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Role of the Adenovirus Early Region 1B Tumor Antigens in Transformation and Lytic Infection

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We have investigated the contribution of each of the two adenovirus type 5 (Ad5) major early region 1b (E1b) proteins in cell transformation and in lytic infection. An Ad5 E1 plasmid, in which the reading frame for the 19-kDa E1b protein was abolished by a stop codon close to the initiation codon, transformed primary baby rat kidney (BRK) cells with an efficiency of about half of that of a wild type Ad5 E1 plasmid, whereas a plasmid with a mutation in the gene for the 58-kDa E1b protein transformed the same primary cells with only one-third of the wild type efficiency. Plasmids containing region E1a only or a plasmid carrying mutations in the genes for major E1b proteins all transformed primary cells with an efficiency of approximately 5% of wild type. To test the effect of the E1b mutations in virion-mediated cell transformation, the mutant E1b regions were introduced into intact viral genomes by overlap recombination and were subsequently used in a transformation assay on BRK cells. The 19 and 58-kDa mutant viruses were found to transform BRK cells with 11 and 25% of the efficiency of wild type virus, respectively. These results suggest that the 19-kDa E1b protein is essential for virus-mediated cell transformation, in agreement with results of others, but not for plasmid-mediated cell transformation. In lytic infection, the 19-kDa mutant virus was some 30-fold reduced in yield on HeLa cells, whereas the 58-kDa mutant virus was 3000-fold reduced in its ability to grow on HeLa cells at low multiplicity of infection, but showed a marked multiplicity-dependent leakiness. The 58-kDa mutant virus was not defective when its growth was assayed on human embryonic kidney (HEK) cells. This may indicate that cellular proteins are expressed in HEK cells that are functionally homologous to the 58-kDa E1b protein.

INTRODUCTION

Human adenoviruses have the capacity to transform rodent cells *in vitro*. The genetic information required to induce transformation is contained within the E1 region (0–11 map units, Graham *et al.*, 1974b; Gallimore *et al.*, 1974), which consists of two transcriptional units: E1a and E1b (Wilson *et al.*, 1979). The two major products encoded by region E1b in both infected and transformed cells appear to have molecular weights of 53–65 kDa and 15–19 kDa, depending on the gel system or molecular weight markers used (Halbert *et al.*, 1979; Schrier *et al.*, 1979; Jochemsen *et al.*, 1980; Esche and Siegmann, 1982). The most commonly used molecular weights for these proteins are 58 and 19 kDa, respectively. In transformed cells, both proteins are made from a single 2.2-kb mRNA by using different translational start codons (Bos *et al.*, 1981). In infected permissive cells an mRNA of 1.0 kb accumulates late in infection, which encodes only the 19-kDa protein. The existence of a third E1b mRNA, encoding a 58-kDa-related peptide, was recently demonstrated by amino acid sequence analysis of an E1b-encoded protein (Anderson *et al.*, 1984) and cDNA cloning (Virtanen *et al.*, 1982). Finally, E1b encodes an mRNA transcribed from an independent promoter which specifies structural protein pIX (Wilson *et al.*, 1979; Aleström *et al.*, 1980).

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Region E1b contributes to cell transformation in several ways. First, it is required for expression of the transformed phenotype, since cells transformed by region E1a only are semitransformed in appearance and grow to lower saturation density than cells containing the entire E1 region (Houweling *et al.*, 1980; Shiroki *et al.*, 1979, 1981). Second, region E1b has a profound effect on the efficiency of focus formation in primary cells, since DNA fragments containing region E1a only transform with much lower efficiency than fragments containing the entire E1 region (Houweling *et al.*, 1980; van den Elsen *et al.*, 1982). Third, region E1b is required for tumorigenicity, since both cells transformed by region E1a only or by E1a and part of E1b manifest a reduced capacity to induce tumors in experimental animals (Shiroki *et al.*, 1979; Jochemsen *et al.*, 1982; Gallimore *et al.*, 1984).

The contribution of each of the two major E1b proteins in transformation, however, is far from clear. DNA transfection studies have shown that the left-terminal *Hin*dIII G fragment (0–8 map units) is sufficient for complete morphological transformation of primary rat kidney (BRK) cells (Graham *et al.*, 1974a; van der Eb and Houweling, 1977). Since cells transformed by this fragment do not express the 58-kDa E1b protein, these results suggested that this protein is not required for complete morphological transformation (Schrier *et al.*, 1979). In contrast, viruses that fail to express the 58-kDa E1b protein are unable to transform a variety of primary cells (Graham *et al.*, 1978; Ho *et al.*, 1982), although DNA extracted from these mutant viruses was capable of transforming rodent cells. This suggested that the 58-kDa protein is required for virus-mediated transformation but not for DNA-mediated transformation (Rowe and Graham, 1983). A similar result, although less pronounced, was obtained by Babiss *et al.* (1984). On the other hand, Mak and Mak (1983) have shown that Ad5*dl*313, a virus which lacks the entire E1b region, does transform BRK cells at high multiplicity of infection, suggesting that the 58-kDa protein is not strictly required for virus-mediated cell transformation.

The role of the 19-kDa E1b protein in transformation is less controversial. Viruses with mutations in the gene for this protein have been shown to be drastically reduced in their capacity to transform a variety of cell types (Chinnadurai, 1983; Pilder *et al.*, 1984; Babiss *et al.*, 1984; Takemori *et al.*, 1984; White *et al.*, 1984; Fukui *et al.*, 1984; Subramanian *et al.*, 1984b). Similar results have been obtained in transfection studies with DNA extracted from the mutant virions (Chinnadurai, 1983; Babiss *et al.*, 1984).

Apart from its role in transformation, E1b has been shown to be required for the efficient production of progeny virus (Harrison *et al.*, 1977; Jones and Shenk, 1979).

To determine the contribution of the two E1b proteins in transformation and lytic infection, and to investigate whether mutations in these proteins might differently affect virion-mediated and DNA fragment-mediated transformation, we have constructed Ad5 region E1 plasmids with specific mutations in the genes coding for the two E1b proteins. These mutant E1 sequences were subsequently introduced into intact viral genomes, so that the effects of the same mutations could be examined in DNA-mediated and virus-mediated transformation, as well as in productive infection. We show here that the 19-kDa protein is required for efficient virus-mediated transformation but not for plasmid-mediated transformation. In contrast, the 58-kDa protein was found to be equally required for both processes. In lytic infection, the 19-kDa mutant virus was slightly defective, whereas the 58-kDa mutant virus was severely restricted but showed a marked multiplicity-dependent leakiness.

MATERIALS AND METHODS

Construction of mutant plasmids. Plasmid p5dlTth was constructed from plasmid p5HindG. This recombinant contains the Ad5 *Hin*dIII G fragment inserted into vector pAT153. The plasmid contains a single *Tth*III I site at nucleotide 2395 of the Ad5 sequence. To create a frame shift mutation at this site, p5HindG was digested to completion with *Tth*III I and made blunt by

incubation with DNA polymerase I (large fragment) in the presence of the four dNTPs. After ligation, the DNA was used to transform *Escherichia coli* HB101. Ampicillin-resistant colonies were selected for the loss of the *Tth*III I site. One such clone was found to have a deletion of 2 bp at the *Tth*III I site by DNA sequence analysis, probably as a result of a small amount of exonuclease in one of the enzymes. This mutant *Hin*dIII G fragment was subsequently reintroduced into a full-length E1 clone (*Xho*I C fragment, 0–15.5 map units) by using standard cloning procedures, yielding p5dlTth.

