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cyt Gene of Adenoviruses 2 and 5 Is an Oncogene for Transforming Function in Early Region E1B and Encodes the E1B 19,000-Molecular-Weight Polypeptide

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A total of 59 cytocidal (*cyt*) mutants were isolated from adenovirus 2 (Ad2) and Ad5. In contrast to the small plaques and adenovirus type of cytopathic effects produced by wild-type *cyt*$^+$ viruses, the *cyt* mutants produced large plaques, and the cytopathic effect was characterized by marked cellular destruction. *cyt* mutants were transformation defective in established rat 3Y1 cells. *cyt*$^+$ revertants and *cyt*$^+$ intragenic recombinants recovered fully the transforming ability of wild-type viruses. Thus, the *cyt* gene is an oncogene responsible for the transforming function of Ad2 and Ad5. Genetic mapping in which we used three Ad5 deletion mutants (*dl*312, *dl*313, and *dl*314) as reference deletions located the *cyt* gene between the 3′ ends of the *dl*314 deletion (nucleotide 1,679) and the *dl*313 deletion (nucleotide 3,625) in region E1B. Restriction endonuclease mapping of these recombinants suggested that the *cyt* gene encodes the region E1B 19,000-molecular-weight (175R) polypeptide (nucleotides 1,711 to 2,236). This was confirmed by DNA sequencing of eight different *cyt* mutants. One of these mutants has a single missense mutant, two mutants have double missense mutations, and five mutants have nonsense mutations. Except for one mutant, these point mutations are not located in any other known region E1B gene. We conclude that the *cyt* gene codes for the E1B 19,000-molecular-weight (175R) polypeptide, that this polypeptide is required for morphological transformation of rat 3Y1 cells, and that simple amino acid substitutions in the protein can be sufficient to produce the *cyt* phenotype.

Takemori and colleagues isolated and characterized the first transformation-defective adenovirus mutants, the cytocidal (*cyt*) mutants of adenovirus 12 (Ad12) (57–59). Wild-type (wt) *cyt*$^+$ viruses produce small plaques, whereas *cyt* mutants produce clear large plaques. In addition, the cytopathic effect (CPE) produced by *cyt* mutants is characterized by extensive cellular destruction. This is remarkable because the adenovirus type of CPE produced by all types of adenoviruses (i.e., rounding and agglutination without lysis of infected cells) has been recognized as being essentially pathognomonic (20, 21). Apparently, the adenovirus type of CPE is due, at least in part, to the function of the viral *cyt* gene, and mutations in this gene result in the alteration of the adenovirus type of CPE (58, 59). Therefore, it is logical to assume that *cyt* mutations occur in all other adenovirus types also. Ad12 *cyt* mutants are of particular interest because they are much less tumorigenic in newborn hamsters than wt *cyt*$^+$ viruses and are also less effective in transformation of cultured cells. Studies on complementation in the production of *cyt*$^+$ CPE and tumorigenicity have indicated that there is only one *cyt* gene and that this gene is essential both for the production of *cyt*$^+$ CPE and for tumorigenicity (57–59). Similar Ad12 mutants (*lt*) have been isolated (63) and have been shown to be both low tumorigenic and defective in inducing cell surface changes which might be necessary for cell transformation.

Several lines of evidence indicate that the adenoviral genes that mediate cellular transformation are located within the left-hand 12% of the adenovirus genome. The region required for transformation corresponds to one of the viral gene blocks expressed early after infection (i.e., region E1; 1.5 to 11.5 map units). This is subdivided into two parts, region E1A (1.5 to 4.5 map units) and region E1B (4.5 to 11.5 map units). Both of these regions encode products required for the productive growth of the virus and for the transformation of rat embryo cells. Ad2 left-end sequences are consistently present and transcribed in transformed rat cells (32, 60).

Accumulating evidence shows that separate establishment (region E1A) and transforming (region E1B) functions are required for oncogenic transformation of primary cells in culture by Ad2 and Ad5 (51, 62). We report here on the isolation of a number of Ad2 and Ad5 *cyt* mutants, on their transforming ability, on *cyt*$^+$ reverse mutation, and on intragenic recombination. In addition, extensive attempts were made to locate the sites of *cyt* mutations by deletion mapping (4, 5), using Ad5 deletion mutants (i.e., *dl*312, *dl*313, and *dl*314) (32) as reference deletions. The *cyt* gene was thus shown to be a transforming oncogene in region E1B encoding the 19,000-molecular-weight (19K) (175R) polypeptide (1). Sequencing studies of the cloned *cyt* mutant DNAs corroborated this conclusion.

MATERIALS AND METHODS

Cells and viruses. The human embryonic kidney (HEK) cells, KB cells, and 293 cells (24) used were maintained in Dulbecco minimal essential medium containing 10% calf or fetal calf serum and were used for plaque assays as described previously (58).

(i) Plaque virus stocks. Trypsinized KB cells (15×10^6 to 20×10^6 cells) in 50 ml of maintenance medium (Dulbecco minimal essential medium containing 2.5% fetal calf serum) were infected with recloned viruses at a multiplicity of infection (MOI) of approximately 10. After 2 days the infected cells were pelleted, suspended in 5 ml of the supernatant, freeze-thawed five times, treated once with

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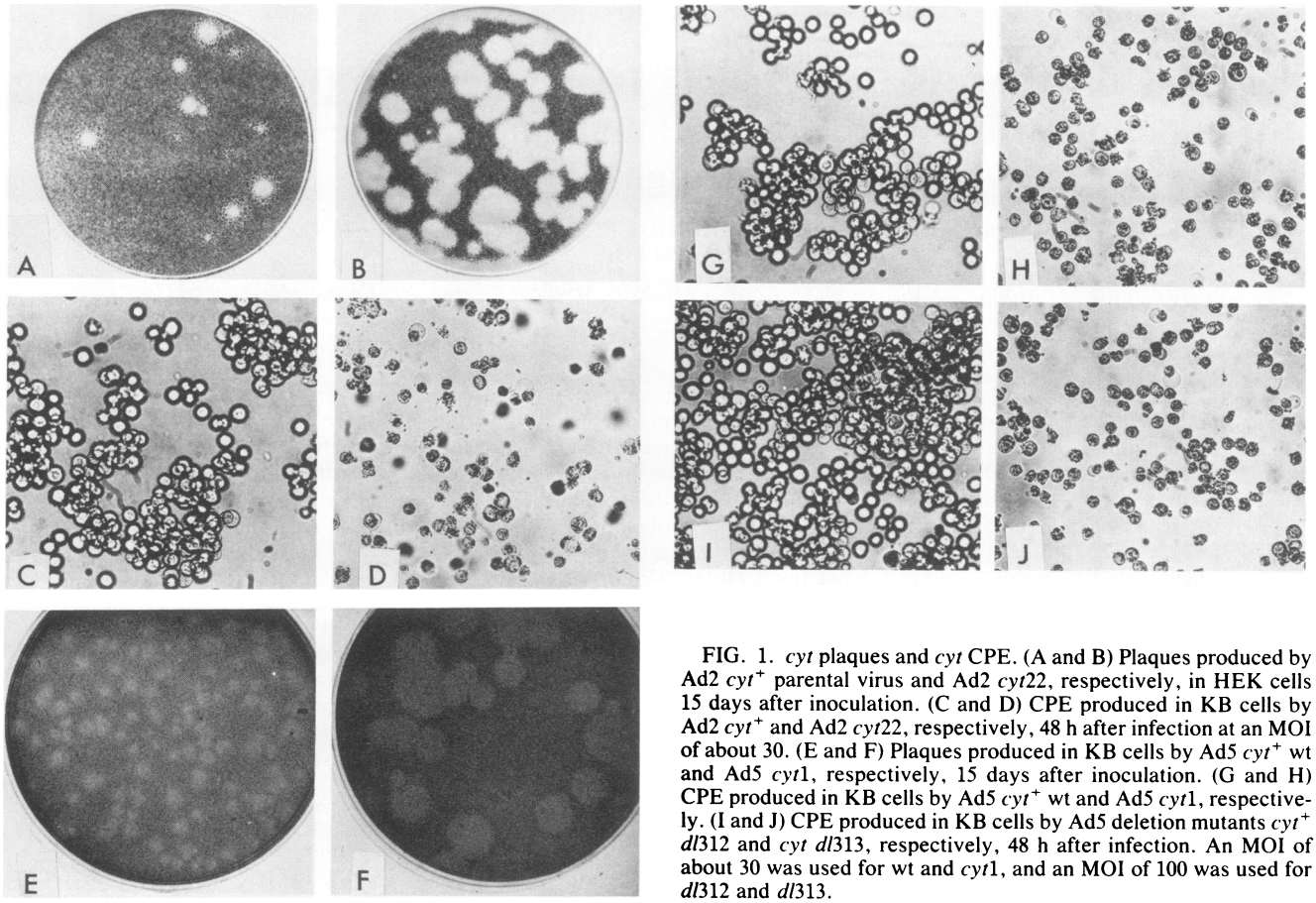

FIG. 1. *cyt* plaques and *cyt* CPE. (A and B) Plaques produced by Ad2 cyt^+ parental virus and Ad2 *cyt*22, respectively, in HEK cells 15 days after inoculation. (C and D) CPE produced in KB cells by Ad2 cyt^+ and Ad2 *cyt*22, respectively, 48 h after infection at an MOI of about 30. (E and F) Plaques produced in KB cells by Ad5 cyt^+ wt and Ad5 *cyt*1, respectively, 15 days after inoculation. (G and H) CPE produced in KB cells by Ad5 cyt^+ wt and Ad5 *cyt*1, respectively. (I and J) CPE produced in KB cells by Ad5 deletion mutants cyt^+ *dl*312 and *cyt dl*313, respectively, 48 h after infection. An MOI of about 30 was used for wt and *cyt*1, and an MOI of 100 was used for *dl*312 and *dl*313.

fluorocarbon, and centrifuged. Glycerol was added to the supernatants to a concentration of 30%, and the stocks were stored at −20°C. Virus stocks of a few Ad2 *cyt* mutants which do not propagate well in KB cells (e.g., *cyt*26, *cyt*30, and *cyt*5) were prepared in 293 cells or HEK cells. Ad5 deletion mutants *dl*312, *dl*313, and *dl*314 (provided by T. Shenk) were recloned and grown into stocks in 293 cells.

