading to low yields of virions (10). The *cyt* mutation has been shown to be recessive, and it has been suggested that the *cyt* mutants may fail to produce a factor which protects the DNA from degradation (10).

Revertants have been isolated from some *cyt* isolates and have been shown to regain the high tumorigenicity, plaque morphology, and lack of DNA degradation during lytic infection (10, 41, 42). The functions for cellular transformation and tumorigenicity are encoded in region E1 of adenoviruses (12, 14, 18, 34); it is possible that the function required for the protection of DNA is also encoded in this region.

Studies of the transcriptional map of Ad12 (28, 37) and those of Ad2 and Ad5 suggest that the organizations of the genomes of these serotypes are structurally similar. Furthermore, Ad12 can complement the functional defects encoded in region 1 of Ad5 and Ad2 (4, 33, 44). In this study, we used Ad5 early mutants which map in region E1 to map by complementation the *cyt* mutation of Ad12. These are the host range mutants (groups I and II) isolated by Harrison et al. (21) and the host range deletion mutants of Jones and Shenk (22). The group I mutants, represented by *hr*1, are defective in viral DNA synthesis (24) and are mapped within 1.3 to 3.7 map units (13, 15). The group II mutants, represented by *hr*6, are DNA positive (24) and are mapped within 6.1 to 8.5 map units (13, 15). The host range deletion mutants *dl*312 and *dl*313, with deletions of nucleotides 448 to 1349 and nucleotides 1334 to 3639, respectively (5, 35), are both DNA negative (22). Results showed that a function which inhibits DNA degradation maps in region E1b of Ad12 and Ad5. Furthermore, the product of this function does not appear to be polypeptide IX (2, 30) or the 53,000- to 58,000-dalton (53K to 58K) polypeptide encoded in region E1b of Ad5 (9, 20, 25, 31, 32). Additional data suggest that a second viral product which positively influences the nuclease activity is also involved.

## MATERIALS AND METHODS

**Cells and virus.** 293 cells were obtained from F. L. Graham (1, 17). The establishment and growth of hamster embryo cell cultures were as previously described (27) except that cells were grown on plastic petri dishes (Falcon Plastics; 150 mm) instead of in glass bottles.

Ad5 host range mutants *hr*1 and *hr*6 were obtained from F. L. Graham (16, 21). Ad5 deletion mutants *dl*312 and *dl*313 were obtained from T. Shenk (22). All Ad5 mutants were grown in 293 cells. Crude stocks of *cyt* mutants H12*cyt*70 and H12*cyt*61 (originally designated *cyt*129 and *cyt*135, respectively) were obtained from N. Takemori (11, 41) and grown as described

previously (10). Ad12 strain 1131 (29), Ad12 strain Huie, and Ad5 were grown in KB cells. The method for virus purification has been described previously (19), and virions purified by CsCl density gradient centrifugation were used throughout this study.

**Infection of cells.** KB cells and hamster embryo cells were infected in suspension at multiplicities of 150 to 300 and 2,000 to 5,000 virions per cell, respectively. 293 cells were infected in monolayer cultures at a multiplicity of 200 virions per cell.

**Size of DNA molecules.** The size of single-stranded DNA molecules was assayed by alkaline sucrose gradient centrifugation (10). Infected cells were labeled with [$^3$H]thymidine (7.5 μCi/μg) at 15 μCi/ml from 14 to 36 h postinfection and then washed once with phosphate-buffered saline. The cells, together with Ad5 virions containing [$^{14}$C]DNA as the 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–0.1% sodium dodecyl sulfate. The gradient was centrifuged at 24,000 rpm for 13 h at 20°C, using 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.

**Restriction endonucleases and electrophoresis.** Endonucleases *Xho*I and *Sal*I were purchased from Bethesda Research Laboratories, Inc. Viral DNA from virions and intracellular DNA from infected cells were prepared as described previously (19, 26). Fragments were separated by electrophoresis through a 1% agarose gel in buffer containing 40 mM Tris, 5 mM sodium acetate, and 1 mM EDTA (pH 7.8) for approximately 10 h at 2 V/cm. The fluorography procedure was as described previously (3, 23).