The plasmid p5dlSacKpn was constructed from the plasmid p5dlSac, described previously (Bernards *et al.*, 1983a). The mutation at the *Kpn*I site was made by cleaving p5dlSac with *Kpn*I, after which the 3′-protruding ends were made blunt by S1 nuclease treatment (see Bos *et al.*, 1981). Subsequently, the plasmid was recircularized with T4 ligase and used to transform *E. coli* HB101. The DNA sequence of one of the *Kpn*I deletion mutants (p5dlSacKpn) revealed that this mutant had a deletion of 92 bp at the *Kpn*I site (Table I).

Construction of mutant viruses. Viruses Ad5*dl*19 and Ad5*dl*58 were made from mutant plasmids p5dlSac and p5dlTth, respectively, by overlap recombination, a detailed protocol which has been described previously (Bernards *et al.*, 1983b, 1984).

Cells and viruses. Monolayers of HeLa cells, KB cells and BRK cells were grown in Eagle's minimum essential medium (MEM) supplemented with 8% newborn calf serum. HEK cells were grown in MEM supplemented with amino acids, vitamins, and 10% fetal calf serum. Growth of viruses on HeLa, KB, and human embryonic kidney (HEK) cells was determined by infecting subconfluent cultures of cells (5.10^5 cells per dish) with 1 or 10 PFU of virus per cell for 1 hr at 37°. Virus was diluted in PBS + 2% fetal calf serum, and cultures were infected with 0.5 ml of virus dilution per 6-cm dish. After infection, cells were washed four times with PBS and incubated for 4 days in 5 ml of MEM + 2% fetal calf serum. At Day 4, cells and medium were collected and freeze-thawed five times to release virus from the cells. Subsequently, cell debris was removed by centrifugation and the virus yield was determined by plaque assay on 293 cells.

Transformation of BRK cells. DNA transfection experiments were performed essentially as described by van der Eb and Graham (1980), the only modification being that cells were treated 4 hr post-transfection with 10% DMSO in PBS for 90 sec. Virus-mediated transformation experiments were performed by infecting subconfluent cultures of BRK cells at a multiplicity of 10 PFU per cell for 1 hr at 37° (0.5 ml per 6-cm dish). Virus was diluted in PBS + 2% fetal calf serum and was inactivated by irradiating the diluted virus stocks with 200 $J \cdot m^{-2}$ of uv light prior to infection.

RESULTS

Construction of Mutant E1b Plasmids and Viruses

In a previous report we have described the construction of two Ad5 E1 plasmids carrying specific mutations in the genes for the 19-kDa (p5dlSac) or 58-kDa (p5dlHind) major E1b proteins (Bernards *et al.*, 1983a; Fig. 1). In both plasmids the lesions consisted of a frame shift mutation downstream from the translational start codon of the E1b gene in question. Since the p5dlHind plasmid retained the coding information for a considerable part of the 58-kDa E1b protein, we constructed a second mutant Ad5 E1 plasmid, p5d1Tth, which carried a more upstream stop codon in the reading frame for the 58-kDa protein (Fig. 1, Table 1). This mutation does not affect the reading frame for the 58-kDa related peptide recently identified by Anderson *et al.* (1984). Additionally, a mutant E1 plasmid p5dlSacKpn was constructed in which the genes for both major E1b proteins were mutated. In this plasmid the translational start codon for the 58-kDa protein (position 2019-2021) was lost as a result of a deletion of 92 bp at the *Kpn*I site at position 2048 (Fig. 1, Table 1).

To investigate the contribution of the 19- and 58-kDa E1b proteins in virion-me-

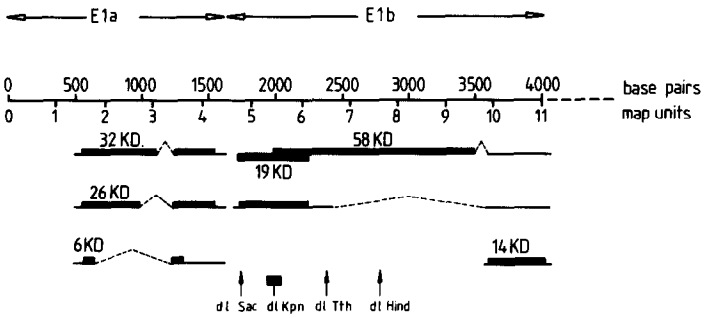


FIG. 1. Schematic representation of the E1 region of Ad5. Solid lines represent DNA segments transcribed into RNA, dotted lines represent introns. Black bars represent protein-coding sequences. The positions of the various deletions and insertions are indicated.

diated cell transformation and in lytic infection, the mutations of p5dlSac and p5dlTth were introduced into an intact viral genome by overlap recombination (Bernards *et al.*, 1983b, 1984). This method involves cotransfection into permissive human cells of a mutant plasmid and a subgenomic fragment of Ad5 DNA which lacks part of the E1 region. Infectious virus can arise only if the two DNA fragments recombine to yield a complete viral genome. Thus, to construct a virus with a mutation in the gene for the 19-kDa E1b protein, p5dlSac (0–15.5 map units) was cotransfected with the Ad5*dl*309 *Xba*I A fragment (3.8–100 map units) into 293 cells in a plaque assay. These cells express the Ad5 E1 gene products allowing efficient replication of the mutant viruses (Aiello *et al.*, 1979). To identify mutant viruses, individual plaques were picked and propagated further in 6-cm dishes of 293 cells. After the development of a cytopathic effect, viral DNA was isolated and digested with restriction endonuclease *Sac*I. Mutant viruses should yield a novel 5411-bp *Sac*I fragment, which arose by fusion of the 1770-bp *Sac*I E fragment and the 3641-bp *Sac*I G fragment. A plaque isolate which manifested the novel *Sac*I cleavage pattern (not shown) was plaque-purified once more and propagated further on 293 cells. This virus was named Ad5*dl*19. A similar procedure was followed to construct Ad5*dl*58,

TABLE 1

NATURE OF THE DELETIONS AND INSERTIONS IN THE E1b REGION OF Ad5

Plasmid	Mutation[a]	Affected protein	Truncated product (predicted)
p5dlSac	−11 (1771–1781)	19 kDa	2 kDa
p5dlHind	+4 (2805)	58 kDa	28.5 kDa
p5dlTth	−2 (2399–2400)	58 kDa	13 kDa
p5dlSacKpn	−11 (1771–1781)	19 kDa	2 kDa
	−92 (1978–2069)	58 kDa	—
p5HpaIE	lacks E1b (1572–4070)	19 kDa	—
		58 kDa	—

Note. Plasmids p5dlSac, p5dlHind and p5HpaI E have been described elsewhere (Bernards *et al.*, 1982, 1983a). The exact nature of the mutations in the plasmids p5dlTth and p5dlSacKpn was determined by DNA sequence analysis. The predicted molecular weights of the truncated products encoded by the mutant E1 plasmids was calculated from the nucleotide sequence of the E1b regions as determined by Bos *et al.* (1981).