(ii) Tests for cyt^+ or cyt^- CPE in KB cells. A total of 10^6 trypsinized KB cells in 4 ml of maintenance medium in tissue culture tubes (1.5 by 12 cm) were inoculated either with plaque virus isolates or other virus samples. The appearance of either the adenovirus type of cyt^+ CPE or *cyt* mutant CPE was examined daily under a microscope. The results were always unequivocal, even in the microplaques produced by plaque virus inocula (Fig. 1).

UV irradiation. Virus stocks diluted in phosphate-buffered saline (PBS) in 60-mm dishes were irradiated with a General Electric 15-W germicidal bulb at a distance of 15 cm for 3 to 4 min for mutagenesis. This yielded, on the average, a 4- to 5-log reduction in virus stock titer. For some transformation assays the virus stocks were diluted in PBS so that the desired number of plaque-forming units was contained in 0.1 to 0.2 ml of the inoculum, and these preparations were irradiated for 2 to 3 min. To obtain a mutant virus with increased transforming ability, Ad2 wt virus (parent of group A mutants) was UV irradiated, and the survivors were grown in HEK cells. The process was repeated, and one cloned virus was selected (parent of group B mutants). After the group B stock was UV irradiated three more times, the cyt^+ parental virus of group C was isolated. No cyt^+ virus with increased transforming ability was isolated.

Transformation assays. Rat 3Y1 cells (2×10^4 to 4×10^4 cells per culture) (33) were infected 1 to 2 days after planting with 5 ml of Dulbecco minimal essential medium containing 10% fetal calf serum and virus at a multiplicity of 40 PFU/cell without removing the medium. Then 1 to 2 days after infection the cultures were fed with Dulbecco minimal essential medium containing 0.2 mM $CaCl_2$ and 7% fetal calf serum, and they were refed with this low-calcium medium twice a week. Counts of foci of transformed cells were made at 5 to 6 weeks.

Isolation of revertants and recombinants. (i) Serial passage method. The serial passage method used has been described previously for the isolation of both Ad12 cyt^+ revertants and Ad12 intragenic cyt^+ recombinants (57, 59). KB cells (10^6 cells in 4 ml of maintenance medium) were infected either with a large *cyt* viral inoculum for reversion or with a mixture of two *cyt* mutants (approximately 100 PFU of each mutant per cell) for intragenic recombination, and serial undiluted passages were continued together with single-infection controls. At each passage the cultures were examined daily for the appearance of cyt^+ CPE, indicating the accumulation of cyt^+ revertants or intragenic recombinants. The cyt^+ revertants or recombinants were isolated by plating onto KB cell plates and isolating the presumptive cyt^+ plaques into KB cells to confirm the cyt^+ phenotype. After

further recloning on KB cell plates, the cyt^+ virus was grown into stock.

(ii) Infectious center method. The infectious center method (7, 27, 40) was used for the isolation of both Ad2 cyt^+ intragenic recombinants and Ad5 interserotypic cyt^+ recombinants between Ad5 deletion mutants (*dl*312, *dl*313, and *dl*314) and Ad2 *cyt* mutants. A total of (10^6) KB cells in 1 ml of maintenance medium were infected with two viruses at a MOI of approximately 100 PFU of each virus per cell. Single-infection controls were also included. After 20 min at room temperature, 4 ml of PBS was added. The infected cells were pelleted and suspended in a dilution of Ad2 antiserum to eliminate extracellular Ad2 *cyt* virus. Then 50 to 100 infected cells were plated onto KB cell monolayers in 60-mm dishes, and after incubation for 1 to 2 h an agar overlay was added. The infectious center plaques produced after 15 to 20 days of incubation were isolated in a KB cell suspension (10^6 cells per 4 ml of maintenance medium). In many cases two to five undiluted serial passages were made before the appearance of cyt^+ CPE, ensuring enough accumulation of cyt^+ viruses for isolation by plating onto KB cell plates. Presumptive Ad2 cyt^+ intragenic recombinant plaques were isolated in KB cell suspensions. For the isolation of interserotypic Ad5 cyt^+ recombinants, the cyt^+ passage virus was neutralized with anti-Ad2 serum to eliminate the majority of Ad2 *cyt* mutants and was plated onto KB cell plates. Ad5 deletion mutants do not produce plaques on KB cell plates. The presumptive Ad5 cyt^+ recombinant plaques were isolated in KB cell suspensions. In both cases, after the cyt^+ phenotype was confirmed by producing cyt^+ CPE in KB cells, the recombinants were recloned on KB cell plates and grown into virus stock.

Restriction endonuclease mapping of recombinants. The crossover sites in recombinants isolated from crosses between Ad2 *cyt* mutants and Ad5 deletion mutants were mapped by restriction endonuclease analysis of viral DNAs (52). ^{32}P-labeled viral DNA was extracted from Hirt supernatants (12), digested with *Eco*RI, *Bam*HI, or *Bgl*II, electrophoresed on 0.8% agarose gels, and autoradiographed after gel drying. In order to analyze the *Nar*I and *Xmn*I sites in region E1, the ^{32}P-labeled *Bgl*II fragment (map units 0 to 9.1) was purified on a 0.8% agarose gel. The fragment was electroeluted, purified on a small DEAE column, and analyzed for *Nar*I or *Xmn*I sites.

DNA sequencing of *cyt* mutants. Standard procedures were used for virus preparation and viral DNA extraction (25). *cyt*6, *cyt*15, and *cyt*7 seed stocks were inoculated into KB cell monolayers ($\sim 10^7$ cells). At 40 h postinfection, the cells were harvested and lysed by repeated freezing and thawing. Two-thirds of the lysed cells were used to inoculate 3 liters of KB cells in suspension. The cells were harvested 40 h postinfection, and the virus was purified by banding twice in CsCl gradients. Viral DNA was extracted, and the *Sma*I fragment (map units 2.76 to 10.77) was cloned into pUC12 (41) by using *Escherichia coli* HB101 as the host. Genomic DNA from *cyt*4, *cyt*5, *cyt*22, *cyt*24, and *cyt*37 was prepared from Hirt supernatants. The *Eco*RI A fragment was purified by agarose gel electrophoresis. This was cleaved with *Sma*I, and the *Sma*I fragment (map units 2.76 to 10.77) was cloned into pUC12. The plasmid clones were 5'-labeled at the unique *Bst*EII site (nucleotide 1,912) in the middle of the E1B 19K gene, and the *Bst*EII-*Xba*I (nucleotides 1,912 to 1,336) and *Bst*EII-*Hin*dIII (nucleotides 1,912 to 2,798) fragments were sequenced by the procedure of Maxam and Gilbert (39).

RESULTS

Isolation of *cyt* mutants of Ad2 and Ad5. Parental virus stocks were diluted to concentrations of 10^9 to 10^{10} PFU/ml in PBS and were UV irradiated to produce a 4- to 5-log reduction in virus stock titer. The survivors were plated on either HEK or human KB cells. Putative mutant plaques were purified by replating. *cyt* mutants produced large, clear, sharp-edged plaques (Fig. 1B and F). All cells in the plaques were dead. In contrast, a few surviving cells could usually be seen in the small, fuzzy-edged plaques of cyt^+ viruses (Fig. 1A and E).

The CPE produced by the isolates was tested by infecting either HEK or KB cells. Parental cyt^+ viruses produced the adenovirus type of CPE (i.e., marked rounding and aggregation of infected cells without cell lysis) (Fig. 1C and G) (20, 21). In contrast, *cyt* mutants produced unequivocal *cyt* CPE characterized by extensive cellular destruction (Fig. 1D and H). *cyt* CPE was not quite evident in 293 cells (HEK cells transformed by sheared Ad5 DNA that retain and express E1) (24), perhaps because of the transformed state of the cells. Thus, *cyt* mutants of Ad2 and Ad5 are similar to the mutants of Ad12 described previously (58, 59). Direct plating of irradiated virus ensured that the mutants were of independent origin. *cyt* mutants were isolated at a mutation frequency of approximately 10^{-4}.