**Hybridization of labeled infected-cell DNA to immobilized viral DNA.** The hybridization of labeled DNA to DNA immobilized on nitrocellulose membrane filters was done as described by Warnaar and Cohen (43). Labeled DNA from infected cells was exhaustively hybridized to excess viral DNA immobilized onto filter disks (2 μg per filter). The hybridized radioactivity was determined by liquid scintillation counting.

## RESULTS

**DNA degradation in KB cells coinfected with an Ad12 *cyt* mutant and host range mutants.** KB cells were infected with *cyt* mutant H12*cyt*70 at a multiplicity of 150 virions per cell and then immediately superinfected with either *hr*1, *hr*6, *dl*312, or *dl*313 of Ad5 at an equal multiplicity. Infected cells were labeled with [$^3$H]thymidine (15 μCi/ml, 7.5 μCi/μg) from 14 to 36 h, and the labeled DNA from cell lysates was analyzed in alkaline sucrose gradients (Fig. 1). Infection with H12*cyt*70 alone produced a small peak of DNA cosedimenting with the marker viral DNA ($^{14}$C-labeled Ad5) and a broad peak of degraded DNA sedimenting slower (Fig. 1a) as described previously (11). Coinfections with *hr*1 and *dl*312 both resulted in a relatively large peak of DNA cosedimenting with the marker and no degraded DNA (Fig. 1b and c). Similar results were obtained in the coinfection with *hr*6 (Fig. 1d). Coinfection of KB cells with *dl*313, however, resulted in even more extensive DNA degradation than in cells infected with H12*cyt*70 alone (Fig. 1e). Therefore, *hr*1, *dl*312, and *hr*6 complemented the DNA degradation function in H12*cyt*70, whereas *dl*313 did not. This suggests that a function altered by the deletion in *dl*313, but not by the mutation in *hr*6 which maps within this deletion, appears to fall in the same complementation group as H12*cyt*70.

**DNA degradation in KB cells infected with region E1 mutants of Ad5.** KB cells were infected with each Ad5 mutant at a multiplicity of 150 virions per cell and assayed for DNA degradation. The region E1a mutants, *hr*1 and *dl*312, produced no significant peak of DNA cosedimenting with the marker and no indication of DNA degradation (Fig. 2a and b), consistent with these mutants being DNA replication deficient (22). *hr*6 produced a large peak of DNA cosedimenting with the marker and no degraded DNA (Fig. 2a), consistent with this mutant being normal in viral DNA synthesis (24). Multiplicities of infection ranging from 25 to 500 virions per cell with *hr*6 still did not produce any DNA degradation (data not shown). *dl*313, however, consistently produced extensive DNA degradation, with no significant amount of DNA cosedimenting with the marker (Fig. 2b), suggesting that DNA degradation is an early function. However, it has been reported that the deletion mutants display a multiplicity-dependent leakiness (35). It is therefore possible that viral DNA synthesis had occurred in the infected cells. To test this possibility, DNA from KB cells infected with *dl*313 at a multiplicity of 200 virions per cell and labeled with [$^3$H]thymidine at various 1-h intervals between 14 and 36 h was hybridized to excess unlabeled Ad5 DNA. No significant amount of radioactivity was found to hybridize (data not shown). *dl*313, therefore, induced extensive degradation of host DNA in the absence of viral DNA synthesis.

**Polypeptides not involved in DNA degradation.** Region E1b of Ad5 codes for several polypeptides (9, 20, 25, 31, 32). Experiments were carried out to identify the polypeptide(s) involved in DNA degradation by complementing Ad5 *dl*313 with cells or viruses that express only some of the polypeptides in region E1b. To interpret the complementation results, it is necessary to demonstrate at first that the function in *dl*313 is recessive, as it is in H12*cyt*70. Figure 3a shows the result of coinfection of KB cells with *dl*312 and *dl*313. Clearly, these two mutants complemented for viral DNA synthesis and prevented DNA degradation.