[a] − = deletion; + = insertion.

a virus containing the mutation of p5dlTth in the gene for the 58-kDa E1b protein.

To determine the expression of the E1b proteins in mutant virus-infected cells, HeLa cells were infected with Ad5*dl*19 and Ad5*dl*58 at a multiplicity of 10 PFU per cell in the presence of 20 μg/ml of cytosine arabinoside, and labeled from 36 to 40 hr postinfection with [^{35}S]methionine. Ad5-specific proteins were precipitated from infected cells with an Ad5 anti-tumor serum which precipitates mainly the E1a proteins and the 58-kDa E1b protein, and with a monoclonal antibody against the 19-kDa E1b protein (kindly provided by Dr. A. Zantema). As can be seen in Fig. 2, Ad5*dl*58-infected cells fail to produce the 58-kDa E1b protein and Ad5*dl*19-infected cells fail to produce the 19-kDa E1b protein, whereas cells infected with Ad5*dl*309, the parental virus used to construct the mutants, produced both E1b products. The level of expression of the 19-kDa protein in the Ad5*dl*58-infected cells was consistently somewhat reduced when compared to Ad5*dl*309-infected cells. A more detailed analysis of the effect of E1b mutations on the expression of other early viral genes will be presented elsewhere. The immunoprecipitation data, together with the altered restriction endonuclease cleavage pattern of the mutant viruses (not shown), clearly demonstrate that the mutant E1b viruses carry the same mutations as those the mutant E1b plasmids used for their construction.

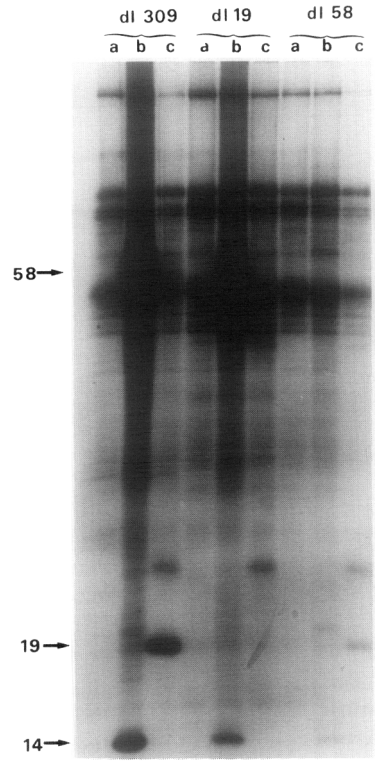


FIG. 2. Polyacrylamide gel electrophoresis of T antigens precipitated from HeLa cells infected with mutant E1b viruses. HeLa cells were infected with 10 PFU per cell of Ad5*dl*309 (left 3 lanes), Ad5*dl*119 (middle 3 lanes), and Ad5*dl*58 (right 3 lanes) in the presence of 20 g/ml of cytosine arabinoside and labeled between 36 and 40 hr postinfection with [^{35}S]methionine as described (Bernards *et al.*, 1982). The sera used were (a) rat pre-immune serum, (b) Ad5 anti-tumor serum, (c) monoclonal antibody against the Ad5 19-kDa E1b protein.

Transformation by Mutant Plasmids and Viruses

To assess the contribution of the 19- and 58-kDa E1b proteins in the process of cell transformation, primary cultures of BRK cells were transfected with the mutant E1b plasmids using the calcium-phosphate coprecipitation technique (van der Eb and Graham, 1980). Foci of morphologically transformed cells were first detected after 2 weeks and counted after 4 weeks. The efficiencies of focus formation of the various mutant plasmids are given in Table 2A. Unexpectedly, the 19-kDa mutant plasmid was found to transform the BRK cells with an efficiency of about half that of wild type E1 plasmid. This result was surprising since viruses with a mutation in the 19-kDa E1b protein have previously been shown to be transformation-defective (Chinnadurai, 1983; Fukui *et al.*, 1984; Pilder *et al.*, 1984; Babiss *et al.*, 1984; White *et al.*, 1984; Subramanian *et al.*, 1984b; Takemori *et al.*, 1984). The two different 58-kDa mutant plasmids both transformed the BRK cells with one-third of wild type efficiency. The 19- + 58-kDa double-mutant plasmid transformed the primary cells with an efficiency which was only slightly higher than that of a plasmid containing

TABLE 2

TRANSFORMATION OF BRK CELLS BY MUTANT PLASMIDS (A) AND MUTANT VIRUSES (B)

A: Plasmid	E1b mutation	Foci/g genome equivalent DNA	% of wild type
p5XhoC	—	15.1 ← 5	100
p5dlSac	19 kDa only	7.3 ← 2.5	48
p5dlTth	58 kDa only	5.0 ← 1.5	33
p5dlHind	58 kDa only	4.5 ← 1.9	30
p5dlSacKpn	19 + 58 kDa	1.1 ← 0.2	7
p5HpaIE	lacks E1b	0.4 ← 0.3	3
B: Virus	**E1b mutation**	**Foci/dish**	**% of wild type**
Ad5*dl*309	—	39 ← 14	100
Ad5*dl*19	19 kDa only	4.4 ← 0.6	11
Ad5*dl*58	58 kDa only	9.6 ← 3.5	25
Ad5*dl*315	lacks E1b	0	0

Note. In each DNA transfection experiment, 5 replicate 6-cm dishes of BRK cells were transfected with 5 μg genome equivalent of DNA (1 μg genome equivalent is equivalent to 1 μg of Ad5 DNA and corresponds to 0.27 μg of Ad5 E1 plasmid). The results given are the average values (←SD) of three transformation experiments. For virus-mediated transformations, 5 replicate dishes (6 cm) of subconfluent BRK cells were infected with 10 PFU of uv-inactivated virus per cell. Data are the average values of three independent experiments.

region E1a only (p5HpaIE, Table 2A). This indicates that the low efficiency of transformation found for the *Hpa*I E fragment is not due to the fact that this fragment lacks the E1A polyadenylation signal (van den Elsen *et al.*, 1982).

Morphologically, the p5*dl*Sac-transformed cells did not differ significantly from wild type E1-transformed cells (Figs. 3A, B) and a similar morphology was found for p5*dl*Hind-transformed cells (Fig. 3C). The morphology of p5*dl*Tth-transformed cells was variable, about half of the foci showing the epithelioid morphology characteristic for adenovirus-transformed cells (Fig. 3D) and the other half of the foci consisted of less densely packed fibroblastic cells (Fig. 3E). Cells transformed by the p5*dl*SacKpn double-mutant plasmid were morphologically much like E1a-transformed cells (Fig. 3F).