As described above, two additional cyt^+ viruses were isolated from the original wt stock and were used to generate additional *cyt* mutants. Thus, *cyt* mutants were isolated in the following four groups, with each group derived from a different parental virus: Ad2 group A, consisting of 10 *cyt* mutants (*cyt*1 through *cyt*10) and 9 *cyt* mutants (*cyt*11 through *cyt*19) that were isolated from wt stock (1446) on HEK and KB cells, respectively; Ad2 group B, consisting of 12 mutants (*cyt*22 through *cyt*33) that were isolated on HEK plates from another cyt^+ clonal stock (1433); Ad2 group C, consisting of 21 mutants (*cyt*34 through *cyt*54) that were isolated on KB plates from a third cyt^+ clonal stock (2854); and Ad5 *cyt* mutant group, consisting of 7 isolates (*cyt*1 through *cyt*7) that were obtained on KB plates from wt Ad5 stock (32).

Some mutants (*cyt*3, *cyt*4, and *cyt*5) isolated from HEK plates propagate poorly in KB cells. Virus stocks of these mutants prepared in KB cells have titers that are 10^2- to 10^3-fold less than the titers of other *cyt* isolates and cyt^+ parental viruses, which usually have titers of 10^{10} to 10^{11} PFU/ml. *cyt*23 and especially *cyt*26 produce *cyt* CPE but fail to propagate in KB cells, although they grow in HEK and 293 cells. These mutants are similar to *cyt kb* mutants of Ad12 (59).

Ad5 host range (*hr*) deletion mutants isolated by Jones and Shenk (32) were tested for CPE in KB cells. Mutants *dl*312 and *dl*314 produced cyt^+ CPE (Fig. 1I). In contrast, 10 independent clonal stocks of *dl*313 produced *cyt* CPE (Fig. 1J). Thus, Ad2 *cyt*26 and Ad5 *dl*313 have similar phenotypes; they produce *cyt* CPE and do not propagate in KB cells, but grow in HEK cells or 293 cells or both.

Transforming ability of *cyt* mutants. The transforming ability of *cyt* mutants, as defined by induction of focus formation in established rat 3Y1 cells, was investigated. Representative results are shown in Table 1. All *cyt* mutants were defective for transformation. Parental cyt^+ viruses induced dense complete foci. In contrast, the few cells transformed by *cyt* mutants usually produced thin incomplete foci. UV-irradiated viruses were also used for transfor-

TABLE 1. Transformation of rat 3Y1 cells with *cyt* mutants

Virus	No. of foci[a]
Ad2 group A	
wt *cyt*$^+$	70
*cyt*1	(1)
*cyt*3	0
*cyt*4	(1)
*cyt*6	0
*cyt*12	(1)
*cyt*15	0
Ad2 group B	
Parental virus *cyt*$^+$	97
Parental virus UV(800)[b]	522
*cyt*22	0
*cyt*22 UV(800)[b]	(2)
*cyt*23	(3)
*cyt*23 UV(800)[b]	(1)
*cyt*24	(4)
*cyt*24 UV(800)[b]	(2)
*cyt*27	(1)
*cyt*33	(3)
Ad2 group C	
Parental virus *cyt*$^+$	66
*cyt*36	0
*cyt*37	0
*cyt*38	0
*cyt*40	0
*cyt*42	0
Ad5	
wt *cyt*$^+$	131
*cyt*1	(2)
*cyt*3	(2)
*cyt*4	(5)
*cyt*5	(2)
*cyt*6	(1)
*cyt*7	(4)
*dl*313	0
*dl*313 UV(160)[b]	0

[a] Number of foci per approximately 4 × 10^4 rat 3Y1 cells infected at a multiplicity of 40 PFU/cell without removing the media and counted 4 to 6 weeks postinfection. Cells transformed with *cyt* mutants usually did not form dense foci. The numbers in parentheses are numbers of incomplete foci of this type.

[b] An appropriate dilution of virus in PBS was UV irradiated for 3 min at a distance of 15 cm (see text). Rat 3Y1 cells (4 × 10^4 cells) were infected at a multiplicity of 800 PFU/cell (before irradiation) without removing the media. For *dl*313, a multiplicity of 160 PFU/cell was used.

mation with the same results; these viruses could be used at higher multiplicities without producing cytotoxicity and were therefore suitable for qualitative tests. The transforming phenotypes of the *cyt*$^+$ and *cyt* viruses are designated Tra$^+$ and Tra$^-$, respectively. The lack of transformation by *cyt* mutants is not the result of extensive cell killing, because rat 3Y1 cell monolayers are not grossly affected by the mutant viruses. Note that the transformation assays were done at an MOI of 40 PFU/cell, so that extensive cell killing would have been obvious.

The group C parental *cyt*$^+$ virus had a tendency (sometimes irregular) to produce morphological transformation distinct from that of wt *cyt*$^+$ virus (Fig. 2A). Besides producing foci similar to wt foci, many highly refractile elongated structures were also seen. This is designated aberrant transformation (Fig. 2B). The group C *cyt*$^+$ virus produced plaques with a red halo on KB plates after long incubation. The red halo was completely absent in all *cyt* mutants and was at best minimal in Ad2 wt *cyt*$^+$, group B *cyt*$^+$, and Ad5 wt *cyt*$^+$ viruses. Red plaque mutants have also been described for simian virus 40 (49). It may be hypothesized that both aberrant transformation and red halo phenotypes are due to a mutation in the *cyt* gene, and both phenotypes are abolished in the *cyt* mutation.

The Ad5 deletion mutant *dl*313 has a *cyt* Tra$^-$ phenotype (Table 1). *dl*312 and *dl*314 are *cyt*$^+$, but are Tra$^-$ in rat 3Y1 cells (data not shown) (32). Experiments were done to investigate whether different *cyt* mutants would complement in the transformation of rat 3Y1 cells. UV-irradiated viruses were used at an MOI of 160 or 400 PFU/cell. No complementation was found in a number of mixed infections of two *cyt* mutants. However, limited complementation (~40 to 50%) was regularly obtained between *dl*312 and 2 of 10 *cyt* mutants tested (*cyt*1 and *cyt*15). However, there was no complementation between these 10 *cyt* mutants and *dl*313 or *dl*314 (data not shown).

Revertants of *cyt* mutants. Although the majority of *cyt* mutants are stable, a few did revert spontaneously after less than 10 serial passages in KB cells. One such mutant was *cyt*5, which was originally isolated in HEK cells. The titers of *cyt*5 stocks prepared in KB cells were at least 100-fold less than the titer of wt virus. Therefore, *cyt*$^+$ revertants have selective advantage in KB cell passages. Accordingly, 10 independent clonal stocks of *cyt*5 grown in HEK cells were used for serial passages in KB cells to isolate independent *cyt*$^+$ revertants. In 9 of 10 cases, *cyt*$^+$ CPE appeared in the seventh passage, and in one case it was seen after 10 passages. *cyt*$^+$ revertants were isolated from the 12th passage. After plaque purification, all revertants showed the wt phenotype for plaque morphology, CPE, and growth in KB cells. Eight of the revertants tested were also Tra$^+$ (Table 2). In similar experiments, *cyt*$^+$ Tra$^+$ revertants were also recovered from *cyt*3 (data not shown). These experiments suggest that the *cyt* and Tra$^-$ phenotypes in *cyt*5 are the consequence of a single lesion, probably a point mutation, in the *cyt* gene.

We also investigated whether *cyt*$^+$ Tra$^+$ revertants could be obtained from a *cyt*$^+$ mutant which is not readily revertible by continuing further the serial passages in KB cells. Nine independent clonal stocks of *cyt*6 were propagated serially in KB cells. *cyt*$^+$ CPE appeared after 13 to 27 passages, and in all cases wt-like *cyt*$^+$ revertants were isolated from passage 30. In contrast to *cyt*$^+$ revertants of *cyt*5, none of the *cyt*$^+$ revertants of *cyt*6 recovered fully the Tra$^+$ phenotype in rat 3Y1 cells, although some revertants

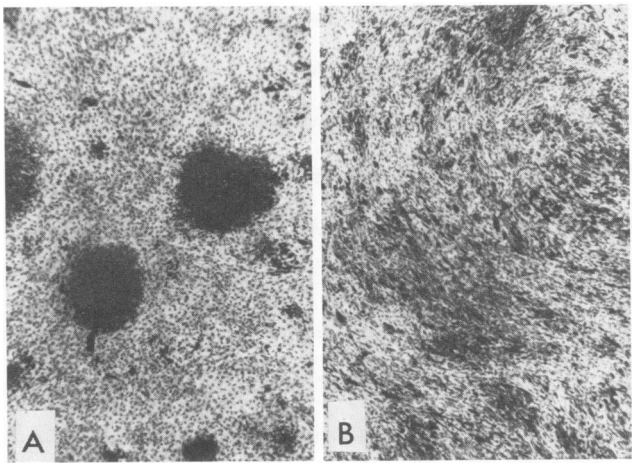

FIG. 2. Transformation of rat 3Y1 cells with wt Ad2 (A) and aberrant transformation with *cyt*$^+$ recombinant 2296 (B). Recombinant 2296 was isolated from a cross between *cyt*26 and *cyt*23.

produced incomplete transformation (Table 2). Two UV-irradiated revertants, revertants 3590 and 3596, produced aberrant transformation when they were tested in Wistar rat embryo cells (~10 passages). These findings were not pursued further.

