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Incomplete Transformation of Rat Cells by a Deletion Mutant of Adenovirus Type 5

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Rat 3Y1 cells were infected with adenovirus type 5 (Ad5) wild type, *dl*312 (deletion of 902 base pairs between 1.5–4.5 map units), and *dl*313 (deletion of 2,350 base pairs between 3.5–10.5 map units). After cultivation for 4 weeks, transformed foci appeared in wild type- and *dl*313-infected cells. No focus was observed in *dl*312- and mock-infected cells. Foci induced by *dl*313 were less dense than those induced by wild type. Cell lines (313Y cells) established from *dl*313-induced foci contained the E1 gene of the *dl*313 genome (E1a only). Cell lines (5WY cells) established from Ad5 wild type-induced foci contained the E1 gene of wild type (E1a and b). The difference between the transcriptional patterns of the E1 gene in 313Y cells and that in 5WY cells was the same as the difference in *dl*313- and wild type-infected cells. Colonies were formed in soft agar culture inoculated with 5WY cells, but no colony was formed after inoculation of 313Y cells. The transformed phenotype of 313Y cells was incomplete compared with that in 5WY cells. In nongrowing 3Y1 cells, *dl*313 and Ad5 wild type induced cellular DNA synthesis but *dl*312 did not. The above results suggest that the E1a gene is functioning in *dl*313-infected but not in *dl*312-infected cells and that such functions as induction of cellular DNA synthesis and transformation of cells are dependent on expression of the Ad5 E1a gene.

The smallest DNA fragments with transforming activity are *Hin*dIII fragment G (the left end, 6.8%) of adenovirus type 12 (Ad12) DNA (16), *Hsu*I fragment G (the left end, 7.3%) of Ad2 or Ad5 DNA (6, 20, 21), and *Hin*dIII fragment IJ (the left end, 8.1%) of Ad7 DNA (15). These DNA fragments of adenoviruses have the capacity to induce complete transformation in the same way as the whole viral DNA. Recently, smaller DNA fragments, such as *Acc*I fragment H (the left end, 4.6%) of Ad12 DNA (19), *Hpa*I fragment E (the left end, 4.5%) of Ad5 DNA (9), and *Hin*dIII fragment I or H (the left end, 4.0%) of Ad3 or Ad7 DNA (3), have been shown to transform cells incompletely or partially. These results mean that the early region 1 (E1a and b) of adenovirus DNA is able to transform cells completely but that the E1a gene alone transforms rat cells incompletely or partially. Ad5 deletion mutants that lack portions of E1a or E1b were isolated by Jones and Shenk (10). One of the mutants, *dl*312, lacks E1a (deletion of 902 base pairs between 1.5–4.5 map units), and the other, *dl*313, lacks E1b (deletion of 2,350 base pairs between 3.5–10.5 map units). Both mutants grow in 293 cells, human embryo kidney (HEK) cells transformed by Ad5 DNA fragments (8), but neither grows in HeLa or HEK cells.

In this report, it is shown that rat cells (3Y1) were transformed incompletely by one of the deletion mutants, *dl*313, but not by the other, *dl*312. Cellular DNA synthesis was induced in nongrowing rat cells by *dl*313 but not by *dl*312. The biological significance of these findings is discussed.

MATERIALS AND METHODS

Cells and virus. HEK, 293 (8), rat 3Y1 (12), and KB cells were cultured in Eagle minimal essential medium (MEM) with 10% calf or fetal calf serum. Ad5 wild type, *dl*312, and *dl*313 (10) were obtained from T. Shenk and propagated in either KB or 293 cells.

Transformation of rat cells. This was carried out as described previously (16, 22). Rat 3Y1 cells in a stationary state were infected with virus at 10 PFU/cell and subcultured at 10^5 cells per dish in Eagle MEM with 10% fetal calf serum. After 3 days, the medium was changed to low calcium (0.1 mM Ca^{2+}) medium. Medium was changed every 4 days thereafter. Transformed foci were counted 2 and 4 weeks after infection.

Growth of transformed cells. Transformed cells were grown in Eagle MEM (0.1 mM Ca^{2+}) supplemented with 2% or 10% fetal calf serum, and growth of transformed cells in soft agar was carried out as de-

scribed previously (16, 19). Seaplaque agarose (Marine Colloids Co.) and a selected lot of fetal calf serum were used, because the results in soft agar depended on the lot of fetal calf serum used.

