Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 2167–2171, April 1986
Genetics

Mutations in the *E1a* gene of adenovirus type 5 alter the tumorigenic properties of transformed cloned rat embryo fibroblast cells

(transforming genes/289-amino acid protein/243-amino acid proteins/13S mRNA unique region)

LEE E. BABISS*†, WEN-SHING LIAW*, STEPHEN G. ZIMMER‡, GABRIEL C. GODMAN§, HAROLD S. GINSBERG*, AND PAUL B. FISHER*¶

Departments of *Microbiology and §Pathology, Comprehensive Cancer Center, Columbia University, College of Physicians and Surgeons, New York, NY 10032; ‡Department of Medical Microbiology and Immunology, University of Kentucky Medical Center, Lexington, KY 40536; and †The Rockefeller University, New York, NY 10021

Contributed by Harold S. Ginsberg, November 20, 1985

ABSTRACT **The adenovirus type 5 mutants H5hr1 and H5dl101 contain modifications in the *E1a* gene affecting the 13S mRNA-encoded 289-amino acid polypeptide and exhibit a cold-sensitive transformation phenotype upon infection of cloned rat embryo fibroblast (CREF) cells. Transformed cell lines expressing solely *E1a* or *E1a* and *E1b* gene products derived from these viruses display enhanced anchorage-independent growth at 37°C versus 32°C and display a cytoskeletal architecture resembling untransformed fibroblastic CREF cells. In contrast, CREF cells transformed by H5wt or the *E1a* and *E1b* region of H5wt grow with similar efficiency in agar at 37°C or 32°C and exhibit an epithelioid morphology that is associated with an altered cytoskeleton. Regardless of the expression or presence of other viral early regions, including *E1b*, *E2a*, and *E4* genes, specific CREF cell lines expressing an altered 289-amino acid protein and a wild-type 12S mRNA-encoded 243-amino acid protein were capable of inducing tumors in nude mice and in immunocompetent syngeneic Fischer rats. In sharp contrast, cells expressing a wild-type 289-amino acid protein were unable to induce tumors in either nude mice or syngeneic rats. The ability to induce tumors did not correlate with alterations in the pattern of viral DNA integration or differential expression of the *E1a* and *E1b* genes, nor was the tumor induction a consequence of unique properties of the immortal parental CREF cell line.**

Complete transformation of continuous rodent cell lines by adenovirus type 5 (Ad5) requires expression of the viral early regions 1a (*E1a*) and 1b (*E1b*) transcriptional units (1–9). While at least six proteins are encoded in the *E1a* and *E1b* gene mRNAs (10, 11), the biochemical function(s) of these proteins in relation to their overall contribution to the transformed cell phenotype is only now beginning to be understood.

The use of viruses containing mutations in the *E1a* gene of Ad5 has clearly shown that expression of both the 13S and 12S mRNAs is required for maintaining the complete transformation of cloned rat embryo fibroblast (CREF) cells (6–9, 12) but not establishment of the transformation event. While viruses that express only the 13S or the 12S E1a mRNAs can transform CREF cells, the cells differ dramatically in their morphology and biological properties (i.e., growth in agar, saturation density), suggesting a possible synergistic relationship between the 13S mRNA-encoded 289-amino acid protein and 12S mRNA-encoded 243-amino acid protein.

We have recently described the isolation of several CREF cell lines transformed by plasmids containing the *E1a* or *E1a* and *E1b* gene sequences from H5sub309 (phenotypically resembles wild-type Ad5), H5dl101 [contains a 5-base-pair (bp) deletion in the *E1a* gene (nucleotides 1008–1012) affecting the 13S mRNA product; see ref. 7], and H5hr1 (contains a deletion of nucleotide 1055 in the *E1a* gene affecting the 13S mRNA product; see refs. 6, 13, and 14). In the presence or absence of viral *E1b* gene expression, it was observed that these mutants transformed CREF cells at a 5- to 10-fold higher efficiency than wild-type Ad5, and cell lines that encoded an altered E1a 289-amino acid polypeptide displayed a cold-sensitive phenotype with respect to maintenance of the transformed cell morphology (6, 7).

While the *E1a* gene alone is capable of immortalizing primary rodent cells (15, 16) and incompletely transforming rat 3YI cells (17), no biological properties of the *E1a*-expressing cells were demonstrated. Furthermore, the relevance of the enhanced transformation efficiency observed in the absence of a normal 289-amino acid E1a protein has yet to be correlated with the overall transformed cell phenotype.