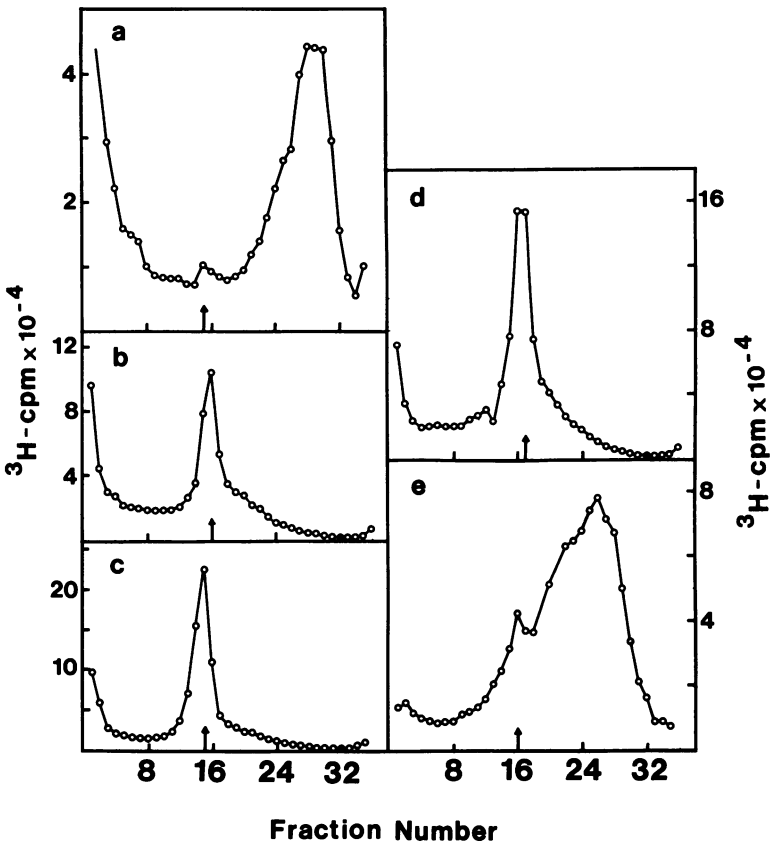


FIG. 1. Sedimentation profiles in alkaline sucrose gradients of intracellular DNA in KB cells singly infected with *cyt* mutant H12*cyt*70 and doubly infected with H12*cyt*70 and various Ad5 mutants. The multiplicity of infection of each virus was 150 virions per cell. Sedimentation is from right to left. Cells infected with: (a) H12*cyt*70, (b) H12*cyt*70 and *hr*1, (c) H12*cyt*70 and *dl*312, (d) H12*cyt*70 and *hr*6, and (e) H12*cyt*70 and *dl*313. Arrows indicate the positions of $^{14}$C-labeled Ad5 marker DNA.

It has been shown that 293 cells do not express polypeptide IX, a structural protein encoded in region E1b, even upon productive infection with *dl*313 (5, 38). Figure 3b shows the result of infection of 293 cells with *dl*313. A large peak of DNA cosedimented with the marker, and relatively little DNA sedimented slower. This suggests that polypeptide IX is not involved in the inhibition of DNA degradation.

Group II host range mutant *hr*6 fails to synthesize the 58K polypeptide encoded in region E1b (25, 32) but synthesizes viral DNA (24). KB cells were coinfected with *hr*6 and *dl*313 at a multiplicity of 150 virions per cell each and assayed for DNA degradation. DNA degradation was not observed (Fig. 3c).

This result could have been due to an inhibition or exclusion of *dl*313 by *hr*6. To determine whether complementation had occurred, we analyzed intracellular viral DNA labeled with [$^3$H]thymidine from 24 to 36 h by restriction endonuclease cleavage. The restriction maps for enzymes *Xho*I and *Sal*I are shown in Fig. 4a. Neither enzyme cuts within the left-hand 12% of the prototype Ad5 DNA where the mutations are located. These terminal fragments should differ between the mutants by the size of the deletion in *dl*313, 7% of the viral genome equivalent. Figure 4b, lanes 1 and 4, represents wild-type Ad5 DNA which should be indistinguishable from *hr*6 DNA. *Xho*I cleavage of the DNA from the coinfected cells shows both fragment C of *hr*6 and fragment C* of *dl*313 (Fig. 4b, lane 2). *Sal*I cleavage shows the presence of fragment B of wild-type Ad5 and an apparent overabundance of fragment C, which indicates the presence of fragment B* of *dl*313 which comigrated. These results demonstrate that *hr*6 and *dl*313 complemented for viral DNA synthesis and that the 58K polypeptide is not required for the prevention of DNA degradation.