The differences in relative transformation efficiency between our 19-kDa mutant plasmid and the 19-kDa mutant viruses studied by others may well be caused by viral genes located outside the transforming region. Alternatively, the different nature of the mutations may be the cause of the different results. To discriminate between these two possibilities, we introduced our mutant E1b plasmids into intact viral genomes and tested the transforming activity of the resulting virions in primary cells. Transformation of rat cells by adenovirus virions is complicated by the fact that these cells are semipermissive for Ad5 (Gallimore, 1974). Therefore, Ad5 may cause extensive cell death in primary cultures of rat cells at high multiplicity of infection, which may reduce the frequency of transformation. To circumvent this problem, we used uv-inactivated virus stocks for transformation experiments with virus (see under Methods). As a control we used Ad5*dl*309, the Ad5 parental virus from which the mutants were derived, as well as Ad5*dl*315, an E1b deletion mutant which was previously shown to be defective in transformation (Jones and Shenk, 1979). The results (Table 2B) indicate that the 19-kDa mutant virus is drastically reduced in its capacity to transform BRK cells when compared to Ad5*dl*309. When the relative efficiency of transformation of the 19-kDa mutant plasmid (50%, Table 2A) is compared to that of the 19-kDa mutant virus

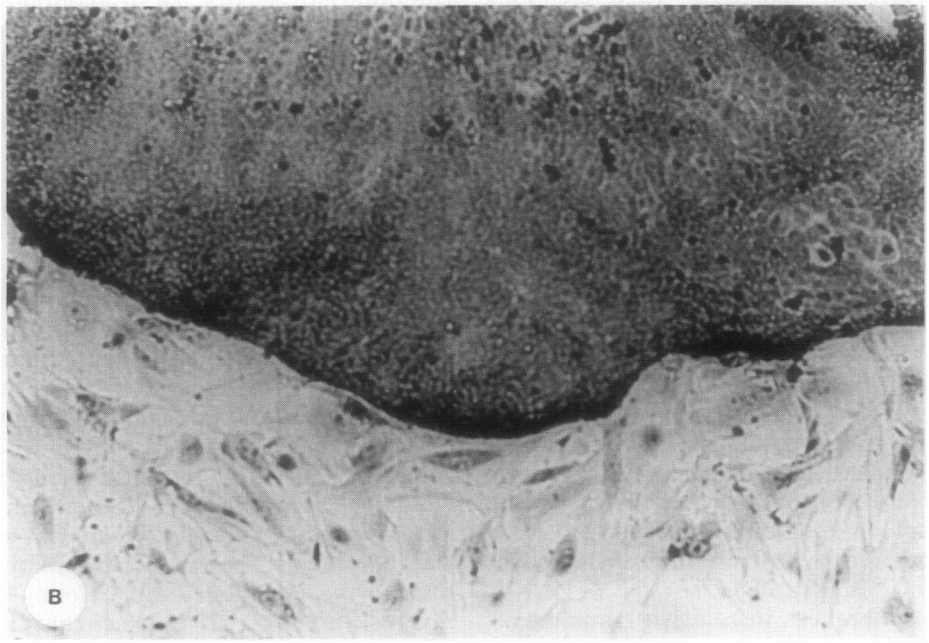
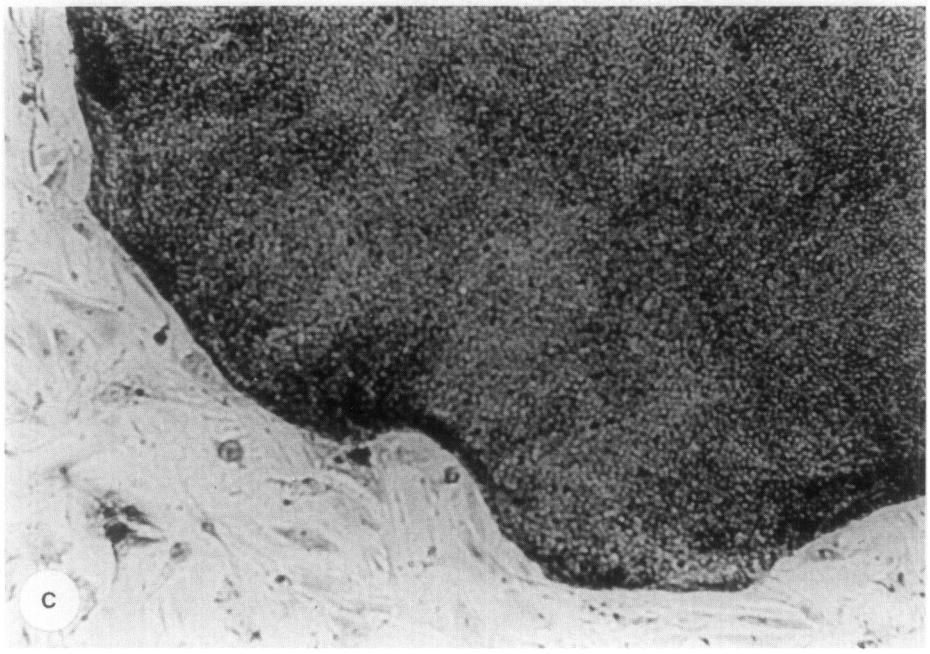


FIG. 3. Photomicrographs of Ad5-transformed cells. Primary foci of transformed BRK cells were fixed 3 weeks after transfection with methanol/acetic acid and Giemsa stained. Magnification ×100. Cells were transformed by (A) p5XhoC; (B) p5dlSac; (C) p5dlHind; (D) and (E) p5dlTth; (F) p5HpaI E.