This paper extends earlier observations showing that H5dl101- or H5hr1-transformed CREF cell lines, which express *E1a* and/or *E1b*, display enhanced anchorage-independent growth at 37°C but not at 32°C (7). It is also demonstrated that the lack of proper 289-amino acid protein function permits H5dl101- and H5hr1-transformed CREF cell lines to induce tumors in nude mice and in immunocompetent syngeneic Fischer rats, regardless of *E1b* gene expression. In contrast, transformed cell lines expressing the wild-type 289-amino acid polypeptide are defective in their ability to form tumors in either nude mice or syngeneic rats. It is demonstrated that the ability to form tumors is not correlated with alterations of the integration sites of the viral DNA sequences, nor does it represent differential transcription of additional viral early gene regions. The significance of these findings and the ability of tumors to form in the absence of *E1b* gene expression will be discussed.

MATERIALS AND METHODS

Cell Cultures and Soft-Agar Cloning. CREF cells (12) and the transformed CREF cell lines described in Table 1 were grown in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum. The isolation and subsequent cloning of the cell lines used in these studies (Table 1) have been described (6, 7, 12). The techniques used to evaluate cloning efficiency in agar have also been described (18).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: CREF, cloned rat embryo fibroblast; Ad5, adenovirus type 5; bp, base pair(s); nmt, nude mouse tumor; Imm, immunocompetent rat tumor.

¶To whom reprint requests should be addressed.

Table 1. *In vitro* and *in vivo* properties of wild-type and mutant Ad5-transformed CREF cells

Cell line	Transforming agent*	Immunofluorescence† 21 kDa	Immunofluorescence† 55 kDa	% agar cloning efficiency‡ 37°C	% agar cloning efficiency‡ 32°C	Tumorigenicity§ Nude mice	Tumorigenicity§ Fischer rats
CREF	None	−	−	<0.001	<0.001	0/6	0/6
wt3A	H5wt	+	+	45 ± 2	38 ± 2	1/9 (90)	0/6
c2	H5wt (0–15.5 m.u.) (*E1a* + *E1b*)	+	−	22 ± 6	24 ± 3	0/3	0/3
d2	H5wt (0–15.5 m.u.) (*E1a* + *E1b*)	+	+	48 ± 3	45 ± 4	0/6	0/6
hr1A2	H5hr1	+	+	69 ± 6	16 ± 3	6/6 (18)	6/6 (42)
g8t	H5hr1 (0–15.5 m.u.) (*E1a* + *E1b*)	+	+	22 ± 2	<0.02	9/9 (44)	0/6
d3t	H5hr1 (0–4.5 m.u.) (*E1a*)	−	−	41 ± 5	6 ± 2	6/6 (14)	3/3 (95)
O1	H5dl101 (0–4.5 m.u.) (*E1a*)	−	−	36 ± 3	5 ± 1	6/6 (27)	6/6 (157)

*H5wt, wild-type Ad5; H5wt (0–15.5 m.u.), transfected CREF clone containing *E1a* and *E1b* region of H5wt; H5hr1, host-range mutant of Ad5 (contains a 1-bp deletion of nucleotide 1055); H5hr1 (0–15.5 m.u.), transfected CREF clone containing *E1a* and *E1b* region of H5hr1; H5hr1 (0–4.5 m.u.), transfected CREF clone containing *E1a* region of H5hr1; H5dl101 (0–4.5 m.u.), transfected CREF clone containing *E1a* region of H5dl101 virus (contains one 5-bp deletion of nucleotides 1008–1012).

†Immunofluorescence staining of transformed CREF cells was performed as described. Antisera raised in rabbits against the *E1b*-encoded 21-kDa and 55-kDa polypeptides was provided by M. Green. +, Presence of the viral antigen; −, absence of the viral antigen.

‡Agar cloning assays were performed as described (18). Approximately 10^3, 5×10^3, or 10^4 cells in low Ca^{2+} medium were prepared in 0.4% Noble agar and were seeded at 37°C or 32°C on 0.8% agar base layers prepared in the same medium. Plates were fed once a week with 2 or 3 ml of 0.4% agar in low Ca^{2+} medium and colonies of >0.1 mm were counted after 21 days. Each value is the mean ± SD of four plates.

§Four-week-old BALB/c nude mice or 6- to 8-week-old Fischer 344 rats were injected with 2×10^6 cells. Results indicate number of animals with tumors per number of animals injected and numbers in parentheses indicate average latency time in days—i.e., first appearance of a palpable tumor.