**DNA degradation in infected hamster embryo**

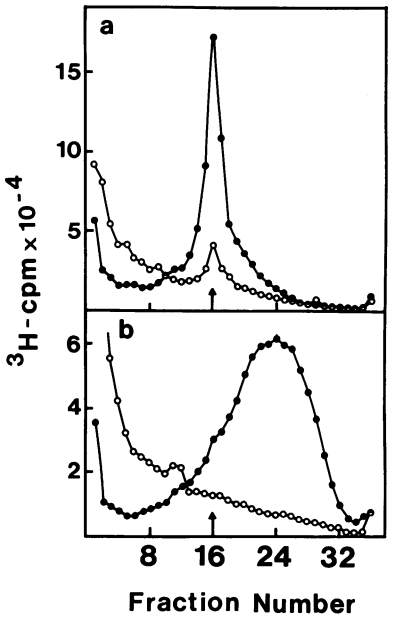


FIG. 2. Sedimentation profiles in alkaline sucrose gradients of intracellular DNA in KB cells infected with various Ad5 mutants at a multiplicity of infection of 200 virions per cell. Sedimentation is from right to left. Cells infected with: (a) *hr*1 (○) and *hr*6 (●) and (b) *dl*312 (○) and *dl*313 (●). Arrows indicate the positions of $^{14}$C-labeled Ad5 marker DNA.

**cells.** Ad5 *dl*313 induced DNA degradation in the absence of viral DNA synthesis (Fig. 2b). It was of interest to determine whether the *cyt* mutants could also induce DNA degradation in the absence of viral DNA synthesis. Hamster cells are nonpermissive for Ad12, and viral DNA is not synthesized in the infected cells (7, 8, 36). These cells are, however, permissive for Ad5.

Primary hamster embryo cells were infected with *cyt* mutants H12*cyt*70 and H12*cyt*61 and Ad12 at a multiplicity of 5,000 virions per cell and with Ad5 and *dl*313 at a multiplicity of 2,000 virions per cell and assayed for DNA degradation.

Ad5 produced a sharp peak of DNA cosedimenting with the marker and no apparent DNA degradation (Fig. 5a), whereas *dl*313 produced a broad peak of degraded DNA and no intact viral DNA (Fig. 5b). These results are similar to that produced in KB cells.

H12*cyt*70, H12*cyt*61, and wild-type Ad12 produced no significant amount of DNA cosedimenting with the marker, but all produced a large peak of smaller DNA fragments (Fig. 5c, d, and e), similar to those produced in *dl*313-infected hamster cells (Fig. 5b). This indicates that both the *cyt* mutants and the wild-type Ad12 induced DNA degradation in hamster cells.

**DNA degradation in KB cells induced by H12*cyt*61.** Mutant H12*cyt*61 is an atypical *cyt* mutant in that it transforms rat embryo cells in vitro as efficiently as does the parental virus Ad12 (Mak and Mak, unpublished data). This unique mutant was therefore further studied in complementation experiments similar to those done with H12*cyt*70 to determine whether there were any differences concerning the function of virus-induced DNA degradation.

KB cells were first infected with H12*cyt*61 at a multiplicity of 150 virions per cell and then immediately superinfected with the parental vi-

