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The Zinc Finger Protein GLI Transforms Primary Cells in Cooperation with Adenovirus E1A

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The *GLI* gene was previously isolated by virtue of its amplification in human glioblastomas. We have now found that *GLI* expression can result in the in vitro transformation of both primary and secondary rodent cells. When coexpressed with adenovirus E1A, the GLI protein functions analogously to RAS, resulting in the formation of dense foci of cells which are tumorigenic in nude mice.

The *GLI* gene has been found to be amplified in several human tumors of mesenchymal origin, including those of the glioblastoma, liposarcoma, rhabdomyosarcoma, and osteosarcoma cell types (11, 23; unpublished observations). Additionally, the *GLI* gene is located at chromosome 12q13.3-14.1, a region that is affected by chromosome translocations in a variety of benign and malignant human tumors, including lipomas, myxoid liposarcomas, uterine leiomyomas, and salivary gland adenomas (2). *GLI* encodes a nuclear protein containing five zinc fingers of the Cys-Cys-His-His type first described in the *Xenopus* transcription factor IIIA (5, 18), suggesting that *GLI* might, like the TFIIIA gene, encode a DNA-binding transcription factor (12). Support for this hypothesis was obtained by isolating several GLI binding sites from the human genome, each of which contained the 9-bp sequence GACCACCCA (14).

In tumors or in drug-resistant cell lines, gene amplification affects large segments of DNA, typically 200 to 2,000 kb. Accordingly, several genes are often coamplified, even though only one of the genes (the target gene) presumably provides a selective growth advantage (reviewed in references 27 and 30). Although *GLI* was the only gene identified within an amplification unit estimated to contain at least 350 kb (11), we were interested in finding independent evidence that *GLI* could promote a neoplastic phenotype. We therefore searched for a morphologic effect when *GLI* was expressed alone or in combination with other oncogenes in primary rodent cells. We found that *GLI* could cooperate with the adenovirus E1A gene products to result in the formation of dense foci of transformed, tumorigenic cells. The results suggest that *GLI* is indeed an oncogene and that GLI gene amplification in human tumor cells may confer a similar selective growth advantage.

Isolation and transformation of rodent cells. Rat embryo fibroblasts (REF) were isolated from 13- to 15-day-old Fisher CDF albino rat embryos as described previously (22). Confluent cultures were trypsinized, and aliquots were stored at −85°C in Dulbecco's modified Eagle medium (DMEM) with D-glucose at 1.0 g/liter, 10% fetal bovine serum (FBS; Hyclone), and 7% dimethylsulfoxide. Cells were thawed and passaged once in culture. Transfections were performed when the cultures were 5 to 10% confluent.

Baby rat kidney (BRK) cells were isolated from 6-day-old Fisher rats as described previously (36) and grown in DMEM plus 10% FBS. The cells isolated from one kidney were divided into five 75-cm^2 flasks, and transformations were performed 24 h later at 15 to 30% confluence.

A *Hin*dIII-*Xba*I restriction fragment containing the entire *GLI* coding region from pGLIK12 (14) was cloned into the *Bam*HI site of the expression vector pLTR-2 (6) after all overhangs were filled with Klenow enzyme. The resulting construct places the *GLI* coding sequence downstream of the Moloney murine leukemia virus long terminal repeat. The vector that expressed the sense strand was termed GLI, and the opposite-orientation vector was called antiGLI. The other plasmids used were E1A, containing the leftmost 1,834 bases of the adenovirus type 5 genome (pC1A; 36); RAS, an expression vector containing a mutant H-*ras* gene (pHO6T1; 29); E7, containing the human papillomavirus type 16 E7 open reading frame linked to an actin promoter (p1434; 20); and MYC, containing the normal human c-*myc* gene linked to a retroviral promoter (pM21; 31).