Tumor Induction in Nude Mice and Syngeneic Fischer Rats. The ability of Ad5-transformed CREF clones to induce tumors in 4-week-old BALB/c nude mice and 6- to 9-week-old immunocompetent Fischer 344 rats was determined by injecting 2×10^6 cells per animal. Monolayer cultures of tumors were established by trypsinization of excised tumors, referred to as nmt (nude mouse tumor) or Imm (immunocompetent rat tumor).

Immunofluorescent Staining. The CREF cell lines described in Table 1 were cultured on glass coverslips, fixed with 10% acetone, treated with antisera raised in rabbits against the E1b 21- and 55-kDa polypeptides (kindly provided by Maurice Green), and stained with fluorescein isothiocyanate-conjugated rabbit anti-IgG. After counterstaining with rhodamine, stained cells were visualized by using an Olympus microscope with an ultraviolet source.

To visualize cytoskeletal structures, cell monolayers on coverslips were fixed with 3.5% formalin containing 0.1% Nonidet P-40, incubated first with rhodamine-conjugated phaloidin (Molecular Probes, Junction City, OR), and then incubated with affinity-purified rabbit polyclonal antiserum to chicken gizzard vinculin (Transformation Research, Framingham, MA). Coverslips mounted in Gelvatol (a polyvinyl alcohol) containing *p*-phenylenediamine (1 mg/ml) were examined in a Leitz Dialux 20 microscope with 2.4 Ploempak filter block and water immersion fluorite objectives and were recorded on Kodak Tri-X-film.

DNA Analysis. High molecular weight DNA was isolated from the transformed CREF cell lines shown in Fig. 2 as described (19). The presence of viral sequences in these DNA samples was determined by DNA filter hybridization analysis as described (12, 20–22).

RNA Analysis. Following the procedure of Hofer and Darnell (23) and Weber *et al.* (24), nuclei were extracted from A2, A2 nmt cell lines, A2 Fischer Imm cell lines, and wt3A cells and labeled *in vitro*. The transcription assay used ≈10^8 nuclei and 250 μCi of [^{32}P]UTP (3000 μCi/mmol; 1 Ci = 37 GBq), which were incubated for 15 min at 30°C. Nuclear RNA was isolated by the guanidinium isothiocyanate method of Ulrich *et al.* (25) and prior to hybridization was cleaved by treatment with 0.2 M NaOH for 15 min on ice (26). The preparation of filters containing "dots" of denatured DNA was described by Kafatos *et al.* (27).

RESULTS

Expression of the Transformed Phenotype in Wild-Type and E1a Mutant Ad5-Transformed CREF Cells. While hybrid-plasmid transfection of CREF cells revealed that stable expression of an altered *E1a* gene product alone could result in a cold-sensitive transformation phenotype (7, 9), these studies did not consider the biological properties of individual cloned cell lines derived from such assays. It was regularly observed that when the unique region of the *E1a*-encoded 289-amino acid protein was altered, the transformed cells in which it was expressed were fibroblasts, resembling the parental CREF cells, in contrast to wild-type Ad5 (H5wt)-transformed CREF cells, which were epithelioid. The cytoskeletal architecture of untransformed-, H5hr1-, and H5wt-transformed CREF cells reflected the observed morphologies. As shown in Fig. 1, H5hr1-transformed CREF cells (cell line A2) displayed a cytoskeletal framework of F-actin fibers and vinculin plaques resembling that of the parental CREF cells, with the minor variations probably accounting for the different morphology between the cell lines. In contrast, H5wt-transformed CREF cells (cell line wt3A) had diminished and disoriented actin-based cytoskeletal structures, in agreement with the observation that these cells were epithelioid (Table 1).

It is noteworthy (see Table 1) that the cloned mutant-transformed CREF cell lines' ability to grow in soft agar is correlated with their ability to express the cold-sensitive