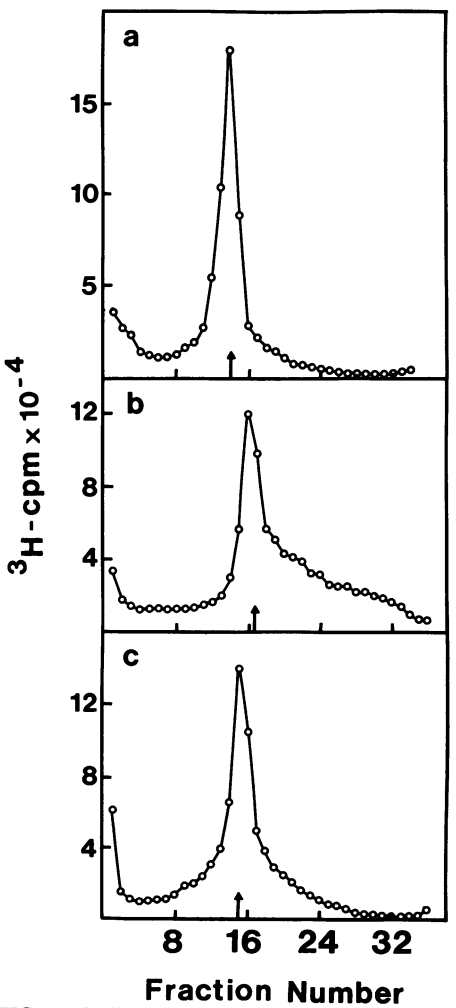


FIG. 3. Sedimentation profiles in alkaline sucrose gradients of intracellular DNA in (a) KB cells coinfected with mutants *dl*313 and *dl*312, (b) 293 cells infected with *dl*313, and (c) KB cells coinfected with *dl*313 and *hr*6. The multiplicity of infection of each virus was 200 virions per cell. Sedimentation is from right to left. Arrows indicate the positions of $^{14}$C-labeled Ad5 marker DNA.