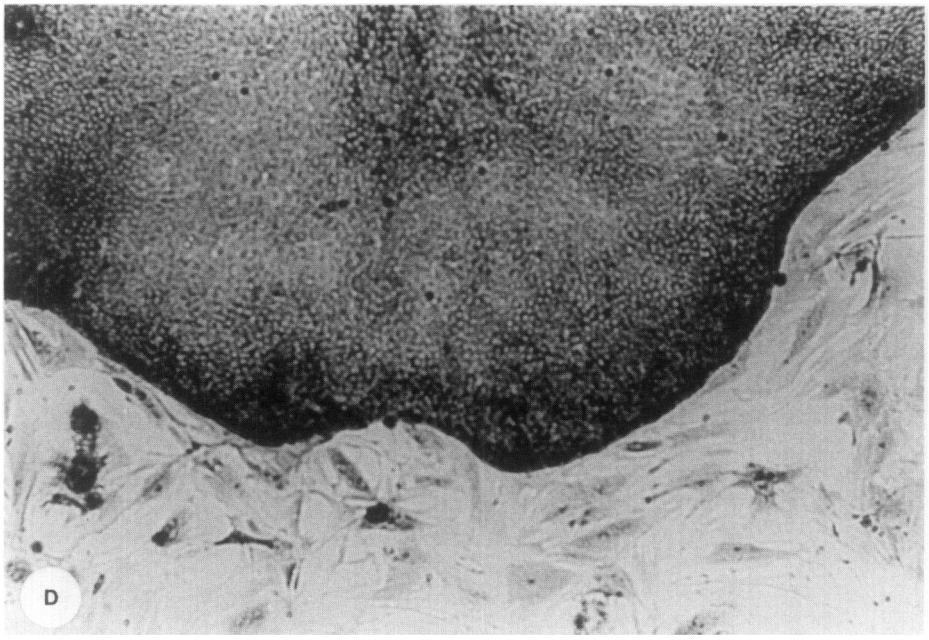
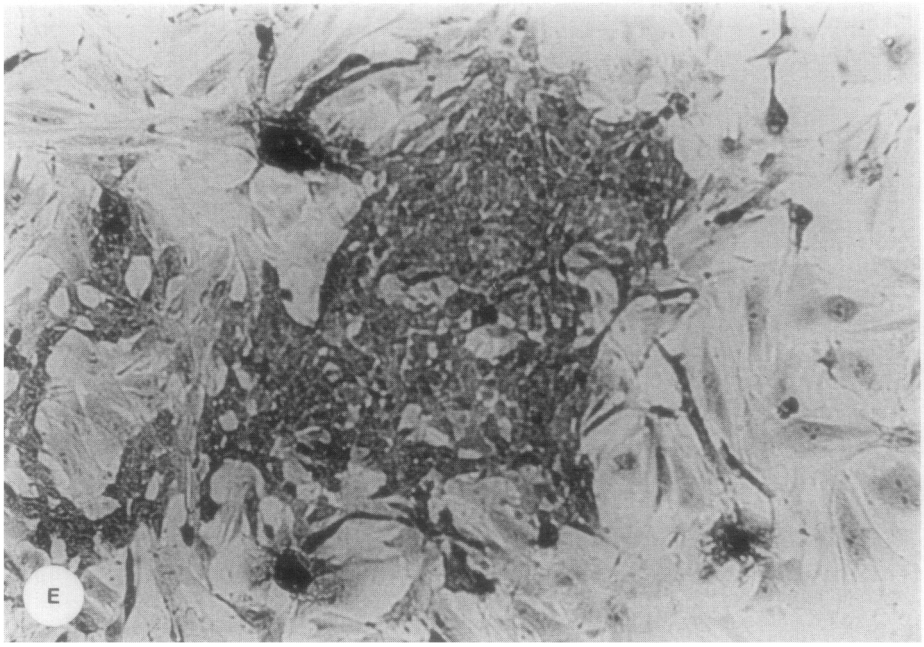
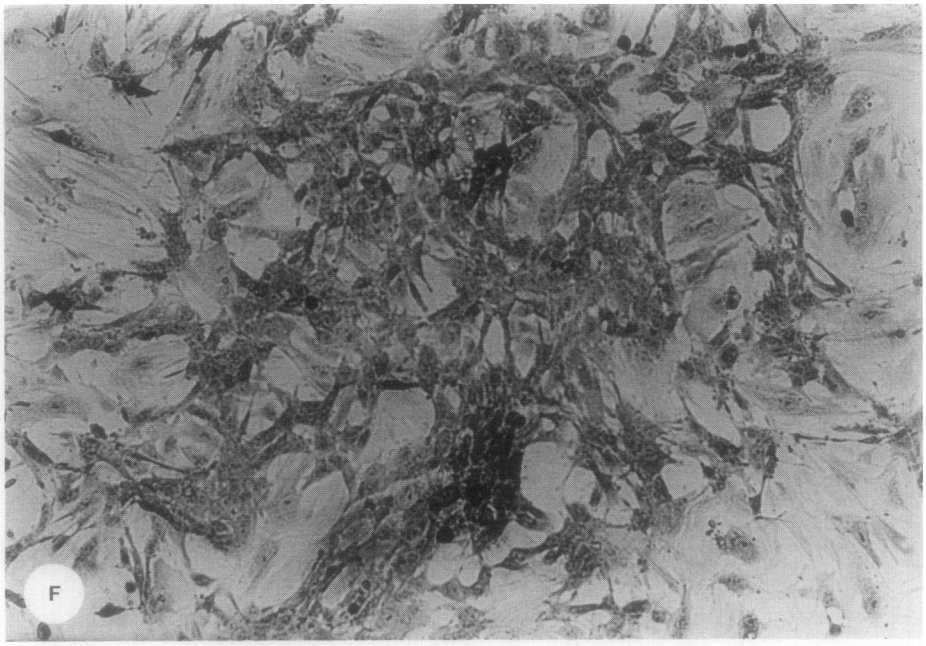


FIG. 3—*Continued.*

(11%, Table 2B) it is evident that the 19-kDa E1b protein is much more important for virus-mediated cell transformation than for plasmid-mediated cell transformation. In contrast, the 58-kDa mutant virus transformed BRK cells with a relative





efficiency similar to that obtained with the 58-kDa mutant plasmid (25 vs 33%, Table 2A, B). The latter result is in conflict with those of Graham *et al.* (1978), who concluded that the 58-kDa E1b protein is essential for virus-mediated cell transfor-

mation. Rather, our present data indicate that the 19-kDa protein plays an important role in virus-mediated cell transformation, which is in good agreement with recent reports showing that 19-kDa mutant viruses were either completely, or almost completely transformation-defective (Chinnadurai, 1983; Fukui *et al.*, 1984; Pilder *et al.*, 1984; Babiss *et al.*, 1984; White *et al.*, 1984; Subramanian, 1984b; Takemori *et al.*, 1984). However, the requirement of the 19-kDa E1b protein in cell transformation is not absolute, since efficient transformation could be obtained with 19-kDa mutant plasmids (Table 2A).

Early Region 1b Proteins in Lytic Infection

The role of the E1b proteins in lytic infection was assessed by comparing the growth potential of Ad5*dl*19 and Ad5*dl*58 in HeLa cells, 293 cells, and HEK cells. The growth potential of the mutants in each cell type was assayed using indirect plaque titration (see under Methods). The yields of each virus are given in Table 3. As expected, Ad5*dl*309 could be propagated with a similar yield on all three cell types. The Ad5*dl*19 mutant showed a slight host-range phenotype, since it replicated some 30 times less efficiently in HeLa cells than the parental strain Ad5*dl*309 (Table 3). Furthermore, the Ad5*dl*19 mutant was different from wild type Ad5*dl*309 virus in that the mutant caused complete cytopathic effect in the HeLa cells within 60 hr postinfection, whereas Ad5*dl*309 needed 4 days to achieve this at the same multiplicity of infection. A third difference between Ad5*dl*19 and Ad5*dl*309 was that Ad5*dl*19 produced much larger plaques on HEK cells than Ad5*dl*309 (not shown). These data suggest that Ad5*dl*19 manifests the cytocidal (*cyt*) phenotype, first described for Ad12 mutants by Takemori *et al.* (1968), which was recently shown to be caused by mutations in the gene for the 19-kDa E1b protein (Pilder *et al.*, 1984; White *et al.*, 1984; Subramanian, 1984a, 1984b; Takemori, 1984; Chinnadurai, 1983).

A more pronounced host-range phenotype was observed with the Ad5*dl*58 mutant. This virus was 4 logs reduced in its growth in HeLa cells, whereas this defective phenotype was not observed in HEK cells (Table 3). Apparently, HEK cells, but not HeLa cells, can provide a cellular function which can complement the Ad5*dl*58 defect. In agreement with the original observation of Jones and Shenk (1979), a partial complementation was also observed in HEK cells for the Ad5 mutant *dl*315 (Table 3). The fact that only partial complementation was observed on HEK cells is probably due to the fact that *dl*315 is more defective for growth in HeLa cells than Ad5*dl*58, since the HEK/HeLa ratio is basically similar for the two mutants (Table 3).