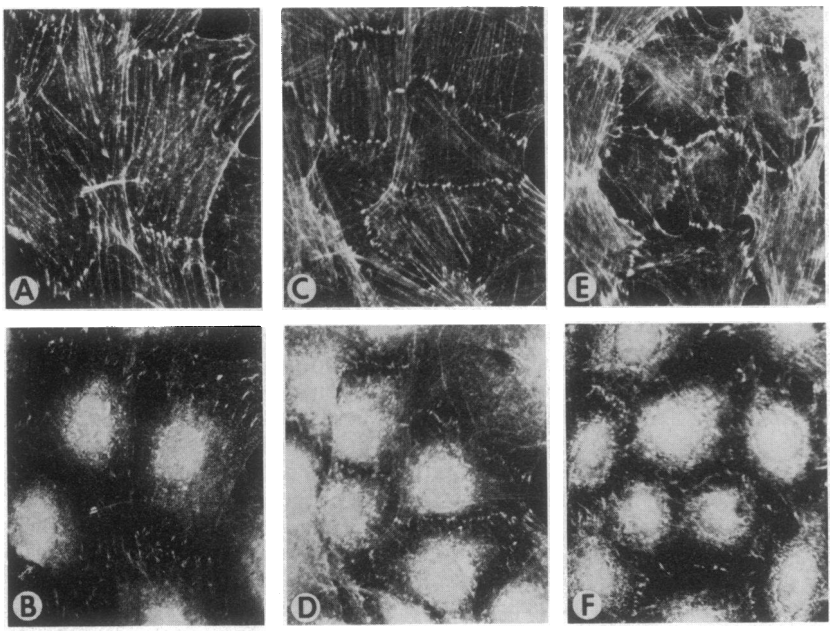

FIG. 1. Distribution of F actin and vinculin by immunofluorescence in CREF (*A* and *B*), Ad5-transformed CREF (wt3A; *E* and *F*), and H5hr1-transformed CREF (A2; *C* and *D*) cell lines. Monolayers at confluence were double-stained with rhodamine-coupled phallotoxin to demonstrate F actin and with rabbit anti-vinculin followed by fluoresceinated goat anti-rabbit immunoglobulin to show sites of vinculin in the same cell. (×480.)

phenotype: E1a-transformed and E1a plus E1b-transformed cells derived from H5hr1 (d3t and g8t) and H5dl101 (O1) or H5hr1 virus-transformed CREF cells (A2) showed reduced cloning efficiencies in soft agar when grown at 32°C as opposed to 37°C. In contrast, cell lines derived from foci produced by H5wt (wt3A), or *E1a* plus *E1b* from H5sub309 (c2 and d2) showed similar cloning efficiencies at 32°C and at 37°C. Furthermore, the colonies that arose at 32°C from cell lines expressing altered E1a 289-amino acid protein (g8t, d3t, O1, and A2) were much smaller than those propagated at 37°C. This is in contrast to the cell lines expressing H5wt E1a proteins, whose colony sizes were similar at either temperature.

To determine the *in vivo* oncogenic potential of the transformed CREF cell lines described in Table 1, 2×10^6 cells were injected subcutaneously into nude mice and 6- to 9-week-old immunocompetent syngeneic Fischer rats. As anticipated, the parental CREF cell line did not induce tumors in either type of animal. Similarly, the wt3A cell line and cell lines expressing *E1a* and *E1b* gene products of H5sub309 (c2 and d2) also did not induce tumors in syngeneic rats; only one tumor developed in nine nude mice injected with wt3A cells, and this tumor regressed. In contrast, all cell lines expressing altered E1a 289-amino acid protein but normal 243-amino acid protein induced tumors in nude mice very efficiently. Surprisingly, tumor induction in syngeneic Fischer rats was also observed in all animals using cell lines A2, O1, and d3t (all were transformed by mutants encoding a truncated 289-amino acid protein), but the appearance of tumors took 2 to 3 times longer than in nude mice studies. It is unclear why the *E1a*- and *E1b*-expressing cell line derived from H5hr1 (g8t) did not induce tumors in Fischer rats.

Viral DNA Integration and RNA Expression in Wild-Type- and E1a Mutant-Transformed CREF Cell Lines. One possible explanation for why lines derived from H5hr1- and H5dl101-transformed foci were able to induce tumors in both nude and syngeneic animals could be that the E1a 289-amino acid protein functions to stabilize the viral DNA in the chromosome. To determine whether tumor induction was correlated with translocation of viral DNA sequences and the possible activation of a formerly unexpressed cellular oncogene, DNA·DNA filter hybridization studies were performed by using high molecular weight DNA isolated from parental transformed cell lines, nmt cell line, and Fischer rat tumor (Imm) cell line (A2, g8t, and O1 cell lines, respectively). As shown in Fig. 2, the pattern of viral DNA integration observed in the parental cell line was always identical to the integration patterns seen in the cell lines derived from tumors. Proliferation of virus- or plasmid-derived transformed CREF cell lines in either nude or syngeneic animals did not appear to alter the integration pattern of the viral sequences and, therefore, suggests that the 289-amino acid protein may not play a role in integration stabilization. Additional DNA·DNA filter hybridizations were performed to confirm that cell line O1 contained only viral *E1a* sequences, while cell line g8t contained only viral *E1a* and *E1b* sequences (data not shown).