It may be noted here that *cyt*26, which was isolated and propagated in HEK cells, did not propagate serially in KB cells in repeated tests, although the *cyt* CPE was produced in the initial infection. When *cyt*26 stock prepared in 293 cells was used for serial propagation without dilution in KB cells, the cyt^+ CPE appeared in the sixth passage, and cyt^+ virus was isolated on KB plates. The cyt^+ revertant fully recovered the Tra^+ phenotype. A probable explanation is that this cyt^+ revertant arose as a result of recombination between the mutant genome and the Ad5 cyt^+ sequences residing in the host cell chromosome in 293 cells, as has been suggested for the revertants of Ad5 *hr* deletion mutants (54).

Crosses between *cyt* mutants. In order to ascertain that the independent *cyt* isolates are not identical, a number of crosses were performed between *cyt* mutants (Table 3). In many crosses cyt^+ recombinants were produced and were detected through passages in KB cells. cyt^+ CPE is dominant over *cyt* CPE, as is the case with Ad12 (58), and therefore the accumulation of recombinants can be recognized by the appearance of cyt^+ CPE in serial passages. Control single infections did not produce cyt^+ CPE; therefore, the appearance of cyt^+ revertants can be excluded. cyt^+ recombinants were obtained by both serial passage and infectious center methods. Thus, it is evident that many (if not all) of the mutations of the *cyt* mutants are not identical and are located at different sites in the *cyt* gene. In a number of mixed infections, no cyt^+ CPE was seen before the accumulation of cyt^+ recombinants. Therefore, there is no complementation between *cyt* mutants with respect to cyt^+ CPE. (Since, however, all possible crosses among all *cyt* mutants could not be tested, the possibility that some kind of complementation may be found cannot be totally excluded.) We conclude that there is probably only one *cyt* gene, as is the case with Ad12 (57–59). Thus, recombination between *cyt* mutants is intragenic (4, 5, 57).

All cyt^+ recombinants isolated in different crosses had recovered Tra^+, as well as other cyt^+ phenotypes. This provides further evidence that the *cyt* and Tra^- phenotypes result from the same mutation. Some cyt^+ recombinants produced aberrant transformation, although the parental cyt^+ virus did not. At present, we do not know the reason for this. The cyt^+ recombinants between two group C *cyt* mutants produced plaques with red halos. The red halo phenotype facilitates greatly the detection of cyt^+ recombinants on KB plates.

Genetic mapping of *cyt* mutants. The *cyt* lesions probably are in region E1, located between 1.5 and 11.5 map units, the transforming region (reviewed in reference 60). In view of the marked homologies in DNA sequences between Ad2 and Ad5 in this region (6, 19) attempts were made to locate the sites of Ad2 *cyt* mutations by using three Ad5 deletions, *dl*312 and *dl*314 (deletions in E1A) and *dl*313 (deletion in E1B) (32), as reference deletions in recombination tests (4, 5). As discussed above, *dl*313 has the *cyt* phenotype, whereas *dl*312 and *dl*314 are cyt^+; this suggested initially that the *cyt* gene might be in region E1B. In the actual crosses the infectious center method was used to detect the production of Ad5 (and in two cases Ad2 also) cyt^+ recombinants which grew as well as wt cyt^+ virus in KB cells. The results are

TABLE 3. cyt^+ recombinants isolated in crosses between *cyt* mutants[a]

Cross or virus	Recombinant Virus	Method[b]	No. of foci[c]
Ad2 parental cyt^+			53, 70, 93
*cyt*22 × *cyt*23	3800	Serial passage (6)	34
*cyt*23 × *cyt*24	3804	Serial passage (6)	68
*cyt*22 × *cyt*24	3788	Serial passage (6)	60
*cyt*26 × *cyt*27	2069	Serial passage (7)	33
*cyt*26 × *cyt*23	2296	Serial passage (7)	>250[d]
*cyt*26 × *cyt*24	2298	Serial passage (7)	>250[d]
*cyt*27 × *cyt*22	2445	Serial passage (10)	103
*cyt*37 × *cyt*38	3258	Serial passage (7)	>200[d]
*cyt*26 × *cyt*30	6960 UV(160)[e]	Infectious center (2)	>100
*cyt*26 × *cyt*3	6718 UV(160)[e]	Infectious center (2)	>100
*cyt*4 × *cyt*3	6713 UV(160)[e]	Infectious center (1)	>150
*cyt*26 × *cyt*3	7092 UV(160)[e]	Infectious center (2)	>55
Ad5 wt cyt^+			125
*cyt*1 × *cyt*4	4307	Serial passage (7)	114
*cyt*6 × *cyt*1	4082	Serial passage (7)	59

[a] Stability tests in single infections were done with all *cyt* mutants to check for the accumulation of revertants. As a representative experiment, after 15 serial passages, *cyt*22, *cyt*23, and *cyt*24 showed no cyt^- CPE and produced no transformed foci, compared with 562 foci produced by wt virus. All virus stocks were UV irradiated and were used for transformation at an MOI of 800 PFU/cell (before UV irradiation).

[b] See text. Serial passage and infectious center methods were used. The numbers in parentheses are numbers of passages in KB cells.

[c] See Table 1, footnote *a*.

[d] The foci produced were aberrant (see Fig. 2B).

[e] UV-irradiated recombinant viruses were used for transformation at an MOI of 160 PFU/cell (before UV irradiation).

TABLE 2. Transformation of rat 3Y1 cells with cyt^+ revertants of mutants Ad2 *cyt*5 and Ad2 *cyt*6[a]

Virus	cyt^+ CPE appeared in passage:[b]	No. of foci[c]
wt cyt^+		95
*cyt*5		(1)
cyt^+ Revertants		
2965	7	116
3601	7	110
3603	7	213
3606	7	171
3616	7	ND[d]
3618	7	ND
3625	7	36
3626	7	32
3627	10	31
3628	7	49
wt cyt^+		144
*cyt*6		0
cyt^+ Revertants		
3590	21	(2)
3596	27	(3)
3587	15	0
3549	13	0
3545	24	0
3555	24	(5)
3583	12	(15)
3579	27	(9)
3581	23	(12)

[a] All cyt^- revertants were isolated from independent clonal stocks of *cyt*5 and *cyt*6 after 12 and 33 serial passages, respectively, in KB cells.

[b] KB cell passage in which cyt^- CPE was first recognized.

[c] See Table 1, footnote *a*.

[d] ND, Not done.

TABLE 4. Recombination between Ad2 *cyt* mutants and Ad5 deletion mutants *dl*312, *dl*313, and *dl*314

Cross[a]	Plaque isolate CPE[b]	cyt^+ Recombinants		
		cyt^+/total (PFU/ml)[c]	cyt^+ Isolate[d]	No. of foci[e]
*cyt*4 × *dl*312	cyt^+ (1)	$>1 \times 10^7/1.2 \times 10^7$	R6487 (5)	UV(400), >180
*cyt*4 × *dl*314	cyt^+ (1)	$>1 \times 10^5/7 \times 10^6$	R6527 (5)	>50
*cyt*4 × *dl*313	*cyt* (5)[f]	$<5 \times 10^2/2 \times 10^6$		
*cyt*6 × *dl*312	cyt^+ (1)	$>6 \times 10^3/2.5 \times 10^8$	R6596 (5)	UV(400), >52
*cyt*6 × *dl*314	cyt^+ (1)	$>1 \times 10^3/1.3 \times 10^8$	R6600 (5)	UV(400), >100
*cyt*6 × *dl*313	*cyt* (1)	$<5/3 \times 10^6$		
*cyt*12 × *dl*312	cyt^+ (1)	$>1 \times 10^5/1.2 \times 10^8$	R6558 (5)	UV(400), >120
*cyt*12 × *dl*314	cyt^+ (1)	$>1 \times 10^5/9 \times 10^7$	R6561 (5)	UV(400), >50
*cyt*12 × *dl*313	*cyt* (1)	$<5/1.8 \times 10^7$		
*cyt*3 × *dl*312	cyt^+ (1)	$1.0 \times 10^6/3 \times 10^7$	R7235 (5)	UV(160), >100
*cyt*3 × *dl*314	cyt^+ (1)	$>3 \times 10^3/1.6 \times 10^8$	R7168 (5)	UV(160), >50
*cyt*3 × *dl*313	*cyt* (1)	$<5 \times 10/1 \times 10^6$		
*cyt*22 × *dl*312	cyt^+ (1)	$>1 \times 10^4/3 \times 10^8$	R7188 (5)	UV(160), >50
*cyt*22 × *dl*314	cyt^+ (2)	$>1 \times 10^6/5.2 \times 10^7$	R7240 (5)	NT[g]
*cyt*22 × *dl*313	*cyt* (4)	$<5 \times 10/1 \times 10^8$		
*cyt*23 × *dl*314	cyt^+ (2)	$>1 \times 10^5/4 \times 10^7$	R7373 (5)	NT
*cyt*23 × *dl*313	*cyt* (4)	$<5 \times 10/1.2 \times 10^7$		
*cyt*24 × *dl*314	cyt^+ (1)	$>1 \times 10^5/6 \times 10^7$	R7365 (5)	NT
*cyt*24 × *dl*313	*cyt* (4)	$<5 \times 10/8 \times 10^7$		
*cyt*27 × *dl*312	cyt^+ (1)	$>1 \times 10^5/1.5 \times 10^8$	R7277 (5)	UV(160), >20
*cyt*27 × *dl*314	cyt^+ (1)	$1 \times 10^3/6 \times 10^8$		
*cyt*27 × *dl*313	*cyt* (4)			
*cyt*30 × *dl*312	cyt^+ (2)	$1 \times 10^6/6 \times 10^7$	R7083 (2)	UV(160), >60
*cyt*30 × *dl*314	cyt^+ (2)	$>1 \times 10^4/2.5 \times 10^7$	R7184 (5)	UV(160), >100
*cyt*30 × *dl*313	*cyt* (3)	No virus propagated		
*cyt*26 × *dl*312	cyt^+ (1)	$1 \times 10^6/1 \times 10^7$	R7078 (5)	UV(160), >60
*cyt*26 × *dl*312	cyt^+ (1)	$>1 \times 10^4/6.5 \times 10^7$	R5832 (2)	UV(400), >400[h]
*cyt*26 × *dl*314	cyt^+ (1)	$3 \times 10^5/1 \times 10^7$	R6580 (5)	UV(400), >50
*cyt*26 × *dl*313	*cyt* (1)	No virus propagated		
*cyt*15 × *dl*312	cyt^+ (2)	$1 \times 10^5/3 \times 10^8$	R6730 (5)	0
*cyt*15 × *dl*314	*cyt* (4)	$<1 \times 10^2/4.5 \times 10^8$		
*cyt*15 × *dl*313	*cyt* (2)	$<1 \times 10^2/1.4 \times 10^8$		
*cyt*1 × *dl*312	cyt^+ (1)	$2 \times 10^4/3 \times 10^7$	R6531 (5)	(6)
*cyt*1 × *dl*314	cyt^+ (1)			
*cyt*1 × *dl*313	*cyt* (5)	$<1 \times 10^2/2 \times 10^6$		
Ad2 cyt^+ × *dl*313	cyt^+ (1)	$1 \times 10^3/4 \times 10^5$	R6573 (5)[i]	UV(200), >150