Transformations were performed by the $CaPO_4$ coprecipitation method or by lipid-mediated transfection. Comparable results were obtained with either technique, although lipofectin was more efficient for BRK cells. Transformations with $CaPO_4$ coprecipitates were performed as previously described (36). Lipid-mediated transfections were performed by using lipofectin (Bethesda Research Laboratories; 8). Briefly, a 75-cm^2 flask of cells was washed twice with Hanks balanced saline and once with Opti-MEM (GIBCO) and then covered with 6 ml of Opti-MEM containing 30 μg of lipofectin and the appropriate plasmid (5 μg for E1A, RAS, MYC, and E7 and 10 μg for GLI and antiGLI). After 15 to 18 h at 37°C, the medium was replaced with DMEM containing 10% FBS. RK3E cells were transformed as described above except that 20 μg of lipofectin and 10 μg of plasmid were used. Cells were fixed and stained with modified Wright stain at 2 to 4 weeks.

Cell lines. Cell lines were isolated by trypsinization of individual foci in glass cylinders. The cell line RK3E was cloned from one focus in a flask of primary kidney cells that had been transfected with E1A alone. The RK3E cell line has been deposited with the American Type Culture Collection (Rockville, Md.).

The other cell lines used in this work included RK3E-RAS, derived by RAS transformation of RK3E cells; RK3E-GLI-D, RK3E-GLI-E, and RK3E-GLI-F, which were independent GLI transformants of RK3E cells; and BRK20, a clone derived by cotransformation of primary BRK cells with plasmids [E1A + GLI]. All lines were grown in DMEM

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TABLE 1. GLI transformation of rodent cells

Cell type	Plasmid(s)	$CaPO_4$	Lipofectin
BRK[a]	[E1A + GLI]	5/80, 5/25 6/48, 4/20	6/50, 5/81, 5/55, 3/39, 2/31 4/76, 4/58, 2/41, 4/50
	[E1A + antiGLI]	0/16, 0/7 0/23, 0/31	0/43, 0/55, 0/52, 0/42, 0/29 0/43
	[E1A + RAS]	10/15	24/56
REF[b]	[E1A + GLI]	9	16, 7, 6, 5, 15, 10, 10
	[E1A + antiGLI]	0	0, 0, 0, 0, 0, 0, 1, 0
	[E1A + RAS]	82	93, 58, 72
	[GLI + RAS]	ND[c]	0, 0
	[GLI + MYC]	ND	0, 0
	[MYC + RAS]	ND	100
	[E7 + GLI]	ND	0, 0
	[E7 + RAS]	ND	58
	GLI	ND	0, 0
RK3E[b]	GLI	ND	17, 24, 35, 36, 18
	RAS	ND	29, 30
	antiGLI	ND	0, 0, 0, 0, 0
	E1A	ND	0, 0
	MYC	ND	0

[a] Results for each flask shown as x/y, where x = dense, transformed foci and y = total foci (see Fig. 1A).

[b] Numbers represent transformed foci per flask. For REF, total foci were not recorded because the higher background growth prevented unambiguous identification of the nondense foci induced by E1A alone.

[c] ND, Not determined.

plus 10% FBS except for RK3E-RAS, which was grown in DMEM plus 5% FBS.

***GLI* cooperates with E1A.** As there are numerous examples of combinations of oncogenes that result in focus formation in primary rodent cells (34), but fewer examples of genes with discernible effects when transfected singly (22), we opted to explore the possibility that *GLI* might cooperate with other gene products to transform primary cells. Therefore, we transfected REF and BRK cells with the GLI vector either alone or in combination with RAS, or with one of the RAS-cooperative oncogene vectors MYC, papillomavirus E7, or E1A from adenovirus type 5.

Primary cells transfected with GLI or [GLI + RAS] failed to form foci (Table 1). As previously reported (9, 24), transfection of E1A alone resulted in the formation of flat, monolayered foci in which cells grew to a higher density than did untransfected cells. Transfection of RAS in combination with E1A resulted in the formation of dense, multilayered foci (24, 36). Similarly, transfection of GLI in combination with E1A resulted in the formation of several dense, multilayered foci (Table 1; Fig. 1A). A vector with *GLI* inserted in the antisense orientation, termed antiGLI, served as a negative control in these experiments (Table 1). Thus, GLI appeared to function analogously to RAS by cooperating with E1A to transform both REF and BRK cells.