To determine whether the ability of some of the cell lines to induce tumors was correlated with the expression of viral genes other than *E1a* or *E1b*, we determined the transcription rate for all the viral early gene regions by labeling nascent RNAs prepared from isolated cell nuclei and hybridizing to dots of DNA on nitrocellulose filters. Since chain initiation in

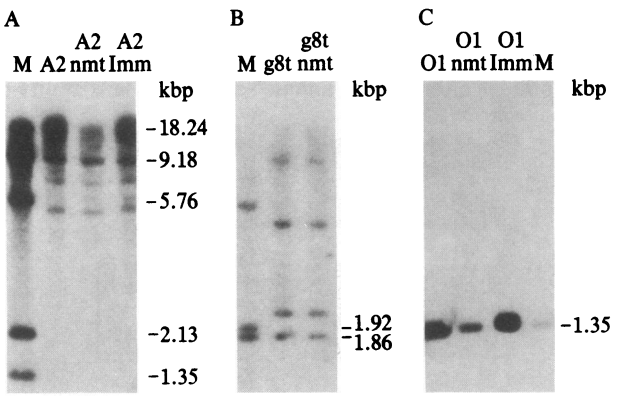

FIG. 2. Analysis of viral DNA sequences in transformed CREF cell lines. A 10-μg portion of cellular DNA isolated from the cloned cell lines indicated was cleaved with *Xba* I (*A*), *Sac* I (*B*), or *Bam*HI and *Xba* I (*C*), and the fragments were separated electrophoretically in 0.6% agarose, transferred to nitrocellulose filters, and hybridized to H5wt DNA labeled with ^{32}P by nick-translation (20–22). The marker lane (M) in *A* represents five genome equivalents of H5wt DNA digested with *Xba* I; the marker lane in *B* pLB212 represents five genome equivalents of pLB212 DNA (7) digested with *Sac* I; the marker lane in *C* represents five genome equivalents of pLB214 (7) digested with *Bam*HI and *Xba* I.

isolated nuclei is inefficient, while chain elongation of previously initiated RNA polymerase II molecules occurs faithfully, this assay is useful for the determination of differential rates of RNA synthesis (23). In these studies, the same amount of total labeled RNA (representing that from approximately equivalent numbers of cell nuclei) from each nuclear sample was used in the hybridization assay so that differential rates among the various cell lines could be measured. The DNA dots included regions of the Ad5 *E1a, E1b, E2a, E3*, and *E4* transcription units, and the cellular genes actin and tubulin. In this analysis (Fig. 3), the transcription rates for all the viral genes and cellular tubulin genes were similar in A2, A2 nmt, A2 Imm, and wt3A cell lines. The reduction by a factor of 2–3 in actin transcription seen in wt3A cells was reproducible in several experiments and with additional H5wt-transformed CREF cell lines, which exhibited epithelioid morphology. Similar results were obtained when the cytoplasmic steady-state levels of the E1a, E2a, and E4 mRNAs were compared in A2, A2 nmt, and A2 Imm cell lines and E1a mRNAs in O1, O1 nmt, and O1 Imm cell lines (data not shown).

DISCUSSION

This study describes the cellular morphology, anchorage-independent growth, and tumor induction in athymic nude mice and in immunocompetent syngeneic Fischer rats of transformed CREF cell lines that differ in their ability to encode the wild-type Ad5 E1a 289-amino acid protein. Mutants of Ad5 (H5hr1 and H5dl101) that contain defects in the gene encoding the E1a 289-amino acid polypeptide, but encode a normal 243-amino acid protein gene, can produce morphological transformation of CREF cells at 37°C but fail to do so efficiently at 32°C (6, 7). Both H5dl101 and H5hr1 transform CREF cells at a 5- to 10-fold higher efficiency than wild-type virus at 37°C and produce transformed foci consisting of predominantly fibroblastic cells, which when cloned resemble uninfected CREF cells. This partially transformed morphology was correlated with the finding that these mutants do not produce alterations in the cytoskeletal architecture within the cell (Fig. 1). Associated with this conditional transformation phenotype, the anchorage-independent growth of cloned cell lines transformed by H5hr1 or H5dl101 E1a or E1a plus E1b DNA are also cold sensitive (Table 1). Furthermore, the ability of the transformed cell lines to induce tumors in nude mice and in syngeneic Fischer rats also appears to occur because the E1a-encoded 289-amino acid polypeptide is altered in its unique sequences. This ability of a mutant E1a 289-amino acid polypeptide to allow tumor induction did not depend on viral *E1b, E2a*, or *E4* expression and was not associated with alterations in the integration pattern of the viral DNA sequences in the transformed cell line.