[a] The infectious center method was used for all crosses (see text).
[b] Infectious center plaques (usually two to four plaques) were isolated and planted into KB cell cultures (10^6 cells), both to check CPE phenotype and to propagate the virus serially. The numbers in parentheses are passage numbers.
[c] Titer of cyt^+ recombinants/total titer in the infected KB cell cultures tested for cyt^+ recombinants.
[d] In each cross two or more independent cyt^+ recombinants were usually isolated from independent infectious centers. Only one representative cyt^+ recombinant is shown for each cross. The numbers in parentheses are serotypes.
[e] See Table 1, footnote *a*.
[f] *cyt* CPE persisted for five serial passages.
[g] NT, Not tested.
[h] Transformation was aberrant.
[i] Control recombination between Ad2 cyt^+ and *dl*313. Ad5 cyt^+ transforming recombinant was obtained.

shown in Table 4. Eight *cyt* mutants recombined with *dl*312 and produced cyt^+ Tra^+ viruses. We conclude that the *cyt* gene is located outside the *dl*312 deletion. In the cross between *cyt*26 and *dl*312 or between *cyt*30 and *dl*312 both Ad5 and Ad2 cyt^+ recombinants were isolated (i.e., since *cyt*26 and *cyt*30 do not propagate in KB cells, we could select for Ad2 cyt^+ recombinants). The Ad2 cyt^+ recombinant R5832 isolated in crosses between *cyt*26 and *dl*312 was the most efficient transforming virus. *cyt* Tra^- mutant 6119 was obtained from R5832 after UV irradiation. In crosses with *dl*314, 10 *cyt* mutants produced cyt^+ recombinants, and 6 of the 10 cyt^+ recombinants tested fully recovered wt transforming ability. Therefore, the *cyt* lesions also lie outside the *dl*314 deletion. In all control single *cyt* infections no cyt^+ virus was recovered from infectious center isolates (data not shown in Table 4), indicating that we had isolated recombinants rather than revertants. In summary, the majority of the *cyt* lesions are rescued by *dl*312 and *dl*314, as the deletions in *dl*312 and *dl*314 in turn are rescued by the *cyt* mutants.

*cyt*1 and *cyt*15 differed from the other mutants because they recombined with *dl*312 to produce cyt^+ recombinants without apparent recovery of the transforming ability, at least for the cyt^+ isolates tested. In crosses with *dl*314, these two mutants did not produce cyt^+ recombinants as regularly as other cyt^+ mutants did. Therefore, *cyt*1 and *cyt*15 may contain one or more mutations that overlap the *dl*314 or *dl*312 deletion or both.

In crosses between *dl*313 and 12 *cyt* mutants, cyt^+ CPE never appeared in KB cells infected with infectious center isolates and in the following KB serial passages (Table 4). Therefore, cyt^+ recombinants were not produced. All attempts to recover cyt^+ recombinants also invariably failed on KB cell plating of serially passaged viruses. In crosses between *dl*313 and *cyt*26 or *cyt*30 the infectious center

isolates could hardly be propagated in KB cells, indicating the failure of both to complement in growth and to recombine.

These crosses indicate clearly that the majority of the *cyt* mutations are probably located between the 3′ ends of the *dl*314 and *dl*313 deletions (nucleotide positions 1,679 to 3,625) (8, 54). A few *cyt* mutants may have lesions extended into the *dl*312 deletion or may be double mutants with lesions in the deletions of both *dl*313 and *dl*312 (or *dl*314).

Restriction endonuclease mapping of the crossover points in *cyt*$^+$ Tra$^+$ and *cyt* Tra$^-$ recombinants. Genetic mapping indicated that most or all *cyt* lesions lie outside the *dl*312 and *dl*314 deletions but within the *dl*313 deletion. This interpretation was further investigated by restriction endonuclease mapping of the crossover site in the *cyt*$^+$ Tra$^+$ recombinants listed in Table 4, as well as in *cyt* Tra$^-$ recombinants isolated after crosses with *dl*312, *dl*314, and *dl*313 (see legend to Fig. 3 for recombinants tested). In these experiments we exploited differences in restriction sites between Ad2 and Ad5 to locate portions of the recombinant genome derived from each parental virus (52). Initial results obtained with *Eco*RI, *Bam*HI, and *Bgl*II established that most *cyt*$^+$ Tra$^+$ recombinants had Ad5 sequences from map unit 25 (the leftmost diagnostic restriction site) to map unit 100 (data not shown). R5832 and R6561 were double and triple recombinants, respectively. All nine *cyt* Tra$^-$ recombinants had Ad5 sequences from map unit 25 to map unit 100. Figure 3 illustrates analyses using *Nar*I and *Xmn*I which are diagnostic of Ad2 and Ad5 in region E1. Figure 3A shows data that are representative of groups of recombinants, Fig. 3B illustrates the proteins thought to be encoded by regions E1A and E1B, Fig. 3C gives the *Nar*I and *Xma*I cleavage maps in Ad2 and Ad5 DNAs, and Fig. 3D presents our interpretation of the results. All *cyt*$^+$ Tra$^+$ recombinants except R6580 have Ad2 sequences at nucleotide 1,302 and Ad5 sequences at nucleotide 2,256; i.e., they lack the Ad5 *Nar*I site and the Ad2 *Xmn*I site at these positions. In the *dl*312 crosses, we concluded that recombination occurred between nucleotide 1,349, the 3′ boundary of the deletion, and nucleotide 2,256, the Ad2 *Xmn*I site. In the *dl*314 crosses, recombination occurred between nucleotide 1,679, the 3′ boundary of the deletion, and nucleotide 2,256. R6580 has Ad5 sequences at both nucleotide 1,302 and nucleotide 2,256. Since *dl*314 did not plaque on KB cells under our conditions, we assumed that R6580 is a double recombinant, as indicated. Significantly, none of the 13 *cyt*$^+$ Tra$^+$ recombinants tested had Ad2 sequences at nucleotide 2,256. In marked contrast, all 10 of the *cyt* Tra$^-$ recombinants examined had Ad2 sequences at nucleotide 2,256, indicating that they recombined somewhere between nucleotide 2,256 and map unit 25.

These results argue strongly that the mapped *cyt* lesions lie to the left of nucleotide 2,256 and to the right of the deletions in *dl*314 (i.e., between nucleotides 1,679 and 2,256). As shown in Fig. 3B, this strongly suggests that the *cyt* gene codes for the region E1B 19K (175R) protein.