The growth defect of adenovirus E1a and E1b mutants has been shown to be not absolute, since the mutants often proved to be far less defective at higher multiplicities of infection were used (Frost and Williams, 1978; Shenk *et al.*, 1979; Ross *et al.*, 1980). To investigate the multiplicity-dependence

TABLE 3

HOST-RANGE PHENOTYPE OF MUTANT VIRUSES

Virus	Mutation	Virus yield assayed on (PFU/ml)			Ratio HEK/HeLa	Ratio 293/HeLa
		293	HeLa	HEK		
Ad5*dl*309	—	2.10^9	6.10^8	2.10^9	3	3.6
Ad5*dl*19	19 kDa	2.10^9	2.10^7	6.10^8	30	110
Ad5*dl*58	58 kDa	6.10^9	6.10^4	2.10^8	3000	10^5
Ad5*dl*315	19 + 58 kDa	4.10^9	3.10^2	4.10^6	13000	10^7

Note. Virus yields were determined by indirect plaque titration on 293 cells.

of Ad5*dl*58, we infected HeLa and KB cells with 1 or 10 PFU per cell and scored the production of progeny virus after 4 days in a plaque assay on 293 cells. As can be seen in Table 4, Ad5*dl*58 was 50-fold less defective on HeLa cells and 100-fold less defective on KB cells when infected at 10 PFU per cell instead of 1 PFU per cell. Furthermore, the degree of defectiveness of Ad5*dl*58 was more pronounced on HeLa cells than on KB cells at both multiplicities tested (Table 4). These results indicate that the 58-kDa E1b protein is required for the efficient production of progeny virus, although its relative contribution is not only dependent on the cell type but also on the multiplicity of infection.

DISCUSSION

We have investigated the roles of the 19- and 58-kDa major E1b tumor antigens of Ad5 in cell transformation and lytic infection. A mutation caused by an early stop codon in the gene coding for the 19-kDa protein resulted in a 50% reduction of transforming activity in DNA fragment-mediated transformation, but in a 90% reduction in virion-mediated transformation, as compared to the respective wild type frequencies. A transformation-defective phenotype for 19-kDa E1b mutant viruses has also been observed by others. The transformation defect appears to be independent of the cell type used since it was not only described for BRK cells (Mak and Mak, 1983; White *et al.*, 1984; Subramanian *et al.*, 1984b), but also for primary rat embryo fibroblasts (Subramanian *et al.*, 1984b; Pilder *et al.*, 1984), a rat embryo fibroblast line (CREF cells) (Pilder *et al.*, 1984; Subramanian *et al.*, 1984b; Babiss *et al.*, 1984) and rat 3Y1 cells (Chinnadurai, 1983; Takemori *et al.*, 1984; Fukui *et al.*, 1984). The combined results suggest that the 19-kDa E1b protein is required for virus-mediated cell transformation, and not for plasmid-mediated cell transformation. This difference is most readily explained in terms of the cytocidal (*cyt*) phenotype manifested by viruses with a mutation in the 19-kDa gene. This *cyt* phenotype is characterized by an accelerated development of cytopathic effect and the degradation of both host cell and viral DNA (Subramanian, 1984a; Takemori *et al.*, 1984; White *et al.*, 1984; Pilder *et al.*, 1984). Since rat cells are semipermissive for Ad5 (Gallimore, 1974), the increased cytolytic activity of 19-kDa mutant viruses may also strongly reduce the probability of transformation of these cells. This notion is supported by the observation of White *et al.* (1984), that a 19-kDa mutant virus caused extensive cell death after infection of BRK cells. Transformation could only be obtained by preventing viral DNA replication by a second mutation. The residual 10% transforming activity observed with our 19-kDa mutant virus can then be explained by the fact that we have used uv-inactivated virus for the transformation assays. The 19-kDa protein is, however, by no means irrelevant for transformation since transformed cells which fail to express this protein are unable to grow in semisolid media (Fukui *et al.*, 1984; Subramanian *et al.*, 1984b; Pilder *et al.*, 1984) and are no longer tumorigenic (Bernards *et al.*, 1983a).

The 58-kDa mutant virus Ad5*dl*58 was found to transform BRK cells at 25% of wild type virus efficiency (Table 2B), and a similar decrease in transformation efficiency was found for the 58-kDa mutant plasmid as compared to wild type Ad5 E1 plasmid (Table 2A). This result suggests that the 58-kDa E1b protein is equally important for virus-mediated and plasmid-mediated cell transformation. Transforming activity of Ad5 viruses with mutations

TABLE 4

HOST-RANGE PHENOTYPE OF Ad5*dl*58 AT DIFFERENT MULTIPLICITIES OF INFECTION

Virus	m.o.i.	Virus yield on (PFU/ml) HeLa	KB
Ad5*dl*309	1 PFU/cell	6.10^8	8.10^8
Ad5*dl*309	10 PFU/cell	1.10^9	8.10^8
Ad5*dl*58	1 PFU/cell	6.10^4	2.10^5
Ad5*dl*58	10 PFU/cell	3.10^6	2.10^7

Note. Virus yields were determined by indirect plaque titration on 293 cells.

in the 58-kDa E1b gene has also been reported by Logan *et al.* (1984) and Babiss *et al.* (1984). The latter authors showed that the efficiency of transformation decreased with decreasing size of the residual 58-kDa truncated product. The observation that Ad5 group II hr mutants, which are defective in the synthesis of the 58kD protein, were unable to transform BRK cells (Graham *et al.*, 1978; Ho *et al.*, 1982; Mak and Mak, 1983) may possibly be explained by the fact that these mutants express only very short truncated fragments of the 58-kDa protein. The finding that DNA extracted from the hr II mutant virions did have transforming activity on BRK cells (Rowe and Graham, 1983) indicates that virus-mediated transformation is more dependent on the size of the 58-kDa truncated product than DNA-mediated transformation. This conclusion is supported by the data obtained by Babiss *et al.* (1984). We consider it unlikely that the higher transformation frequencies of 58-kDa mutant viruses obtained by us are due to the fact that we have used uv-inactivated virus for our transformation studies, since (1) uv inactivation does not seem to alter the transformation frequency of wild type virus (cf. Graham *et al.*, 1978), and (2) uv inactivation did not unveil any transforming activity of the transformation defective mutant Ad5*dl*315 (Table 2B).

Our results indicate that the 58-kDa protein is more crucial in determining the transformed morphology than the 19-kDa protein since (1) cells transformed by 19-kDa mutant plasmids were very similar in morphology to wild type EI-transformed cells, and (2) 50% of the foci transformed by a plasmid with an early stop codon in the gene for the 58-kDa protein (p5*dl*Tth, 13-kDa truncated product) consisted of cells with a semitransformed morphology (Fig. 3). The observation that p5*dl*HindIII-transformed cells (28.5-kDa truncated product of the 58-kDa protein) were fully transformed in morphology suggests that cell morphology is also dependent on the size of the 58-kDa truncated protein.

In lytic infection, the yield of the 19-kDa mutant virus on HeLa cells was only some 30-fold lower than that of the wild type Ad5*dl*309 virus. A similar result was recently obtained by Pilder *et al.* (1984) who found that the Ad5 mutant *dl*337 was 10-fold reduced in its growth in HeLa cells and by Subramanian *et al.* (1984a) who showed that a similar mutant was 100-fold reduced in KB cells. These results indicate that the 19-kDa E1b protein is not strictly required for virus growth, in spite of the fact that 19-kDa mutant viruses cause extensive degradation of both cellular and viral DNA in infected cells (Subramanian *et al.*, 1984a; Takemori *et al.*, 1984; White *et al.*, 1984; Pilder *et al.*, 1984).