That GLI and RAS both cooperated with E1A suggested that the cellular components which mediate the GLI and RAS effects could function in the same pathway. However, GLI failed to transform in other traditional RAS assays. Transfection of GLI into the RAS-transformable, established cell line NIH 3T3 did not result in foci (not shown); also, GLI failed to cooperate with the RAS-cooperative oncogene vectors MYC and papillomavirus E7 (Table 1). Another difference between GLI and RAS was the cell morphology of lines derived from GLI- or RAS-induced foci. While [E1A + RAS]-transformed cells were refractile and rounded as previously reported (24, 36), [GLI + E1A]-transformed cells grew to a high cell density, but the cells were not rounded or refractile (Fig. 1B to D).

GLI transforms an E1A-expressing cell line. The cotransformation assay was limited by the small number (typically 4 to 10) of dense foci per flask (Table 1). An established cell line that transformed more efficiently would obviate the need for the isolation of primary cells and might prove helpful in future studies of *GLI* function. On the basis of the results presented above, it seemed that a cell line which stably expressed E1A might be transformed by subsequent transfection of the GLI expression vector. Therefore, a number of BRK foci resulting from E1A transfection were cloned by trypsinization in glass cylinders and expanded in culture. One line, termed RK3E, passaged rapidly in culture yet showed density-dependent inhibition of growth, analogous to established cell lines such as NIH 3T3. Karyotype analysis, performed at approximately 20 doublings after cloning, revealed the cells to be euploid with an elongation of one chromosome 5q, as the only abnormality (not shown). This cell line formed dense foci when transfected with RAS or GLI but not when transfected with E1A, antiGLI, or MYC (Fig. 2; Table 1). In contrast to the cotransformation assay in primary cells, in which [E1A + RAS] induced foci more efficiently than did [E1A + GLI], this cell line yielded approximately 30 foci per flask when either the RAS or GLI plasmid was used. The cell lines derived from GLI- or RAS-induced RK3E foci were morphologically similar to those derived by cotransfection of [E1A + GLI] or [E1A + RAS], respectively, into primary cells.

GLI-transformed cells are tumorigenic. To further characterize the GLI-induced phenotype in these lines, 5×10^6 cells per site were injected subcutaneously into nude mice. The E1A-expressing line RK3E failed to form tumors at 9 weeks, while one RAS transformant and two independent GLI transformants of RK3E, as well as an [E1A + GLI] cotransformant of primary BRK cells, all formed tumors at each injection site (Table 2). GLI-induced tumors were generally greater than 1 cm in diameter 3 weeks after injection. RK3E-RAS cells formed 2-cm-diameter tumors at 14 days.

GLI is expressed in transformed foci. Western analysis detected a 150-kDa species in three GLI tranformants of RK3E cells and in an [E1A + GLI] cotransformant of primary BRK cells (Fig. 3). The 150-kDa species comigrated with the endogenous GLI protein expressed in the human glioblastoma multiform-derived cell line D259 MG (3) (Fig. 3, lane 1), which contains a 75-fold amplification of the *GLI* locus (14). Thus, transformed rat cells expressed levels of GLI protein which were comparable to that found in human tumor cells with *GLI* amplification.

Because the GLI vector encodes a DNA-binding protein that could act as a transcription factor, it was possible that GLI-E1A cooperation resulted from transactivation of E1A expression by GLI. This possibility was tested by examining the levels of E1A expression in GLI transformants relative to nontransformants. Northern (RNA) blot analysis of total cellular RNA isolated from an [E1A + GLI] cotransformant of BRK cells and from two GLI transformants of RK3E cells revealed equivalent or reduced levels of E1A transcripts relative to those found in the parent RK3E cells and RAS-transformed RK3E cells (data not shown).