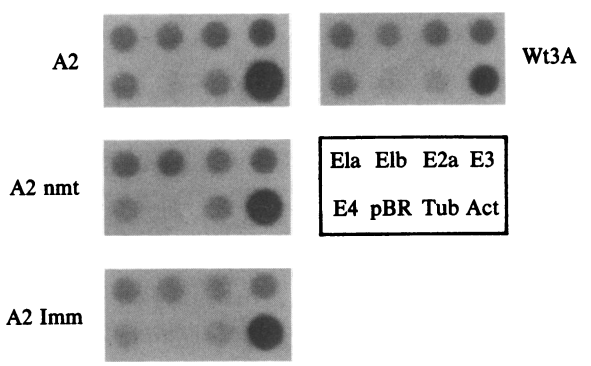

FIG. 3. Dot hybridization of RNA labeled in nuclei isolated from cell lines A2, A2 nmt, A2 Imm, and wt3A. Nuclear RNA was labeled *in vitro* with [^{32}P]UTP as described in the text, and RNA containing 5×10^6 cpm was hybridized to a dot containing 7 μg of E1a (0.0–4.5 m.u.), E1b (7.9–9.6 m.u.), E2a (60.1–63 m.u.), E3 (72.3–75 m.u.), E4 (93–100 m.u.), chicken actin cDNA (Act), rat tubulin cDNA (Tub), and pBR322 (pBR) DNA.

A simple interpretation of the tumorigenicity studies would suggest that the ability of E1a mutant virus-transformed CREF cells to induce tumors in both nude and syngeneic animals is a consequence not of the virus, but of the CREF cells. CREF cells were derived from a primary culture of Fischer rat embryo cells and were selected for their ability to remain at confluence for extended periods in culture and for their high frequency of transformation by H5wt. While it is an established cell line, it does not display any phenotype that resembles a partially transformed (*E1a* expressing) cell line. Since neither CREF cells nor H5wt-transformed CREF cells are able to induce tumors in nude mice or syngeneic rats, the data (Table 1) are in accord with similar studies using H5wt-transformed 3Y1 or BRK cells (see ref. 28 for review). Therefore, the ability of H5hr1 or H5dl101 E1a-expressing CREF cell lines to induce tumors appears to be related to viral *E1a* gene expression and not only to the cell line used.

Of particular interest was the observation that cells expressing altered E1a 289-amino acid protein can induce tumors in syngeneic animals regardless of *E1b* gene expression. Previous studies using Ad5 and Ad12 hybrid plasmids suggested that the oncogenic potential of a transformed cell in athymic nude mice was determined by *E1b* gene expression, whereas the ability to escape T-lymphocyte immunity in syngeneic rats was determined by *E1a* gene expression (29). This ability to evade the immune defenses of the host was demonstrated for Ad12 but not for Ad5 (29, 30). The situation in the CREF system may be different since preliminary studies indicate that both CREF cells and Ad5-transformed CREF cells (both wild type and mutant) display low levels of RT1 (class I major histocompatibility complex antigens of rat origin) expression as indicated by immunofluorescence and ^{51}Cr-release assays using cytotoxic T cells directed against the *RT1^l* haplotype (unpublished data). Our results suggest that the lack of a functional 13S mRNA gene product permits these virus-transformed CREF cells to escape the host-cell defense mechanisms. This is further supported by our tumorigenicity studies using E1a mutant virus H5dl520-transformed CREF cells (9), which do not synthesize any 13S mRNA or its protein product but can induce tumors in both nude mice and syngeneic rats (unpublished observations). Whether lack of expression of the E1a-encoded 289-amino acid protein also relates to these mutant viruses being able to transform CREF cells at a higher efficiency is not clear at this time.

A possible interpretation of the ability of E1a mutant-transformed CREF cell lines to induce tumors in syngeneic rats in the absence of viral E1b expression could be that the 12S mRNA-encoded 243-amino acid protein alone enhances cellular p53 expression and/or activates some cellular oncogene to complement the E1b defect. Houweling *et al.* (15) have demonstrated that the *Hpa* I–E (0–4.5 m.u.) region of Ad5 can immortalize primary baby rat kidney cells. In addition, E1a has been shown to complement the T24 (Ha-*ras*) oncogene resulting in the neoplastic transformation of primary rat embryo cells (31). Both the immortalization and T24 oncogene complementation functions of E1a are found with other genes, including v-*myc* (31), c-*myc* (31), n-*myc* (32, 33) polyoma large T antigen (16), and the cellular tumor antigen p53 (34–36). In the case of p53, overexpression of this cellular tumor antigen in the established rat cell line Rat-1 results in the acquisition of tumorigenic potential by these cells without dramatic alterations in cellular morphology (37). In addition, it has been widely established that the E1a 289-amino acid protein can play several roles relative to