Detection of the viral genome in transformed cells. The viral genome in transformed cells was examined by spot hybridization qualitatively (5). Cellular DNA was extracted from transformed or control cells, heat denatured, spread in a spot on a membrane filter, and fixed. The Ad5 wild type whole DNA, *Xba*I fragment E (0–3.8 map units), and *Bgl*II-*Xba*I fragment B (3.8–9.4 map units) of Ad5 DNA were labeled with ^{32}P by nick translation and used for DNA-DNA hybridization, which was detected by autoradiography.

DNA-DNA hybridization. DNA-DNA hybridization was carried out on membrane filters as described previously (18), using viral DNA from purified Ad5 virions and cellular DNA from 3Y1 cells.

S1 mapping. S1 mapping of cytoplasmic mRNA's from infected and transformed cells was carried out as described by Berk and Sharp (2).

RESULTS

Transformation of rat 3Y1 cells by Ad5 wild type or deletion mutants. When 3Y1 cells were infected with Ad5 wild type or *dl*313, transformed foci became visible 4 weeks after infection. No focus was observed in 3Y1 cells infected with *dl*312 or mock infected (Fig. 1, Table 1). Since distinct or weak degeneration of cells was observed in wild type- or *dl*313-infected cells and not in *dl*312-infected or mock-infected cells, the morphology of background cells is different among these cultures. The foci induced by wild type were denser than those induced by *dl*313 (Fig. 2).

Transformed cells were subcultured from foci and designated 5WY-1 to -4 cells from Ad5 wild type-transformed foci or 313Y-1 to -10 cells from *dl*313-transformed foci. About half of the 313Y cells had a fibroblastic appearance, and the remaining cells showed polygonal or epithelial morphology. In contrast, 5WY cells showed polygonal or triangular morphology. The size of each 313Y cell was larger than that of each 5WY cell.

No difference could be detected in the growth rate and saturation density between 313Y and 5WY cells, due to the difference in their growth patterns (Table 2). The growth of 5WY cells seemed to be more rapid than that of 313Y cells. However, 5WY cells showed a tendency to be detached easily from the dish. After cultivation for 7 days, most of the 5WY cells were detached from the dish, whereas 313Y cells remained in a confluent state in the dish. In soft agar, 5WY cells formed small colonies, but 313Y cells did not (Table 3). However, 313Y cells after pro-

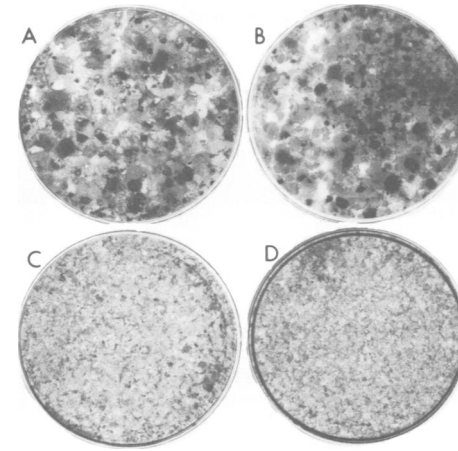

FIG. 1. *Transformed foci induced in rat cells. Rat 3Y1 cells were infected at 10 PFU/cell with Ad5 wild type, dl312, and dl313 and incubated at 37°C. Cells were stained with Giemsa 4 weeks after infection. Many transformed foci were densely stained in cells infected with Ad5 wild type (A) and dl313 (B). No transformed focus was observed in cells infected with dl312 (C) or medium only (D).*

TABLE 1. *Transformation of 3Y1 cells*[a]

Infected with:	No. of foci per dish at:			
	2 weeks		4 weeks	
	Expt 1	Expt 2	Expt 1	Expt 2
Ad5 wild type	0	0	15	20
*dl*312	0	0	0	0
*dl*313	15	20	15	20
Mock-infected	0	0	0	0

[a] Rat 3Y1 cells were infected and the transformation assay was carried out as described in the text. Two and four weeks after infection, the number of foci per dish (average of 10 dishes) was counted. Experiments were repeated, and the results are shown separately.