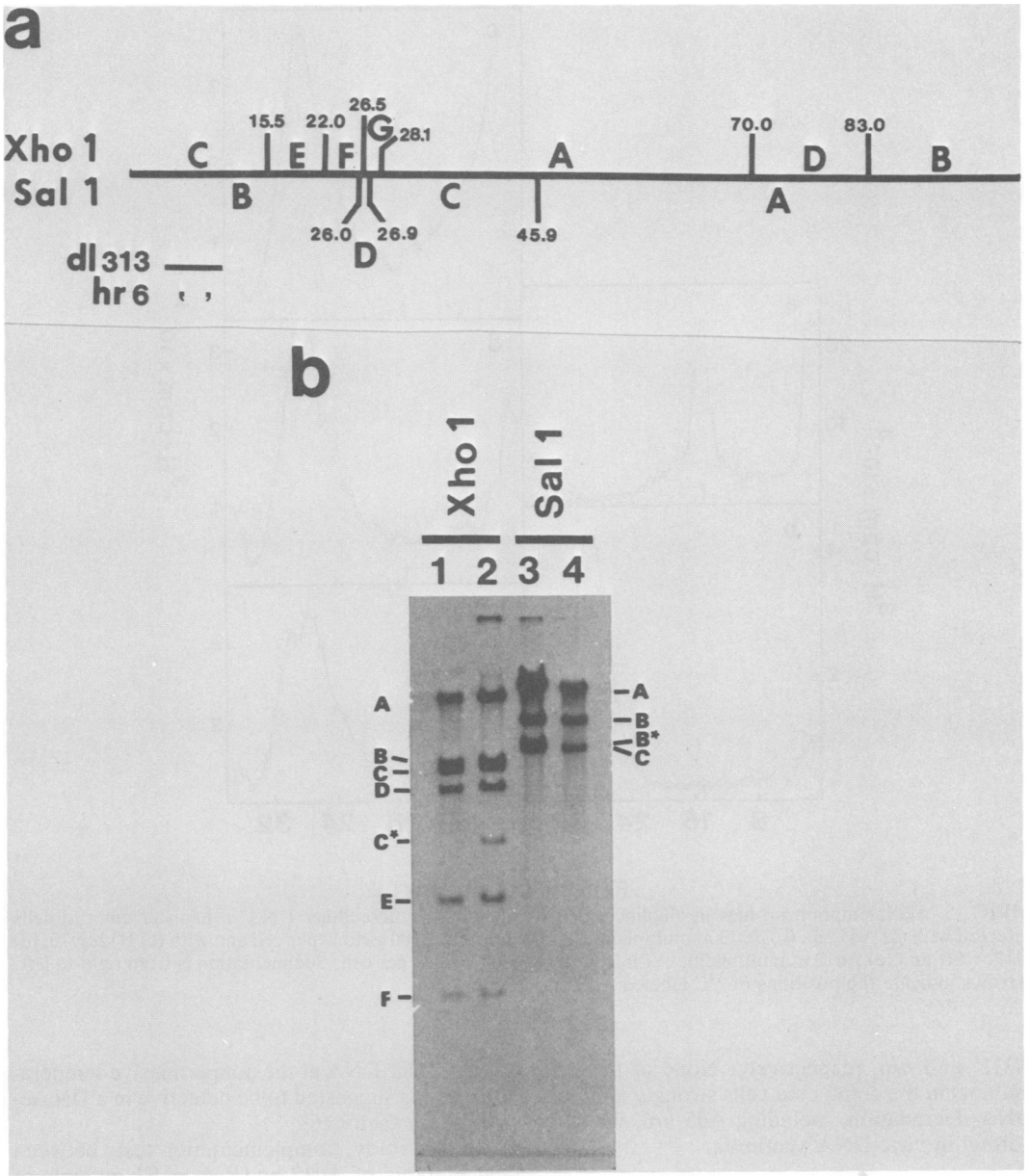


FIG. 4. (a) Restriction map of Ad5 DNA for endonucleases *Xho*I and *Sal*I, showing locations of deleted sequences in *dl*313 and the mutation in *hr*6 in region E1 of the genome. (b) Fluorogram of restricted DNA from Ad5 virions (lanes 1 and 4) and from KB cells coinfected with mutants *hr*6 and *dl*313 each at a multiplicity of infection of 200 virions per cell (lanes 2 and 3). Infected cells were labeled with [$^3$H]thymidine (15 μCi/ml, 7.5 μCi/μg) from 24 to 36 h postinfection. Asterisks denote fragments of *dl*313 DNA reduced in size by the deletion.

rus Ad12 (strain 1131) or with wild-type Ad12 (strain Huie) at an equal multiplicity. Superinfection with strain 1131 did not inhibit DNA degradation (Fig. 6b). Similar results were obtained with strain Huie (data not shown). To investigate the possibility that superinfection by the wild-type viruses was being interfered with by the mutant, the reverse order of infections was also done. Similar results (data not shown) were obtained. This suggests that the mutation in H12*cyt*61 is dominant.

Coinfection of KB cells with H12*cyt*61 and mutants of Ad5 was also examined. Figure 6c, d, and e shows the results of coinfection with *hr*1,