The 58-kDa mutant virus *dl*58 showed a decrease of 4 logs in its ability to grow in HeLa and KB cells at low multiplicity of infection. A severe host-range phenotype was also reported for other Ad5 E1b mutants such as *hr*6 and *hr*7 (Harrison *et al.*, 1977; Ho *et al.*, 1982) which are also defective in expression of the 58-kDa protein (Lassam *et al.*, 1979). A less drastic growth defect on HeLa cells (100-fold reduced) was recently reported for the Ad5 58-kDa mutant *dl*338 (Logan *et al.*, 1984). The difference in HeLa cell growth potential between our mutant *dl*58 and *dl*338 may also be due to the fact that the two mutants contain the coding information for 58-kDa truncated products of different size (13 and 28.5 kDa, respectively). The marked multiplicity-dependence of the *dl*58 mutant viruses in HeLa cells may be explained by assuming that a high concentration of a partially defective truncated product may restore its biological activity. It should be noted, however, that truncated forms of the 58-kDa protein have never been detected. However, the finding that some rats or hamsters injected with cells transformed by the Ad5 *Hin*dIII G fragment (containing the coding information for about half of the 58-kDa protein) produce antibodies directed against the 58-kDa protein, indicate that such truncated products do indeed exist (Rowe *et al.*, 1984; Zantema *et al.*, 1985).

The observation that 58-kDa E1b mutants, including our mutant Ad5*dl*58, can grow rather efficiently in HEK cells has been interpreted to indicate that these embryonic cells express cellular functions that can complement the 58-kDa defect (Jones

and Shenk, 1979). It is tempting to speculate that this factor belongs to the group of cellular proto-oncogenes, some of which have been shown to be expressed at a high level in embryonic tissues (Müller *et al.*, 1982). The exact nature of the complementing agent, however, is as yet unknown.

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REFERENCES

AIELLO, L., GUILFOYLE, R., HUEBNER, K., and WEINMANN, R. (1979). Adenovirus 5 DNA sequences present and RNA sequences transcribed in transformed human embryo kidney cells (HEK-Ad-5 or 293). *Virology* **94**, 460–469.

ALESTRÖM, P., AKUSJÄRVI, G., PERRICAUDET, M., MATHEWS, M. B., KLESSIG, D. F., and PETTERSSON, U. (1980). Structure of the gene for polypeptide IX and its unspliced messenger RNA. *Cell* **19**, 671–681.

ANDERSON, C. W., SCHMITT, R. C., SMART, J. E., and LEWIS, J. B. (1984). Early region E1 of adenovirus 2 encodes two coterminal proteins of 495 and 155 amino acid residues. *J. Virol.* **50**, 387–396.

BABISS, L. E., FISHER, P. B., and GINSBERG, H. S. (1984). Effect on transformation of mutations in the early region 1b-encoded 21- and 55-kilodalton proteins of adenovirus 5. *J. Virol.* **52**, 389–395.

BERNARDS, R., DE LEEUW, M. G. W., VAESSEN, M. J., HOUWELING, A., and VAN DER EB, A. J. (1984). Oncogenicity by adenovirus is not determined by the transforming region only. *J. Virol.* **50**, 847–853.

BERNARDS, R., HOUWELING, A., SCHRIER, P. I., BOS, J. L., and VAN DER EB, A. J. (1982). Characterization of cells transformed by Ad5/Ad12 hybrid early region 1 plasmids. *Virology* **120**, 422–432.

BERNARDS, R., SCHRIER, P. I., BOS, J. L., and VAN DER EB, A. J. (1983a). Role of adenovirus types 5 and 12 early region 1b tumor antigens in oncogenic transformation. *Virology* **127**, 45–53.

BERNARDS, R., VAESSEN, M. J., SUSSENBACH, J. S., and VAN DER EB, A. J. (1983b). Construction and characterization of an adenovirus type 5/adenovirus type 12 recombinant virus. *Virology* **131**, 30–38.

BOS, J. L., POLDER, L. J., BERNARDS, R., SCHRIER, P. I., VAN DEN ELSEN, P. J., VAN DER EB, A. J., and VAN ORMONDT, H. (1981). The 2.2 kb mRNA of human Ad12 and Ad5 codes for two tumor antigens starting at different AUG triplets. *Cell* **27**, 121–131.

CHINNADURAI, G. (1983). Adenovirus 2 lp^+ locus codes for a 19 kd tumor antigen that plays an essential role in cell transformation. *Cell* **33**, 759–766.

ESCHE, H., and SIEGMANN, B. (1982). Expression of early viral gene products in adenovirus type 12-infected and -transformed cells. *J. Gen. Virol.* **60**, 99–113.

FROST, E., and WILLIAMS, J. (1978). Mapping temperature-sensitive and host-range mutations of adenovirus type 5 by marker rescue. *Virology* **91**, 39–50.

FUKUI, Y., SAITO, I., SHIROKI, K., and SHIMOJO, H. (1984). Isolation of transformation-defective, replication-nondefective early region 1b mutants of adenovirus 12. *J. Virol.* **49**, 154–161.

GALLIMORE, P. H. (1974). Interactions of adenovirus type 2 with rat embryo cells: Permissiveness, transformation and in vitro characterization of adenovirus type 2-transformed rat embryo cells. *J. Gen Virol.* **25**, 263–273.

GALLIMORE, P., BYRD, P., GRAND, R., WHITTAKER, J., BREIDING, D., and WILLIAMS, J. (1984). An examination of the transforming and tumor-inducing capacity of a number of adenovirus 12 early-region 1, hostrange mutants and cells transformed by subgenomic fragments of Ad12 E1 region. *In* "Cancer Cells," Vol. 2, (G. F. Van De Woude, A. J. Levine, W. C. Topp, and J. D. Watson, eds.), "Oncogenes and Viral Genes" pp. 519–527. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.

GALLIMORE, P. H., SHARP, P. A., and SAMBROOK, J. (1974). Viral DNA in transformed cells: II. A study of the sequences of adenovirus 2 DNA in nine lines of transformed cells using specific fragments of the viral genome. *J. Mol. Biol.* **89**, 49–72.

GRAHAM, F. L., ABRAHAMS, P. J., MULDER, C., HEIJNEKER, H. L., WARNAAR, S. O., DE VRIES, F. A. J., FIERS, W., and VAN DER EB, A. J. (1974a). Studies on in vitro transformation by DNA and DNA fragments on human adenoviruses and simian virus 40. *Cold Spring Harbor Symp. Quant. Biol.* **39**, 637–650.

GRAHAM, F. L., HARRISON, T., and WILLIAMS, J. (1978). Defective transforming capacity of adenovirus type 5 host-range mutants. *Virology* **86**, 10–21.

GRAHAM, F. L., VAN DER EB, A. J., and HEIJNEKER, H. L. (1974b). Size and location of the transforming region in human adenovirus type 5 DNA. *Nature (London)* **251**, 687–691.