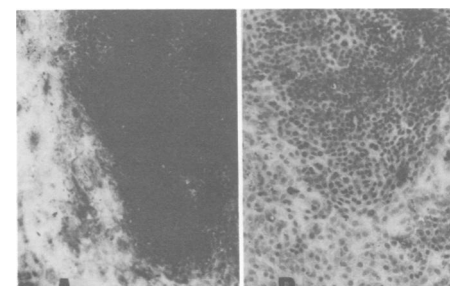

FIG. 2. *Appearance of foci induced by Ad5 wild type and dl313. Transformed foci induced in 3Y1 cells by Ad5 wild type (A) and dl313 (B) were stained by Giemsa and photographed under a microscope.*

TABLE 2. *Saturation density*[a]

Transformed cells	Saturation density (× 10^4 cells/dish) with fetal calf serum at:	
	2%	10%
5WY-1	75	350
5WY-4	80	370
313Y-1	55	250
313Y-2	80	450
313Y-3	80	450
12WY-2 (control)[b]	1,200	1,000
3Y1 (control)	55	280

[a] Cells shown above were cultured at 10^5 cells per dish in Eagle MEM with 2 or 10% fetal calf serum. The number of cells per dish (an average of three dishes) was counted at intervals of 24 h. Medium was changed at intervals of 3 to 4 days. In 4 to 8 days, the number of cells reached a plateau, as shown in the table.

[b] Rat 3Y1 cells transformed with Ad12 wild type. Since 12WY-2 cells had a high saturation density, the cells were used as a positive control.

TABLE 3. *Colony formation in soft agar culture*[a]

Transformed cells	Colony formation[b]
5WY-1	++
5WY-2	+
5WY-3	+
5WY-4	+
313Y-1	−
313Y-2	±
313Y-3	±
313Y-4	±
12WY-2[c]	+++

[a] Cells shown above were cultured in soft agar at 10^4 cells per dish. Colonies were observed at 3 and 4 weeks after seeding. The number of colonies was 10 to 50 per dish (plating efficiency, 2×10^{-2} to 5×10^{-2}) for 5WY-1 to -4 cells.

[b] Colony diameter: +++, >1 mm; ++, 0.1 to 0.5 mm; +, small, <0.1 mm; ±, tiny, hardly visible with naked eye; −, no colony.

[c] Rat 3Y1 cells transformed with Ad12 wild type. Since 12WY-2 cells were able to form large colonies, the cells were included as a positive control.

longed passages in vitro seemed to form tiny colonies.

Viral genome and transcription in transformed cells. It was determined by spot hybridization that 313Y cells had the *dl*313 genome, since DNA from 313Y cells could be hybridized with whole viral DNA (Fig. 3C) and the left-end fragment (3.8%, Fig. 3A) but not with the fragment between 3.8–9.4 map units (Fig. 3B). DNA from 5WY cells could be hybridized with all probe DNAs (Fig. 3, Table 4), showing that the cells do contain sequences of both probes used in Fig. 3A and B. From the intensity of the autoradiographic spots, it was estimated that 313Y-1 to -6 cells contained one to five copies of the viral genome per cell (Table 4). The genome in 313Y cells did not contain the whole portion of the E1b gene, and the cells seemed to be transformed incompletely by the *dl*313 genome (E1a is present but E1b is mostly deleted), as suggested by the difference in transformed phenotype between 313Y and 5WY cells. Viral RNAs in transformed cells were analyzed by the S1 mapping method (Fig. 4). Two major species of mRNA (660 + 375 nucleotides and 485 + 375 nucleotides) were transcribed by the E1a gene, and a major species of mRNA (1,800 + 485 nucleotides) was transcribed by the E1b gene in 5WY cells, as well as in wild type-infected KB cells. In contrast, a major species of mRNA (660 + 100 + 440 nucleotides) was transcribed by the portion of the viral genome consisting of the E1a and the downstream part of the E1b gene in 313Y cells, as well as in *dl*313-infected KB cells. The results indicated that the viral genome was transcribed in 5WY or 313Y cells, but the pattern of transcription was different between 5WY and 313Y cells, just as it was between wild type- and *dl*313-infected KB cells.

Induction of cellular DNA synthesis in nongrowing 3Y1 cells. Confluent monolayers of 3Y1 cells were infected with Ad5 wild type, *dl*312, and *dl*313 at 10 PFU/cell and labeled with [^{3}H]thymidine for 2 h at intervals of 8 h. Induction of DNA synthesis was observed in Ad5 wild type- and *dl*313-infected cells but not in *dl*312- and mock-infected cells (Fig. 5). In wild type- and *dl*313-infected cells, most of the synthesized DNA was cellular but not viral, as shown by DNA-DNA hybridization (Table 5). The results showed that Ad5 wild type and *dl*313 induced cellular DNA synthesis in nongrowing 3Y1 cells, but *dl*312 did not.