Cooperation of two nuclear oncogenes. In vitro transformation methods have allowed classification of oncogenes into functional groups on the basis of their ability to cooperate with other oncogenes. When transfected into primary rodent cells, genes encoding the nuclear proteins Myc, N-Myc,

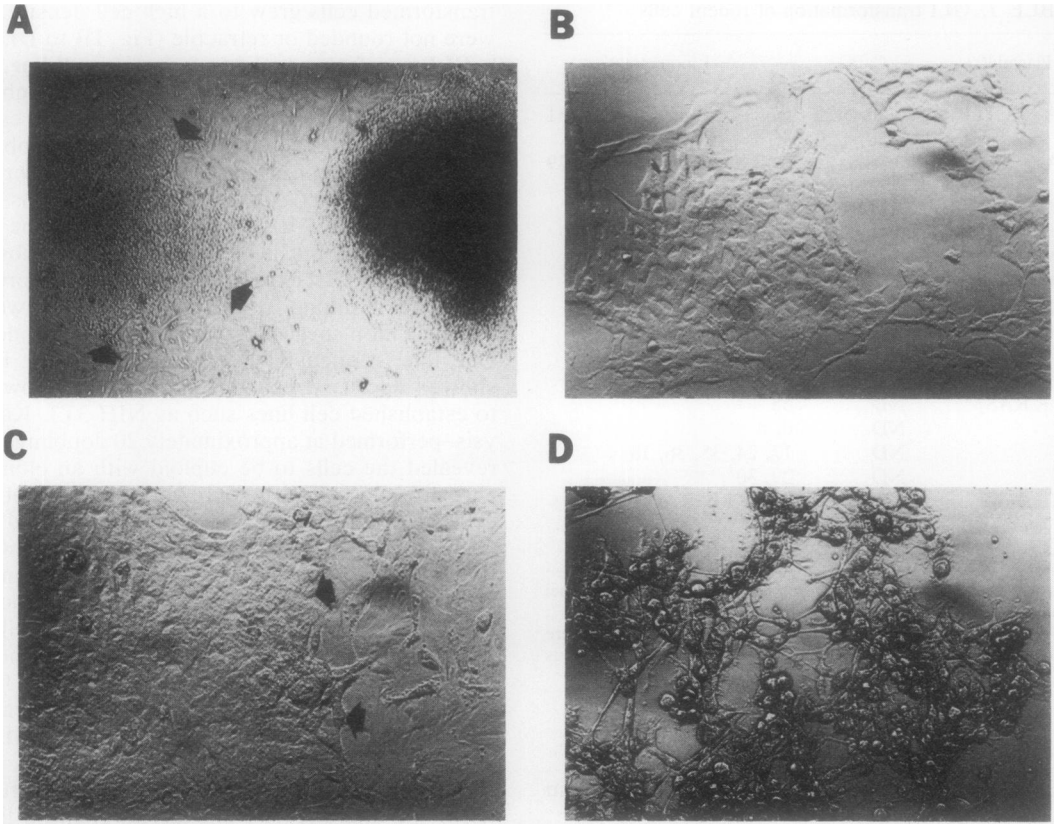

FIG. 1. Primary rodent cells transformed with [E1A + GLI]. (A) Low-power phase-contrast view of two adjacent foci in a flask of BRK cells transfected with [E1A + GLI]. The monolayered focus on the left (arrowed) displayed the morphology typical of E1A-transfected cells (9, 24). GLI cooperated with E1A to transform a subset of the foci, resulting in a dense, multilayered morphology, as shown on the right. In panels B and D, BRK foci induced by [E1A + GLI] or [E1A + RAS] were cloned and passaged in culture. These lines manifested their increased growth potential at confluence, resulting in a dense, multilayered culture. However, when grown at subconfluence, the high-power microscopic appearance of [E1A + GLI]-transformed cells (B) was similar to that of cells transformed by E1A alone (an E1A-induced focus [arrowed] is shown adjacent to normal BRK cells in panel C). In contrast, [E1A + RAS]-transformed cells appeared refractile and acquired a more rounded morphology (D), as previously reported (24, 36).

L-Myc, p53, C-Jun, or the polyomavirus large T antigen, papillomavirus E7, or adenovirus E1A can each cooperate with *ras*, resulting in tumorigenic foci of transformed cells (28, 34, 35). Conversely, oncogenes encoding the cytoplasmic proteins RAS, SRC, or polyomavirus middle T antigen cooperate with the nuclear protein MYC. That classification of oncogenes into functional groups generally reflects subcellular localization has suggested that members within one group may function in related signal transduction pathways. The GLI-E1A cotransformation results described here provide an exception to the generality that nuclear oncogenes cooperate with a cytoplasmic protein to transform primary cells (for some other exceptions, see references 1 and 10) and suggest that the mechanism by which *GLI* transforms may be different from that of other nuclear oncogenes.