transcription: activate expression of early viral genes (38–40), enhance the rate of transcription of cellular genes (41), and suppress the expression of simian virus 40 enhancer-dependent transcription units (42). While no transcriptional function has been ascribed to the 243-amino acid protein, our nuclear transcription data suggest that expression of cellular actin can be enhanced in its presence (Fig. 2). Our findings are also in agreement with the studies of Rowe and Graham (43) using H5hr50 (which does not encode detectable E1b polypeptides), in which they showed that the mutant-transformed BRK cells induce tumors in newborn hamsters.

It has recently been suggested that additional Ad12 early gene regions may play a role in the process of tumor formation in newborn hamsters (44) and in newborn Fischer rats (45). While our studies demonstrate that tumor induction can occur only when defective 13S mRNA and normal 12S mRNA *E1a* sequences are present, we cannot rule out that the decreased latent period observed with the A2 cell line in Fischer rats is due to the possible *E4* gene expression in A2 cells as compared to cells transformed with DNA containing only the *E1a* genes (d3t and O1 cell lines). This hypothesis is being tested by using additional H5hr1 virus-transformed cell lines that differ in their ability to express the viral *E4* gene.

As shown in Table 1, the c2 and the d2 cell lines each displayed equal efficiency in forming macroscopic colonies in soft agar at 32°C and 37°C. However, since cell line c2 did not produce detectable E1b-encoded 55-kDa protein, as determined using a polyclonal serum against the wild-type 55-kDa protein, its overall efficiency of colony formation in soft agar was decreased. This is in agreement with our earlier studies, which showed that as deletions approached the NH_2-terminal end of the E1b 55-kDa polypeptide gene, transformation frequency and cloning efficiency in soft agar was reduced (7).

The inability of cell line g8t to induce tumors in syngeneic Fischer rats, while it displayed high oncogenicity in nude mice, was somewhat surprising (Table 1). One possible explanation is that this finding may represent a feature unique to this transformed cell clone, and additional clones expressing the H5hr1 *E1a* gene and wild-type *E1b* gene products would be tumorigenic in syngeneic rats. Alternatively, this may be a function of *E1b* gene expression altering the ability of the 243-amino acid polypeptide from interacting with some cellular factor to allow tumor formation and evasion from the host cell immune defenses.

We thank Ms. Ellen Reynolds for assistance in the preparation of this manuscript. This work was supported by National Cancer Institute Grants CA-35675 (P.B.F.), CA-33434 (S.G.Z.), CTR1432 and HL15832 (G.C.G.), and AI-12052 (H.S.G.). L.E.B. was supported by a Damon Runyon–Walter Winchell Postdoctoral Fellowship (DRG-794).