DNA sequence of E1B 19K gene in *cyt* mutants. In order to substantiate the genetic mapping and restriction enzyme mapping of the *cyt* gene, we determined the DNA sequence of the region between the E1B promoter region (nucleotide ~1,600) and nucleotide 2,256 (the *Xmn*I site) for eight mutants. The area sequenced represents the boundaries of the *cyt* gene (including the promoter), as indicated by other mapping experiments. The sequence data for two mutants are shown in Fig. 4. *cyt*6 has a point mutation at nucleotide 1,840 which changes the 44th codon in the E1B 19K (175R) gene from lysine to glutamic acid. *cyt*7 has two point mutations, one at nucleotide 1,994 which changes the 95th codon from phenylalanine to cysteine and the other at nucleotide 2,006 which changes the 99th codon from isoleucine to threonine. Table 5 summarizes the sequence results for all eight mutants. All other sequences matched the previously published sequence (19) exactly. *cyt*15 is a double missense mutant. *cyt*4 is an insertion-deletion mutant such that there is a TAA at the 96th codon in E1B 19K. The other four mutants have simple nonsense mutations. Significantly, except for *cyt*15, all of the point mutations in the E1B 19K (175R) gene lie outside the genes for E1B 55K (495R) and for the putative 14K (95R) polypeptide coded off the *l* strand. These results suggest that the point mutations in the E1B 19K (175R) gene are responsible for the *cyt* Tra$^-$ phenotypes of *cyt*6 and *cyt*7. Presumably, most or all of the other *cyt* mutants also have mutations in the gene for the E1B 19K (175R) polypeptide (Fig. 3). The nonsense mutation in *cyt*15 is also a missense mutation in E1B 55K. This may explain why, among all eight mutants that we sequenced, only *cyt*15 cannot grow on KB cells.

DISCUSSION

Recent studies have demonstrated that oncogenic transformation of primary cells in culture requires at least two separate and complementing functions (51, 62). The first, an establishment function, is concerned with immortalization of cells, whereas the second, the transforming function, is required for full expression of an oncogenic phenotype. The viral or cellular *myc* gene, adenovirus early region E1A (29), and the amino-terminal 40% of the polyoma large-T antigen (47) provide the same establishment function. The cloned polyoma middle-T antigen, Ad2 region E1B, and human *ras* genes transform primary cells only following cotransfer with Ad2 E1A (51). In contrast, both polyoma middle-T antigen and human *ras* genes readily transform a variety of established cell lines (9, 22, 47, 61), although attempts to transform established 3Y1 cells with Ad5 E1B alone have failed thus far (62). Possibly cellular establishment genes are activated in these established cell lines.

In the present study 59 independent *cyt* mutants of Ad2 and Ad5 were examined for transforming ability in established rat 3Y1 cells. All except one mutant, Ad2 *cyt*2, were found to be transformation defective. Ad2 *cyt*2 was less defective for transformation. Both *cyt*$^+$ revertants and *cyt*$^+$ intragenic recombinants obtained in crosses between independent *cyt* isolates recovered fully the transforming ability of the wt virus. Deletion mapping experiments on Ad2 *cyt* mutants were performed by using Ad5 region E1A deletion mutants *dl*312 (nucleotides 448 to 1,349) and *dl*314 (nucleotides 1,335 to 1,679) and region E1B deletion mutant *dl*313 (nucleotides 1,275 to 3,625) (8, 32, 54) as reference deletions. Our results showed that the *cyt* mutants recombined with both *dl*312 and *dl*314 to produce intertypic *cyt*$^+$ Tra$^+$ recombinants, whereas no *cyt*$^+$ recombinants were obtained in crosses between *dl*313 and *cyt* mutants (Table 4). Thus, the sites of *cyt* mutations were located in region E1B between the 3′ ends of *dl*314 and *dl*313 (nucleotide positions 1,679 to 3,625). Restriction endonuclease analyses of *cyt*$^+$ intertypic recombinants between *cyt* mutants and deletion mutants *dl*312 and *dl*314 corroborated this conclusion. Furthermore, sequencing studies of the cloned mutant DNAs located the sites of point mutations in the region of E1B encoding the 19K (175R) polypeptide and, except for *cyt*15, not overlapping the region encoding the 55K (495R) polypeptide. UV irradiation induced a TA → CG transition at position 1,840 in *cyt*6, whereas a TA → GC transversion at position 1,994 and

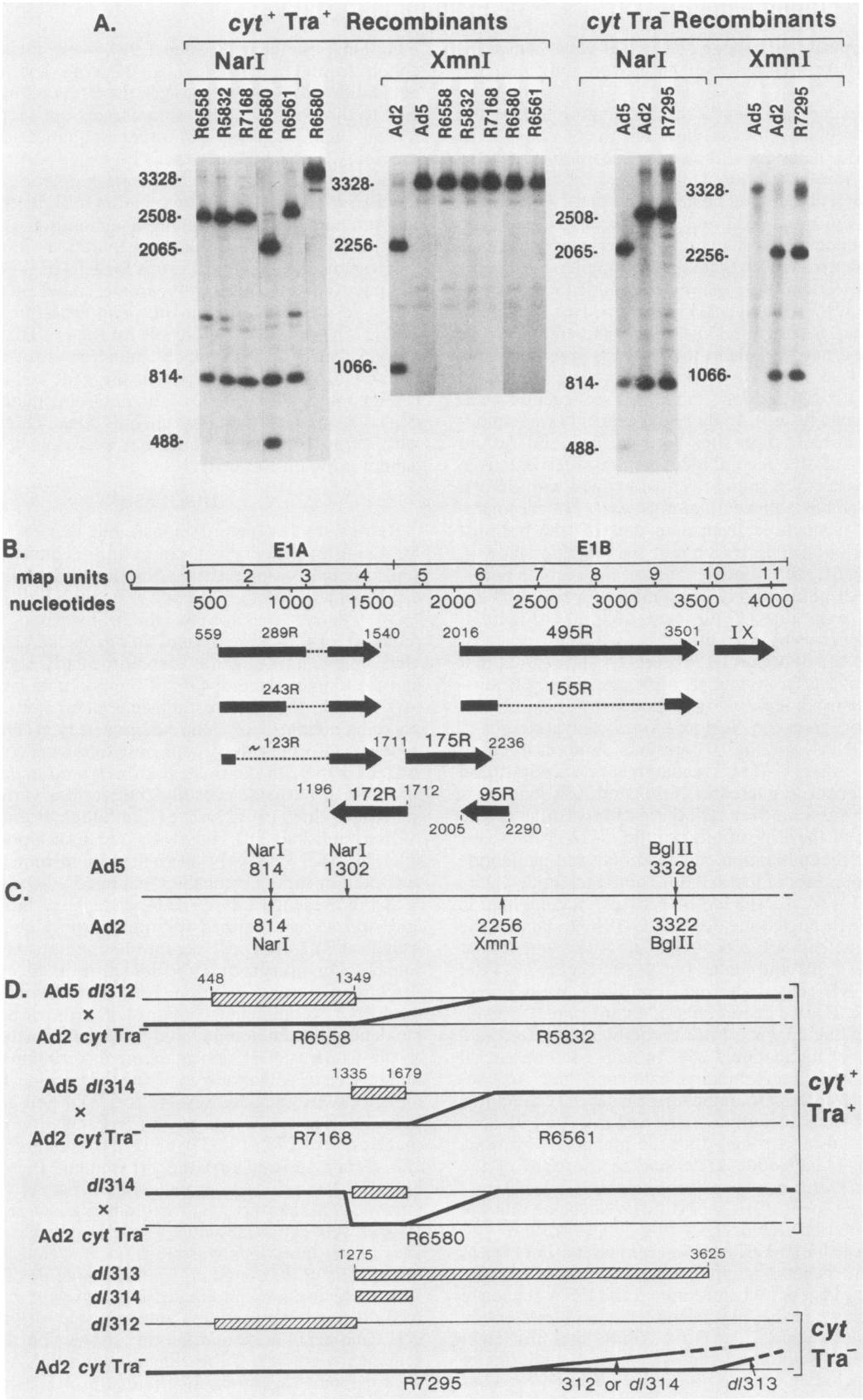

FIG. 3. Restriction endonuclease (*Nar*I, *Xmn*I) mapping of the sites of recombination in region E1 between Ad5 deletion mutants and Ad2 *cyt* Tra⁻ mutants. (A) *Nar*I and *Xmn*I cleavage patterns of the ^{32}P-labeled *Bgl*II fragment (nucleotides 1 to 3,322) of Ad5 and Ad2 wt and selected recombinants. (B) Schematic representation of region E1, indicating the E1A and E1B proteins transcribed off the *r* strand, as well as

a TA → CG transition at position 2,006 were generated in *cyt7*. UV irradiation induces many types of base substitutions, although GC → AT transitions predominate in the *lacI* gene of *E. coli* (42).

Altogether, our study showed that the *cyt* gene of Ad2 and Ad5 is an oncogene for transforming function in region E1B that probably encodes the 19K (175R) polypeptide. Figure 5 shows the amino acid sequences of E1B 19K from Ad2, Ad7, and Ad12; the stippled areas indicate conserved sequences. Ad2, Ad7, and Ad12 are members of adenovirus groups C, B, and A, respectively. Figure 5 shows the secondary structure of Ad2 E1B 19K as predicted by the method of Chou and Fasman (13, 14). The locations of the *cyt* mutations are indicated. Three nonsense mutations are very near the N terminus of E1B 19K. Therefore, E1B 19K is clearly not required for growth on KB cells. The *cyt5* nonsense mutation is toward the C terminus of E1B 19K, and this suggests that the C-terminal sequences are required for E1B 19K function. The missense mutations are interesting. The *cyt15* substitution at amino acid 34 should preclude the β-turn at that position. The *cyt7* substitutions are in the only substantial hydrophobic domain in E1B 19K, whereas *cyt6* has a basic → acidic substitution.