The well-characterized adenovirus E1A gene codes for proteins that are multifunctional, with functions including cooperation with RAS or adenovirus E1B in transformation, activation, or repression of transcription from various promoters, and induction of DNA synthesis (4, 16, 17, 19, 24, 32). In vitro mutagenesis has allowed assignment of certain of these functions to specific regions of the proteins (33, 36). In the future, these mutant E1A constructs could be used to compare GLI cooperativity with other functions such as RAS or E1B cooperativity.

RK3E transformation assay. E1A is unique among the members of the nuclear group of oncogenes in that transfection of E1A alone into primary rodent cells results in the formation of foci (9, 24), while transfection of the other nuclear oncogenes typically results in no morphological phenotype (35). That *GLI* should cooperate specifically with E1A may reflect the greater activity of E1A in vitro compared with the other members of the nuclear group. Thus far, *GLI* and *ras* are the only nonviral genes that have been shown to cooperate with E1A. Just as a variety of oncogenes have been isolated by using transformation of established rodent lines such as NIH 3T3 (references in reference 34), transformation of the E1A-expressing cell line RK3E may also prove useful for identifying oncogenes, especially since the GLI vector failed to transform NIH 3T3 cells.

GLI function. By analogy with genes encoding other zinc finger proteins, *GLI* may encode a transcription factor (7, 15). If GLI transforms by binding to DNA and altering the expression of other genes, then a subset of GLI binding sites is likely to represent the regulatory elements of these genes. A polymerase chain reaction approach has recently been used to isolate GLI binding sites from the human genome, each of which contained a 23- to 25-bp sequence protected from DNase I when bound to GLI (13, 14). Because the five GLI zinc fingers bind to relatively long sequence elements,