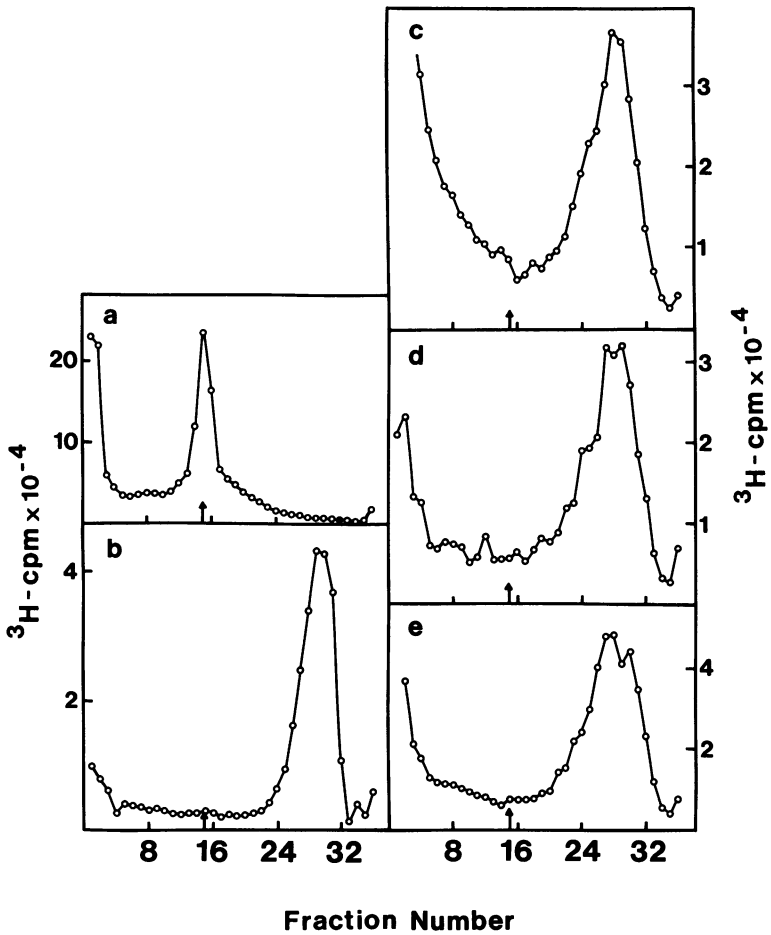


FIG. 5. Sedimentation profiles in alkaline sucrose gradients of intracellular DNA in hamster embryo cells infected with (a) Ad5 and (b) *dl*313 at multiplicities of infection of 2,000 virions per cell and with (c) H12*cyt*70, (d) H12*cyt*61, and (e) Ad12 at multiplicities of infection of 5,000 virions per cell. Sedimentation is from right to left. Arrows indicate the positions of $^{14}$C-labeled Ad5 marker DNA.

*dl*312, and *hr*6, respectively. None of the Ad5 mutants in the coinfected cells strongly inhibited DNA degradation, including Ad5 *hr*6, which is normal in viral DNA synthesis.

## DISCUSSION

We reported earlier that infection of KB cells by *cyt* mutants of Ad12 leads to extensive DNA degradation (10). The result of the *cyt* mutation may be (i) the overproduction of an endonuclease, (ii) the failure to produce a DNase inhibitor, or (iii) the failure to produce a factor that protects the DNA from degradation. Coinfection of cells by a *cyt* mutant and wild-type Ad12 can prevent the degradation of DNA (10), suggesting that a diffusible substance can control the production of or inhibit the action of the DNase. The Ad2 temperature-sensitive early mutant H2*ts*111 also induces the degradation of viral and cellular DNA at the nonpermissive temperature and is suggested to be defective in a DNase-inhibitory factor (6).

In this study, complementation tests between *cyt* mutants of Ad12 and region E1 mutants of Ad5 were employed in mapping a viral function involved in the prevention of degradation of intracellular DNA. Region E1a mutants of Ad5 did not induce DNA degradation in single infection of KB cells and were able to complement this function in H12*cyt*70. *dl*313, on the other hand, failed to complement H12*cyt*70 and in single infection of KB cells induced extensive degradation of host DNA. These results strongly suggest that both Ad12 and Ad5 encode functionally related DNase-inhibitory factors in region E1b of the viral genome.

It is unlikely that the deficiency in the DNase-inhibitory factor in *dl*313 is a secondary effect of

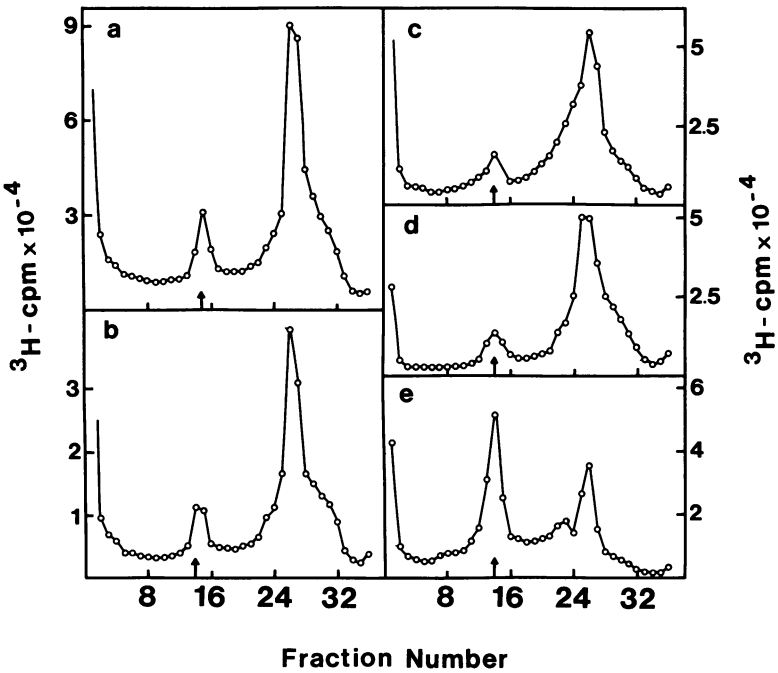


Fig. 6. Sedimentation profiles in alkaline sucrose gradients of intracellular DNA in KB cells singly infected with *cyt* mutant H12*cyt*61 and doubly infected with H12*cyt*61 and various wild-type strains of Ad12 and mutants of Ad5. The multiplicity of infection of each virus was 150 virions per cell. Sedimentation is from right to left. Cells infected with: (a) H12*cyt*61, (b) H12*cyt*61 and Ad12 strain 1131, (c) H12*cyt*61 and *hr*1, (d) H12*cyt*61 and *dl*312, and (e) H12*cyt*61 and *hr*6. Arrows indicate the positions of $^{14}$C-labeled Ad5 marker DNA.