When isolating virion DNA for cloning and sequencing, we noticed that the DNA was intact in some mutants but was extensively degraded in others. Without exception, the DNA from missense mutants was intact, and the DNA from nonsense mutants was degraded (unpublished data). Nonsense mutant DNA in Hirt supernatants was also very degraded. This appears to be a manifestation of the DNA degradation phenotype of Ad12 *cyt* mutants (34; see below). Since some of the nonsense mutations are very near the N terminus, these results establish that the absence of E1B 19K function leads to DNA degradation.

We anticipate that other adenovirus mutants are *cyt* mutants. Ad5 deletion mutant *dl*313 has a *cyt* phenotype, as previously found through complementation tests in inhibition of DNA degradation (34), and this was confirmed in this study by the appearance of *cyt* CPE in *dl*313-infected KB cells (Fig. 1). Ad2 large-plaque (*lp*) mutants (11) may or may not also be *cyt* mutants. The substitution mutant Ad5 *sub*315 lacks 1,780 base pairs (map units 3.5 to 8.5) and is transformation defective (32). The deletion overlaps the *dl*313 deletion, and therefore *sub*315 may also be a *cyt* mutant. The Ad2 DNA-negative temperature-sensitive mutant H2*ts*111, which degrades viral and cellular DNAs at the nonpermissive temperature, has been shown to contain a probable *cyt* mutation (56). Ad5 mutant *dl*342 is a deletion of 1 base pair (GC) at nucleotide 2,179 and is located in the coding sequences of both the E1B 19K polypeptide and the E1B 58K polypeptide (53). It may be anticipated that *dl*342 has a *cyt* phenotype and is transformation defective.

Previous studies have shown that the *cyt* mutants of Ad12 are much less tumorigenic in newborn hamsters than wt cyt^+ viruses (57–59, 63). Ezoe et al. (16) discovered that newly synthesized viral and cellular DNAs were extensively degraded in Ad12 *cyt* mutant-infected cells. The cyt^+ viral function involved in the inhibition of *cyt* DNA degradation has been assigned through complementation tests to a product of E1B, probably the 19K (175R) polypeptide (34). Four Ad12 *cyt* mutants showed much reduced capacity to transform primary baby rat kidney cells, whereas one mutant (H12*cyt*61) transformed as efficiently as wt cyt^+ virus (38). Thus, the Ad12 *cyt* gene is also a transforming oncogene in region E1B. Two Ad12 insertion mutants (*in*205B and *in*205C) and a deletion mutant (*dl*205) in the E1B 19K polypeptide-coding sequence with no mutation in the 54K polypeptide were isolated (17). All of these mutants showed reduced capacity for transformation in rat 3Y1 cells and produced tumors in baby hamsters very inefficiently. Probably these mutants are also *cyt* mutants.

In the majority of the Ad2 and Ad5 *cyt* mutants, the transforming function is unequivocally defective. However, Ad12 *cyt*61 transformed baby rat kidney cells as efficiently as wt cyt^+ virus (38). Also, Ad12 *cyt*143 produced tumors in baby hamsters as well as the wt virus (58). The transforming function of Ad12 is generally more efficient than that of Ad5 and Ad2. This fact may partially explain the apparent normal transforming function of a few *cyt* mutants of Ad12. However, whether the transforming function and the cyt^+ function of the 19K polypeptide (i.e., the *cyt* gene product) may be separable functions remains to be studied. Whether there is a modifying but not abolishing mutation in the *cyt* gene (e.g., increased transforming function or alteration of transformation pattern) will be the subject of further studies.

Our sequencing studies located the *cyt* mutation sites in region E1B encoding the 19K (175R) polypeptide but outside the coding region of the 55K (495R) polypeptide. It seems probable that Ad2 *cyt* mutations can occur without a concomitant mutation in the 55K (495R) polypeptide. Thus, both in the Ad12 mutants of Fukui et al. (17) described above and in Ad2 *cyt* mutants, mutations in the 19K (175R) polypeptide without mutations in the 55K (495R) polypeptide seem to result in defective transforming function; that is, *cyt* mutants are transformation defective even when the 55K (495R) polypeptide is intact. On the other hand, Ad5 host range mutant *hr*6 (26), with a mutation located between 6.1 and 9.0 map units (18), fails to synthesize the 55K (495R) polypeptide encoded in region E1B (35, 50). *hr*6 appears to be cyt^+ in DNA degradation inhibition tests (34), but is transformation defective (23). Therefore, a mutation in the 55K (495R) polypeptide with an intact *cyt* gene also results in defective transforming function. An intriguing question is whether *cyt* mutants and mutants of the 55K (495R) polypeptide complement in transformation (i.e., whether the two mutant groups thus signal the existence of two functional units within the

possible 14K and 23K proteins transcribed off the *l* strand. The nucleotide numbers of initiation and termination codons are given. (C) *Nar*I and *Xmn*I cleavage maps of region E1 of Ad5 and Ad2. (D) Deduced structures of the recombinants. The numbers on the cross-hatched bars indicate the boundaries of the deletions. The crossovers could have occurred anywhere within the boundaries of the diagonal lines. The Ad2 *cyt* and Ad5 *dl* mutants used to generate the recombinants are indicated in Table 4. Seven cyt^+ Tra^+ recombinants isolated from crosses with *dl*312 were analyzed, but only representative data are shown; that is, R6558 represents R6487, R6596, R7078, and R7235. R5832 has two crossover sites, one between nucleotides 1,349 and 2,256 and the second between nucleotide 2,256 and map unit 25. Six cyt^+ Tra^+ recombinants isolated from crosses with *dl*314 were analyzed. The data shown for R7168 represent R6257 and R7184. R6561 is a triple recombinant, with one crossover as shown, a second crossover between map units 29 and 41, and a third crossover between map units 41 and 59. R6580, a presumptive double recombinant as shown, gave the same results as R6600. Nine *cyt* Tra^- recombinants were analyzed, which were isolated from crosses with *dl*312, *dl*314, or *dl*313. The results shown for R7295 are the same as those obtained with R7286, R7088, R6224, R7295, R7288, R7178, R7176, R7172, and R6161.

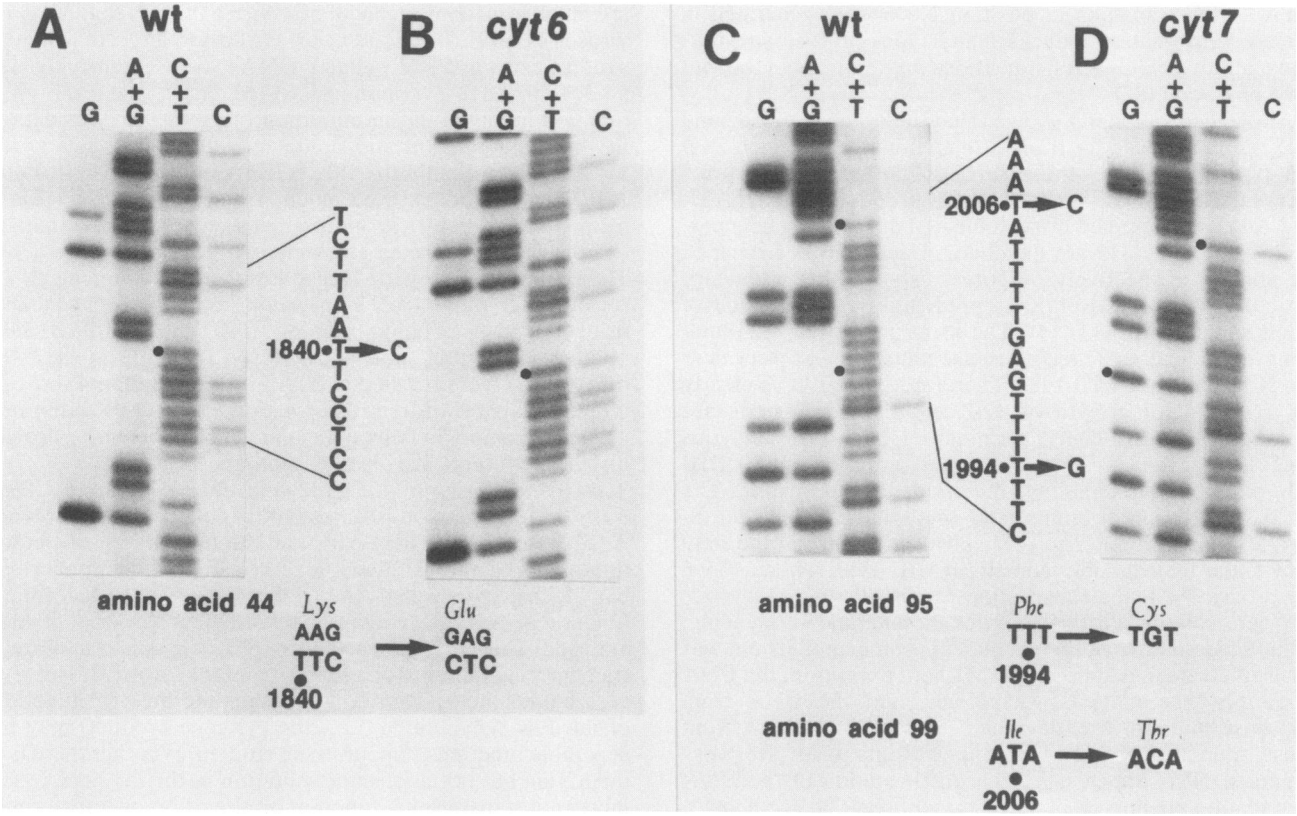

FIG. 4. DNA sequence showing the point mutations in *cyt*6 and *cyt*7. (A and B) Corresponding sequences on the *r* strands of wt and *cyt*6, respectively. (C and D) Corresponding sequences on the *l* strands of wt and *cyt*7, respectively. The dots indicate the mutations.

transforming oncogene of region E1B). Much more study on the mutants of the 55K (495R) polypeptide is needed to solve this question.