DISCUSSION

A mutant, *dl*312, lacks 902 base pairs between 1.5 and 4.5 map units (E1a), and the other mutant, *dl*313, lacks 2,350 base pairs between 3.5 and 10.5 map units (E1b) (10). In 3Y1 cells infected with *dl*313 at 10 PFU/cell, the E1a gene was expressed, but the E1b gene was not. In the cells, cellular DNA synthesis was induced immediately after infection, and transformed foci appeared after incubation for 4 weeks. In 3Y1 cells infected with *dl*312 at 10 PFU/cell, neither morphological alteration nor induction of cellular DNA synthesis was observed, possibly due to the lack of E1a and E1b gene expression. In the cells, no transformed focus appeared after long incubation. This observation indicates that the E1a gene is expressed without expression of the

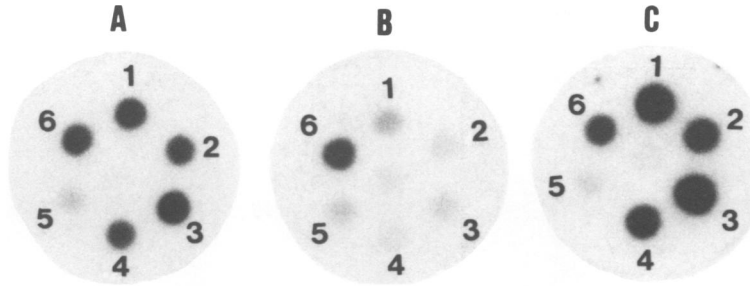

FIG. 3. *Detection of viral DNA in transformed cell DNA. Twenty micrograms each of denatured DNA from 313Y (1-4), 5WY-1 (6), 12WY-1 (rat 3Y1 cells transformed with Ad12 wild type) (5), and 3Y1 cells (central spot) were spotted and hybridized with the nick-translated probes, Ad5 whole DNA (10^8 cpm/μg) (C), XbaI fragment E (0-3.8 map units) (A), or BglII-XbaI fragment B (3.8-9.6 map units) of Ad5 DNA (B) (spot hybridization). The filters were washed, dried, and exposed to an X-ray film for autoradiography.*

TABLE 4. *Detection of viral DNA in six transformed cells by spot hybridization*

DNA from:	^{32}P-Ad5 viral DNA[a]		
	Whole DNA	0-3.8 map units	3.8-9.4 map units
313Y-1 cells	++	+	−
313Y-2 cells	+	++	−
313Y-3 cells	++	+	−
313Y-4 cells	+	++	−
313Y-5 cells	+	+	−
313Y-6 cells	++	+	−
5WY-1 cells	±	+	+
5WY-2 cells	+	++	++
3Y1 cells	−	−	−
Ad5 DNA (control)[b]			
0	−	−	−
0.5	±	±	±
1	+	+	+
2	+	+	+
3	+	+	+
5	++	++	++
10	+++	+++	+++

[a] +++, ++, +, ±, −: the intensity of a spot, corresponding to the number of viral DNA copies.
[b] Number of viral DNA copies per spot.

E1b gene and it is involved in cellular DNA synthesis and in transformation of cells. It also shows that the E1b gene is not expressed without expression of the E1a gene and is involved in neither cellular DNA synthesis nor appearance of transformed foci. This notion is in agreement with the pre-early function of the E1a gene, which regulates the transcription of the other early genes (1, 11).

Although foci of transformed cells appeared in 3Y1 cells infected with both *dl*313 and Ad5 wild type, some differences were observed between cells transformed by *dl*313 and Ad5 wild type. The differences included morphology of foci and cells and growth in soft agar cultures. Taken together, it can be said that the transformed phenotype of cells induced by *dl*313 is incomplete (or partial) compared with that of cells induced by Ad5 wild type. It has been reported that the transformed phenotype of cells induced by the E1 gene (E1a + b) is complete, whereas that of cells induced by the E1a gene only is incomplete or partial (19, 20). Since *dl*313 has the E1a gene and lacks the E1b gene, cells transformed by *dl*313 must be similar to cells transformed by the E1a gene only.