1. Gallimore, P. H., Sharp, P. A. & Sambrook, J. (1974) *J. Mol. Biol.* **89,** 49–72.
2. Graham, F. L., Abrahams, P. J., Mulder, C., Heijneker, H. L., Warnaar, S. O., deVries, F. A. J., Fiers, W. & van der Eb, A. J. (1974) *Cold Spring Harbor Symp. Quant. Biol.* **39,** 637–650.
3. van der Eb, A. J., Mulder, C., Graham, F. L. & Houweling, A. (1977) *Gene* **2,** 115–132.
4. Jones, N. & Shenk, T. (1979) *Cell* **17,** 683–689.
5. Shiroki, K., Maruyama, K., Saito, I., Fukui, Y. & Shimojo, H. (1981) *Virology* **38,** 1048–1054.
6. Babiss, L. E., Ginsberg, H. S. & Fisher, P. B. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 1352–1356.
7. Babiss, L. E., Fisher, P. B. & Ginsberg, H. S. (1984) *J. Virol.* **49,** 731–740.
8. Montell, C., Courtois, G., Eng, C. & Berk, A. J. (1984) *Cell* **36,** 951–961.
9. Haley, K. P., Overhauser, J., Babiss, L. E., Ginsberg, H. S. & Jones, N. C. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 5734–5738.
10. Smart, J., Lewis, J., Mathews, M., Harter, M. & Anderson, C. (1981) *Virology* **112,** 703–713.
11. Anderson, C. W., Schmitt, R. C., Smart, J. & Lewis, J. B. (1984) *J. Virol.* **50,** 387–396.
12. Fisher, P. B., Babiss, L. E., Weinstein, I. B. & Ginsberg, H. S. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 3527–3531.
13. Graham, F. L., Harrison, T. J. & Williams, J. F. (1978) *Virology* **86,** 10–21.
14. Ricciardi, R. P., Jones, R. L., Cepko, C. L., Sharp, P. A. & Roberts, B. E. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 6121–6125.
15. Houweling, A., van den Elsen, P. J. & van der Eb, A. J. (1980) *Virology* **105,** 537–550.
16. Ruley, H. E. (1983) *Nature (London)* **304,** 602–606.
17. Shiroki, K., Handa, H., Shimojo, H., Yano, S., Ojima, S. & Fujinaga, K. (1977) *Virology* **82,** 462–471.
18. Fisher, P. B., Goldstein, N. I. & Weinstein, I. B. (1979) *Cancer Res.* **39,** 3051–3057.
19. Dorsch-Hasler, K., Fisher, P. B., Weinstein, I. B. & Ginsberg, H. S. (1980) *J. Virol.* **34,** 305–314.
20. Rigby, P. W. J., Dieckmann, M., Rhodes, C. & Berg, P. (1977) *J. Mol. Biol.* **113,** 237–251.
21. Southern, E. M. (1975) *J. Mol. Biol.* **98,** 503–517.
22. Wahl, G. M., Stern, M. & Stark, G. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 3683–3687.
23. Hofer, E. & Darnell, J. E., Jr. (1981) *Cell* **23,** 585–593.
24. Weber, J., Jelinek, W. & Darnell, J. E., Jr. (1977) *Cell* **10,** 611–616.
25. Ulrich, A., Shine, J., Chirgwin, J., Picket, R., Tischer, E., Rutter, W. J. & Goodman, H. M. (1977) *Science* **196,** 1313–1316.
26. Nevins, J. R. (1980) *Methods Enzymol.* **65,** 768–785.
27. Kafatos, F. C., Jones, C. W. & Efstratiadis, A. (1979) *Nucleic Acids Res.* **7,** 1541–1551.
28. Graham, F. L. (1984) in *The Adenoviruses*, ed. Ginsberg, H. S. (Plenum, New York), pp. 339–398.
29. Bernards, R., Schrier, P. I., Houweling, A., Bos, J. L., van der Eb, A. J., Zijlstra, M. & Melief, C. J. M. (1983) *Nature (London)* **305,** 776–779.
30. Schrier, P. I., Bernards, R., Vaessen, R. T. M. J., Houweling, A. & van der Eb, A. J. (1983) *Nature (London)* **305,** 771–775.
31. Land, H., Parada, L. & Weinberg, R. A. (1982) *Cell* **29,** 161–169.
32. Schwab, M., Varmus, H. E. & Bishop, J. M. (1985) *Nature (London)* **316,** 160–162.
33. Yancopoulos, G. D., Nisen, P. D., Tesfaye, A., Kohl, N. E., Goldfarb, M. P. & Alt, F. W. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 5455–5459.
34. Eliyahu, D., Raz, A., Gruss, P., Givol, D. & Oren, M. (1984) *Nature (London)* **312,** 646–649.
35. Parada, L., Land, H., Weinberg, R. A., Wolf, D. & Rotter, V. (1984) *Nature (London)* **312,** 649–651.
36. Jenkins, J. R., Rudge, K. & Currie, G. (1984) *Nature (London)* **312,** 651–654.
37. Eliyahu, D., Michalovitz, D. & Oren, M. (1985) *Nature (London)* **316,** 158–160.
38. Jones, N. C. & Shenk, T. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 3665–3669.
39. Berk, A. J., Lee, F., Harrison, T., Williams, J. & Sharp, P. A. (1979) *Cell* **17,** 935–944.
40. Nevins, J. R. (1981) *Cell* **26,** 213–220.
41. Stein, R. & Ziff, E. B. (1984) *Mol. Cell. Biol.* **4,** 2792–2801.
42. Borrelli, E., Hen, R. & Chambon, P. (1984) *Nature (London)* **312,** 608–612.
43. Rowe, D. T. & Graham, F. L. (1983) *J. Virol.* **46,** 1039–1044.
44. Bernards, R., de Leevw, M. G. W., Vassen, M.-J., Houweling, A. & van der Eb, A. J. (1984) *J. Virol.* **50,** 847–853.
45. Shiroki, K., Hashimoto, S., Saito, I., Fukui, Y., Fukui, Y., Kato, H. & Shimojo, H. (1984) *J. Virol.* **50,** 854–863.