Rat 3Y1 cells transformed by a DNA-minus temperature-sensitive mutant, Ad12 *ts*401, displayed a temperature-sensitive response in transformed phenotype. An active *ts*401 gene function is required both for maintenance of transformation and for the expression of the 60K polypeptide in transformed cells (36, 37). Whether *ts*401 maps in region E1B of Ad12 and thus encodes a temperature-sensitive 60K polypeptide remains to be determined.

It is noteworthy that some of the cyt^+ recombinants showed an unexpected phenotype. Ad2 intertypic cyt^+ recombinant 5832 isolated in the cross between Ad5 *dl*312 and Ad2 *cyt*26 turned out to be the most efficient transforming virus with a tendency to aberrant transformation (Table 4). Ad5 genomic sequences between map units 6.2 and 24.8 are recombined into Ad2 *cyt*26. Recombinant 5832 showed a less marked cytotoxic effect in rat 3Y1 cells. An Ad5 recombinant *dl*A with a similar phenotype has been reported previously (55).

Aberrant transformation produces, besides foci of the standard type, many highly refractile elongated structures (Fig. 2) and originally was observed in transformation with Ad2 group C parental cyt^+ virus. It was often seen in transformation with cyt^+ recombinants obtained in crosses between *cyt* mutants from nonaberrant transforming parental cyt^+ virus belonging to Ad2 group B. The occurrence of aberrant transformation is sometimes irregular for reasons which have not been determined thus far.

E1A mutants were used to study the establishment function of the E1A oncogene. The transcription of E1B is defective in H5*hr*1 (26) but is normal in both H5*hr*440 (55) and H5*in*500 (10). Transformation of baby rat kidney cells is defective in both *hr*440 and *in*500 and abortive in H5*hr*1. Therefore, expression of E1B only is insufficient for transformation in the absence of the establishment function of E1A. All three E1A mutants have a mutational lesion in the 289R protein encoded by the 13S mRNA, which is essential for the establishment function. Ralston and Bishop (45) discovered that the proteins encoded by the oncogenes *myc*, *myb*, and E1A are structurally related and that a string of

TABLE 5. Mutations in Ad2 *cyt* mutants

Mutant	Nucleotide mutated	Mutant codon in E1B 19K	Codon wt in E1B 19K
*cyt*22	1,721	Amber (4)[a]	Trp
*cyt*15	1,774	Tyr (22)	Asn
	1,812	Phe (34)	Ser
*cyt*24	1,788	Umber (26)	Trp
*cyt*37	1,794	Umber (28)	Trp
*cyt*6	1,840	Glu (44)	Lys
*cyt*7	1,994	Cys (95)	Phe
	2,006	Thr (99)	Ile
*cyt*5	2,079	Umber (123)	Trp
*cyt*4	Delete 1,997–2,021, insert 9 base pairs	Ochre (96)	

[a] The numbers in parentheses are codon numbers.

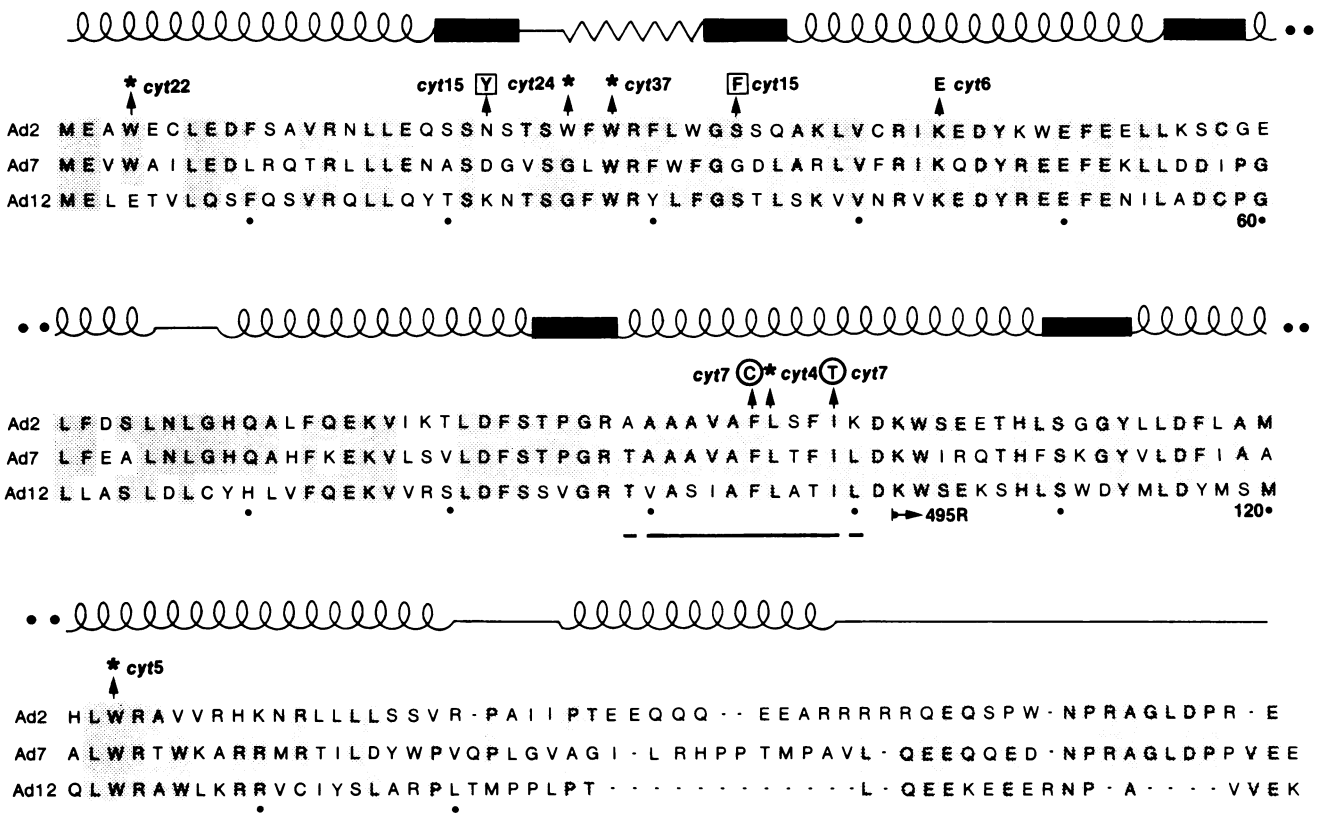

FIG. 5. Schematic representation of the E1B 19K (175R) proteins from Ad2 (group C), Ad7 (group B), and Ad12 (group A). See references 6 and 15. The mutations in eight Ad2 *cyt* mutants are indicated. The asterisks indicate nonsense mutations. *cyt*16 and *cyt*7 are double missense mutants, and *cyt*6 is a single missense mutant. The stippled areas indicate conserved regions. Also shown is the secondary structure as predicted by the method of Chou and Fasman (13, 14). The underlined area (amino acids 89 to 100) is the only hydrophobic domain.

homologous residues are shared by v-*myc* and the 289R protein of Ad5. Ad5 mutants whose lesions are within this region of homology, such as H5*hr*440 or H5*in*500, are transformation defective. In contrast, E1A mutant H5*hr*1 (48), whose lesion is outside this region of homology, retains transforming ability, although the phenotype is cold sensitive in cells transformed by H5*hr*1 (3, 28). However, the process of transformation by the E1A mutant that only expresses the 243 protein has been shown to be cold sensitive (43).

The protein encoded by the oncogenes (including E1A) for the establishment function are located in the nuclei of transformed cells (31, 64). In contrast, the products of oncogenes (including E1B) for the transforming function are associated with membranes (44), including the cell surface (30). Although the concentration of lectin required to agglutinate Ad12 *cyt* mutant-infected cells was not significantly different from that required for uninfected cells, cells infected with Ad12 *cyt*$^+$ wt virus agglutinated in the presence of a lower concentration of lectin. Besides, the Ad12 *cyt* mutants were ineffective in inducing transplantation immunity. Thus, the Ad12 *cyt* mutants are defective in inducing the cell surface changes which might be necessary for cell transformation (63). The adenovirus type of CPE (i.e., marked cellular rounding and agglutination) may suggest by itself the change of infected cell surface and is produced by the *cyt* gene product. It seems possible that the molecular mechanism underlying the production of *cyt*$^+$ CPE in productive infection and transformation of nonpermissive cells may turn out to be closely related if not identical.

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