Jones and Shenk (10) reported that *dl*313 did not induce transformed foci in primary culture of rat embryo or rat brain cells. The difference between their results and the present results may be due to different cells used for transformation. Rat 3Y1 cells are very sensitive to cell transformation, and the use of the cells enabled us to detect incomplete or atypical transformation. Graham et al. (7) reported that Ad5 host range I (hr-1) mutants induced no focus in rat embryo and rat embryo brain cells, but did induce foci of atypically transformed cells in baby rat kidney cell cultures. However, it was difficult to subculture the transformed cells. In contrast to the atypically transformed cells, cells incompletely transformed by Ad5 *dl*313 grew well in subculture. The difference between hr-1 mutant-transformed and *dl*313-transformed cells may be due to the mutated E1a gene products of hr-1 and to the E1a gene products of *dl*313. The difference may also be due to the nature of the 3Y1 cell line compared with the primary culture of rat kidney cells. Houweling et al. (9) reported partial transformation of primary baby rat kidney cells by the leftmost 4.5% fragment of Ad5 DNA. The partially transformed cells and incompletely transformed cells described here are similar in phenotype, suggesting the presence of the E1a gene function and the lack of the E1b gene function in both cell lines.

Immunoprecipitation of tumor antigens in cells transformed by fragments of Ad5 DNA shows nine kinds of T antigens, 65,000-dalton

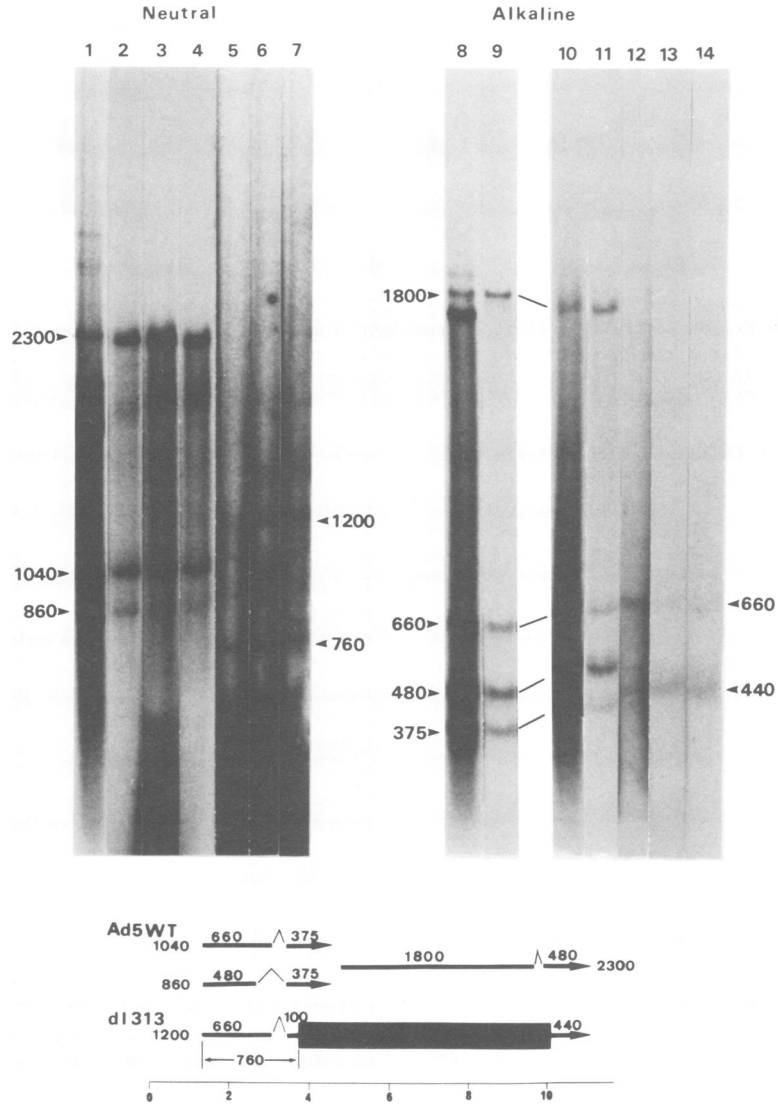

FIG. 4. *Transcriptional patterns of the E1a region in infected and transformed cells. Cytoplasmic RNA was extracted from KB cells infected with either Ad5 wild type or dl313 in the presence of cytosine arabinoside (20 μg/ml) at 16 h postinfection and from transformed cells (5WY-1, 313Y-1, and 313Y-2 cells). These RNAs were hybridized with either ^{32}P-labeled Ad5 DNA or XhoI fragment C (0–15.5 map units) of Ad5 DNA. After hybridization, the samples were treated with nuclease S1 and analyzed by either neutral or alkaline electrophoresis in 1.4% agarose gel. Autoradiograms of the gels were prepared. RNAs from wild type-infected KB (1, 8) and 5WY-1 cells (3, 10) were hybridized with Ad5 DNA. RNAs from wild type-infected KB (2, 9), 5WY-1 (4, 11), dl313-infected KB (5, 12), 313Y-1 (6, 13), and 313Y-2 cells (7, 14) were hybridized with XhoI fragment C of Ad5 DNA. Patterns of RNA transcribed by the E1 region are deduced and depicted under the gel.*