the deletion in region E1b, since *dl*313 appears to express normal or increased amounts of proteins encoded by all early regions except region E1 of the viral genome (32). The failure of H12*cyt*70 and Ad5*dl*313 to complement each other in the prevention of DNA degradation is not due to incompatibility of the E1b products, since wild-type Ad12 has been shown to complement the replication of Ad5 *dl*313 and group II host range mutants (33, 44). Thus, the defect in the *cyt* mutant must lie in region E1b of Ad12.

At least three polypeptides have been identified as being encoded in region E1b of Ad5: polypeptide IX, an 18 to 19K polypeptide, and a 53 to 58K polypeptide (2, 9, 20, 25, 31, 32). Ad5 *dl*313 did not induce DNA degradation in 293 cells which do not express polypeptide IX from the integrated viral sequences, even during productive infection with *dl*313 (5, 38). *hr*6 did not induce DNA degradation and was able to complement *dl*313 in this function in KB cells. *hr*6, a group II mutant, is clearly defective in the synthesis of at least the 58K polypeptide (25, 32). Thus, these two polypeptides are not required for the inhibition of DNA degradation. This leaves the 19K polypeptides and any other polypeptides encoded in region E1b as candidates for the DNase-inhibitory factor. It is of interest that a preliminary analysis by immunoprecipitation with antitumor sera of polypeptides induced in KB cells infected with either H12*cyt*62 or H12*cyt*68 indicates that these two *cyt* mutants fail to induce a 19K polypeptide found upon infection of KB cells by wild-type Ad12 (H. Galet, unpublished data).

Mutant H12*cyt*61 was dominant in inducing DNA degradation. This mutant is also atypical compared with other *cyt* mutants in having wild-type transforming capability (Mak and Mak, unpublished data). Several points can be deduced from these results. The mutation in H12*cyt*61 probably lies outside the transforming region, a location different from that of H12*cyt*70 and *dl*313. The dominant characteristic indicates that the endonuclease or its inducer is viral encoded. It appears that the mutation in H12*cyt*61 prevents the normal E1b factor from controlling the expression or activity of this endonuclease or its inducer.

The observation that wild-type Ad12 induced the degradation of DNA in hamster embryo cells is in keeping with earlier reports that Ad12 induces chromosomal aberrations in hamster cells (39, 40, 46, 47). In a study in which the

ability to induce chromosomal aberrations between wild-type Ad12 and *cyt* mutants was compared, no significant differences were found (39). Inactivation of virus by UV irradiation suggests also that adenovirus-induced chromosomal aberrations are probably due to the action of an enzyme controlled by viral genes (45). The most direct explanation for these observations, based on the results of this study, is that the DNase-inhibitory factor in region E1b of Ad12 is not functionally expressed in nonpermissive hamster cells. In support of this view, it has been reported that Ad12 complements the growth of the group II host range mutants of *Ad5* in HeLa cells but not in the hamster cell line BHK-21 (33). In addition, hybridization of cytoplasmic RNA from Ad12-infected cells to *Eco*RI fragment C (left 16% of the genome) showed that approximately 30% of the sequences expressed in productively infected KB cells are not expressed in abortively infected BHK-21 cells (28).

The induction of DNA degradation by *dl*313 in KB cells and by *Ad*12 in hamster embryo cell demonstrates that viral DNA synthesis is not required for its occurrence. This implies that the viral product which affects the nuclease activity is an early viral function. The possibility remains, however, that this product, under the control of the region E1b inhibitory factor, is normally expressed late. A defective inhibitory factor could result in its early expression.

## ACKNOWLEDGMENTS

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