HALBERT, D. N., SPECTOR, D. J., and RASKAS, H. J. (1979). In vitro translation products specified by the transforming region of adenovirus type 2. *J. Virol.* **31**, 621–629.

HARRISON, T., GRAHAM, F., and WILLIAMS, J. (1977). Host-range mutants of adenovirus type 5 defective for growth in HeLa cells. *Virology* **77**, 319–329.

HO, Y.-S., GALOS, R., and WILLIAMS, J. (1982). Isolation of type 5 adenovirus mutants with a cold-sensitive host range phenotype: Genetic evidence of an adenovirus transformation maintenance function. *Virology* **122**, 109–124.

HOUWELING, A., VAN DEN ELSEN, P. J., and VAN DER EB, A. J. (1980). Partial transformation of primary rat cells by the leftmost 4.5% fragment of adenovirus 5 DNA. *Virology* **10**, 537-550.

JOCHEMSEN, H., DANIELS, G. S. G., HERTOGHS, J. J. L., SCHRIER, P. I., VAN DEN ELSEN, P. J., and VAN DER EB, A. J. (1982). Identification of adenovirus type 12 products involved in transformation and oncogenesis. *Virology* **122**, 15-28.

JOCHEMSEN, H., DANIELS, G. S. G., LUPKER, J. H., and VAN DER EB, A. J. (1980). Identification and mapping of early gene products of adenovirus type 12. *Virology* **105**, 551-563.

JONES, N., and SHENK, T. (1979). Isolation of adenovirus type 5 host range deletion mutants defective for transformation of rat embryo cells. *Cell* **17**, 683-689.

LASSAM, N. J., BAILEY, S. T., and GRAHAM, F. L. (1979). Tumor antigens of human Ad5 in transformed cells and in cells infected with transformation-defective host-range mutants. *Cell* **18**, 781-791.

LOGAN, J., PILDER, S., and SHENK, T. (1984). Functional analysis of adenovirus type-5 early region 1B. *In* "Cancer Cells," Vol. 2, "Oncogenes and Viral Genes" (G. F. Van de Woude, A. J. Levine, W. C. Topp, and J. D. Watson, eds.), pp. 527-532. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.

MAK, I., and MAK, S. (1983). Transformation of rat cells by *cyt* mutants of adenovirus type 12 and mutants of adenovirus type 5. *J. Virol.* **45**, 1107-1117.

MÜLLER, R., SLAMON, D. J., TREMBLAY, J. M., CLINE, M. J., and VERMA, I. M. (1982). Differential expression of cellular oncogenes during pre- and post-natal development of the mouse. *Nature (London)* **299**, 640-644.

PILDER, S., LOGAN, J., and SHENK, T. (1984). Deletion of the gene encoding the adenovirus 5 early region 1b 21,000-molecular weight polypeptide leads to degradation of viral and host cell DNA. *J. Virol.* **52**, 664-671.

ROSS, S. R., LEVINE, A. J., GALOS, R. S., WILLIAMS, J., and SHENK, T. (1980). Early viral proteins in HeLa cells infected with adenovirus type 5 host-range mutants. *Virology* **103**, 475-492.

ROWE, D. T., and GRAHAM, F. L. (1983). Transformations of rodent cells by DNA extracted from transformation-defective adenovirus mutants. *J. Virol.* **46**, 1039-1044.

SCHRIER, P. I., VAN DEN ELSEN, P. J., HERTOGHS, J. J. L., and VAN DER EB, A. J. (1979). Characterization of tumor antigens in cells transformed by fragments of adenovirus type 5 DNA. *Virology* **99**, 372-385.

SHENK, T., JONES, N., COLBY, W., and FOWLKES, D. (1979). Functional analysis of adenovirus type 5 host-range deletion mutants defective for transformation of rat embryo cells. *Cold Spring Harbor Symp. Quant. Biol.* **44**, 367-375.

SHIROKI, K., MARUYAMA, K., SAITO, I., FUKUI, Y., and SHIMOJO, H. (1981). Incomplete transformation of rat cells by a deletion of adenovirus type 5. *J. Virol.* **38**, 1048-1054.

SHIROKI, K., SHIMOJO, H., SAWADA, Y., UEMIZU, Y., and FUJINAGA, K. (1979). Incomplete transformation of rat cells by a small fragment of Ad12 DNA. *Virology* **95**, 127-136.

SUBRAMANIAN, T., KUPPUSWAMY, M., GYSBERS, J., MAK, S., and CHINNADURAI, G. (1984a). 19kDa tumor antigen coded by early region E1b of adenovirus 2 is required for efficient synthesis and for protection of viral DNA. *J. Biol. Chem.* **259**, 11777-11783.

SUBRAMANIAN, T., KUPPUSWAMY, M., MAK, S., and CHINNADURAI, G. (1984b). Adenovirus cyt^+ locus, which controls cell transformation and tumorigenicity, is an allele of lp^+ locus, which codes for a 19-kilodalton tumor antigen. *J. Virol.* **50**, 336-343.

TAKEMORI, N., CLARADAS, C., BHAT, B., CONLEY, A. J., and WOLD, W. S. M. (1984). Cyt gene of adenovirus 2 and 5 is an oncogene for transforming function in early region E1b and encodes the E1b 19000 molecular weight polypeptide. *J. Virol.* **52**, 793-805.

VAN DEN ELSEN, P. J., DE PATER, S., HOUWELING, A., VAN DER VEER, J., AND VAN DER EB, A. J. (1982). The relationship between region E1a and E1b of human adenoviruses in cell transformation. *Gene* **18**, 175-185.

VAN DER EB, A. J., and GRAHAM, F. L. (1980). Assay of transforming activity of tumor virus DNA. *In* "Methods in Enzymology" (L. Grossman and K. Moldave, eds.), Vol. 65, pp. 826-839. Academic Press, New York.

VAN DER EB, A. J., and HOUWELING, A. (1977). Transformation with specific fragments of adenovirus DNAs. II. Analysis of the viral DNA sequences present in cells transformed with a 7% fragment of adenovirus 5 DNA. *Gene* **2**, 133-146.

VIRTANEN, A., PETTERSSON, U., LE MOULLEC, J. M., TIOLLAIS, P., and PERRICAUDET, M. (1982). Different mRNA structures are transcribed from the transforming region of highly- and non-oncogenic human adenoviruses. *Nature (London)* **295**, 705-707.

WHITE, E., GRODZICKER, T., and STILLMAN, B. W. (1984). Mutations in the gene encoding the adenovirus early region 1b 19000 molecular weight tumor antigen cause degradation of chromosomal DNA. *J. Virol.* **52**, 410-419.

WILSON, M., FRASER, N., and DARNELL, J. (1979). Mapping of RNA initiation sites by high doses of UV irradiation. Evidence of three independent promoters within the left 11% of the Ad2 genome. *Virology* **94**, 175-184.

ZANTEMA, A., FRANSEN, J. A. M., DAVIS-OLIVIER, A., RAMAEKERS, F. C. S., VOOIJS, G. P., DELEYS, B., and VAN DER EB, A. J. (1985). Localization of the E1B proteins of adenovirus 5 in transformed cells, as revealed by interaction with monoclonal antibodies. *Virology* **142**, 44-58.