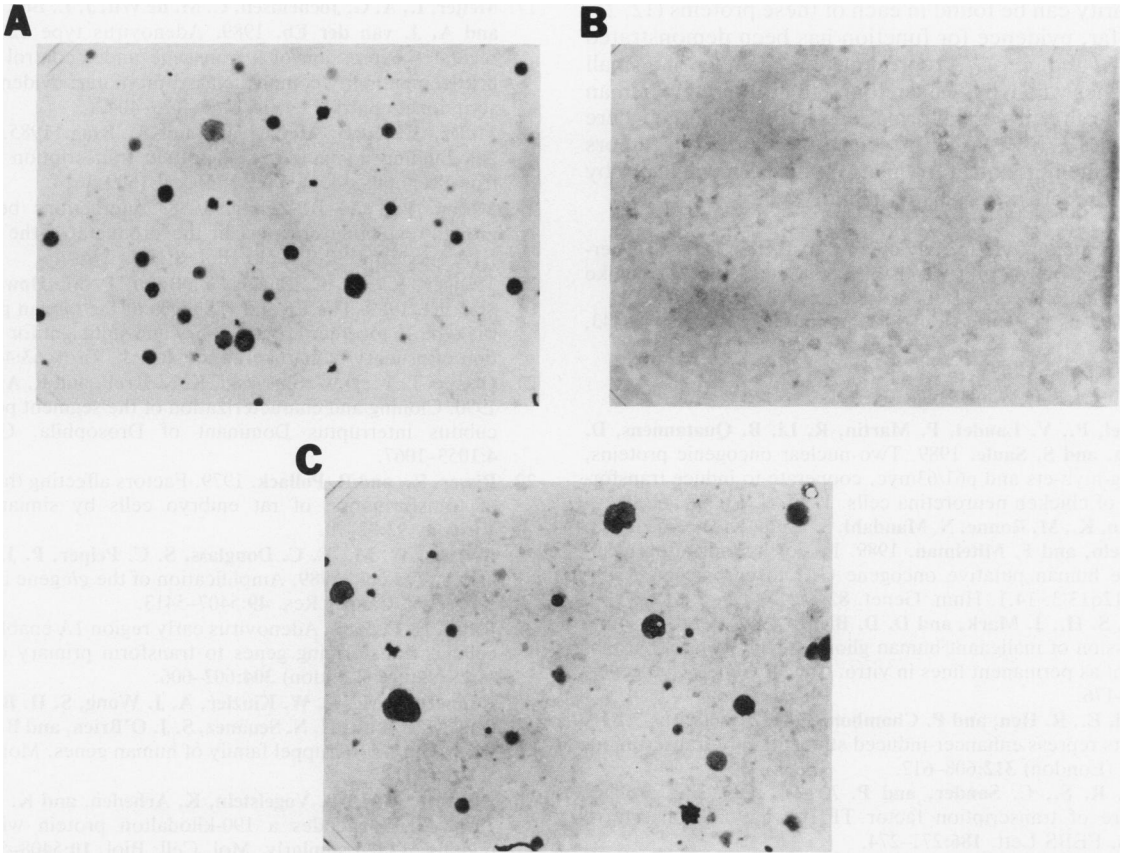

FIG. 2. GLI transformation of an established cell line. The cell line RK3E was cloned from an E1A-induced BRK focus. After expansion in culture, this line transformed efficiently with either GLI (A) or RAS (C) but showed no background of foci without transfection or when transfected with antiGLI (B), MYC, or E1A (see Table 1).

the subset of genes with which GLI interacts may be more easily identified than those interacting with previously described nuclear oncoproteins. As a variety of cellular polypeptides have been shown to bind to the E1A proteins, including the retinoblastoma gene product (37), the identification of downstream cellular components which mediate *GLI*-E1A cotransformation may be an approachable problem.

GLI is the prototype of a family of zinc finger protein-encoding genes that includes the human genes *GLI2* and *GLI3*, the mouse gene *mgli*, and the *Drosophila* segment polarity gene cubitus interruptus dominant (ci^D) (12, 21, 25, 26); each of these genes codes for a zinc finger region that is 77 to 99% similar to that of GLI. In addition, other regions of

TABLE 2. Tumorgenicity of GLI-transformed cell lines in nude mice[a]

Cells	No. of tumors/ no. of sites injected
RK3E	0/4
RK3E-RAS	2/2
RK3E-GLI-D	4/4
RK3E-GLI-E	4/4
BRK20	2/2

[a] For each line, 5×10^6 cells were injected into both flanks of one or two nude mice.

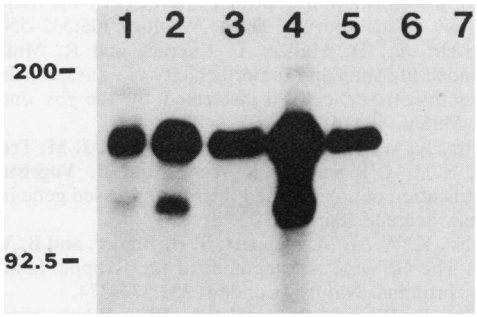

FIG. 3. Western immunoblot analysis of GLI transformants. An affinity-purified rabbit antibody against GLI was used in conjunction with ^{125}I-radiolabeled goat antibodies to detect GLI protein as described previously (14). The GLI antibody identified a 150-kDa species in rat cell lines derived from GLI-induced foci (lanes 2 to 5) and in the human tumor-derived line D259 MG, which contains a 75-fold amplification of the *GLI* locus (lane 1). Molecular weight markers are indicated on the left in kilodaltons. Lanes: 1, human D259 MG tumor cells grown as a mouse xenograft; 2 to 4, RK3E-GLI-D, RK3E-GLI-E, and RK3E-GLI-F, respectively, which were independent GLI transformants of RK3E cells; 5, BRK20, derived by cotransformation of primary BRK cells with [E1A + GLI]; 6, RK3E-RAS, derived by RAS transformation of RK3E cells; 7, RK3E.

GLI similarity can be found in each of these proteins (12, 25, 26). Thus far, evidence for function has been demonstrated only for *GLI* and ci^D. *GLI* represents one member of a small group of genes which have been found to be altered in human tumors. The in vitro transformation assays described here suggest that *GLI* is a target of gene amplification in tumors and should facilitate a future analysis of the mechanism by which *GLI* affects a neoplastic phenotype.

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