(65K), 52K, 42–34K, 25K, 19K, 18K, 17K, 16K, and 15K polypeptides (13). Of these polypeptides, 34–42K polypeptides seem to be coded by the E1a gene, because they are detected in cells transformed by the leftmost 4.5% of Ad5 DNA. Protein synthesis in vitro shows that three size classes of Ad5 E1a mRNA direct synthesis of at least five polypeptides: 42 and 54K polypeptides directed by 0.9 kilobase mRNA, 48 and 58K polypeptides directed by 1.1 kilobase mRNA, and a 28K polypeptide directed by 0.4 kilobase mRNA (4). The above data show that the E1a

TABLE 5. *Viral and cellular DNA in infected cells*

Infected cells	Input DNA (cpm)	DNA hybridized[a]		
		Viral	Cellular	Blank
Wild type-infected 3Y1 cells	97,000	1,650 (1.7)	11,000 (11.3)	400
*dl*313-infected 3Y1 cells	76,000	440 (0.46)	9,550 (9.9)	450
3Y1 cells	28,000	470 (1.6)	3,300 (11.8)	380
Wild type-infected HEK cells	46,000	18,600 (40.0)	2,500 (5.4)	330

[a] Radioactivity (cpm) hybridized per filter. Percentage of hybridized DNA (cpm) to input DNA (cpm) is given in parentheses.

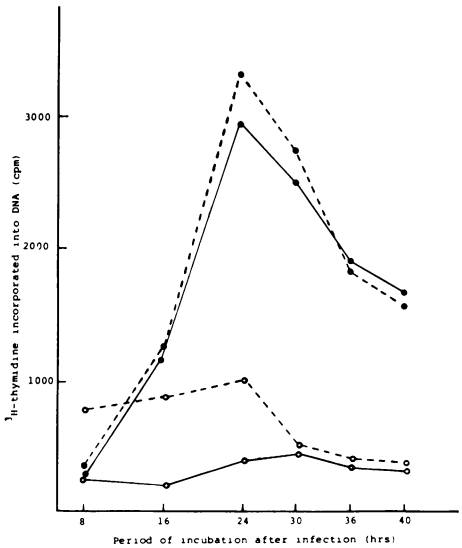

FIG. 5. *The time course of DNA synthesis after infection of 3Y1 cells. Confluent monolayers of 3Y1 cells were maintained in Eagle MEM without serum for 3 to 4 days. Then the cells were infected at 10 PFU/cell with Ad5 wild type, dl312, and dl313 and incubated. At intervals of 8 h, the cells were labeled with [^{3}H]thymidine (2 μCi per dish) for 60 min and harvested. Acid-insoluble radioactivity in each dish was counted and expressed in counts per minute per dish. Symbols: ●——●, Ad5 wild type; ●- - -●, dl313; ○——○, dl312; ○- - -○, medium without virus.*

gene of Ad5 is responsible for incomplete transformation. Since many polypeptides are coded by the E1a gene, it is difficult at present to determine which of these polypeptides is responsible for incomplete transformation. The probable candidates are 34-42K polypeptides (13) or some of the 42, 48, 54, and 58K polypeptides (4), since a similar cluster of polypeptides, 35 to 40K, is responsible for Ad12 incomplete transformation (17) and is coded by the E1a gene of Ad12 (14).

The E1a gene of *dl*313 induces altered products, 40 and 36K polypeptides (4). The present data suggest that the E1a gene is expressed in *dl*313-infected cells and that the altered gene products are functionally intact in inducing incomplete transformation, although their molecular weights are lower. It is conceivable that the E1a gene is involved not only in expression of other viral early genes (1, 11) but also in expression of cellular genes. In rat 3Y1 cells, the E1a gene of *dl*313 may modify expression of cellular genes, may induce cellular DNA synthesis, and may consequently lead cells to incomplete transformation. Further studies are required to substantiate the speculation.

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