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Role of Adenovirus E1B Proteins in Transformation: Altered Organization of Intermediate Filaments in Transformed Cells That Express the 19-Kilodalton Protein

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Cooperation of the nuclear oncogene E1A with the E1B oncogene is required for transformation of primary cells. Expression vectors were constructed to produce the 19-kilodalton (19K) and 55K E1B proteins under the direction of heterologous promoters in order to investigate the role of individual E1B proteins in transformation. Coexpression of E1A and either the 19K or 55K E1B gene products was sufficient for the formation of transformed foci in primary rat cells at half the frequency of an intact E1B gene, suggesting that the 19K and 55K proteins function via independent pathways in transformation. Furthermore, the effects of Ha-*ras* and the E1B 19K gene product were additive when cotransfected with E1A, suggesting that the 19K protein functions in transformation by a mechanism independent from that of *ras* as well. Although expression of E1A and either E1B protein was sufficient for the subsequent growth of cells in long-term culture, the 19K protein was required to support growth in semisolid media. As the 19K protein has been shown to associate with and disrupt intermediate filaments (IFs) when transiently expressed with plasmid vectors (E. White and R. Cipriani, Proc. Natl. Acad. Sci. USA, 86:9886–9890, 1989), the organization of IFs in transformed cells was investigated. Primary rat cells transformed by plasmids encoding E1A plus the E1B 19K protein showed gross perturbations of IFs, whereas cell lines transformed by plasmids encoding E1A plus the E1B 55K protein or E1A plus Ha-*ras* did not. These results suggest that an intact IF cytoskeleton may inhibit anchorage-independent growth and that the E1B 19K protein can overcome this inhibition by disrupting the IF cytoskeleton.

The left 11% of the adenovirus chromosome, which encompasses early region 1 (E1), imparts the ability of this DNA tumor virus to transform primary rodent cells (20, 24). E1 is subdivided into two transcription units, E1A and E1B. The proteins encoded within the E1A gene are potent transactivators of transcription (reviewed in reference 5) and are able to stimulate cells to enter S phase (31, 48) and progress through mitosis (67). The mechanism by which E1A alters cell metabolism to promote transformation is not known but is probably related to the ability of E1A to bind to the product of the retinoblastoma tumor suppressor gene (62). This idea was substantiated by a mutational analysis of the E1A gene which has demonstrated a direct correlation between retinoblastoma tumor suppressor gene product binding and transforming ability of E1A (63). The overall consequence of E1A expression is stimulated cell cycle progression, whereupon E1A imparts the ability of otherwise quiescent cells to become immortal (26, 42). E1A-immortalized cells have extended growth potential over their primary cell counterparts but are not completely transformed in that they generally have a flattened shape, grow slowly and not to a high density, are anchorage dependent, and are not tumorigenic. Full manifestation of the transformed phenotype requires expression of a second, cooperating oncogene, such as the E1B gene (reviewed in references 12 and 18), or other cellular or viral oncogene products such as Ha-*ras*, polyomavirus middle T antigen (42, 66), or $pp60^{c\text{-}src}$ (45). Upon complete transformation, cells possess a very rounded shape, grow rapidly and to a high density, display anchorage-independent growth, and are often tumorigenic.

The function of some cytoplasmically localized oncoproteins has been emerging over the years. The *ras*-encoded proteins, for example, belong to the class of membrane-bound GTP-binding proteins and are presumed to exert their effects by intervening in signal transduction pathways (reviewed in reference 3). $pp60^{c\text{-}src}$ possesses tyrosine kinase activity, localizes to the inner surface of the plasma membrane, and is thought to function by altering the phosphorylation state of cellular proteins (reviewed in reference 8). Polyomavirus middle T antigen is also localized to the cytoplasm and forms a complex with $pp60^{c\text{-}src}$ (17), resulting in stimulation of *src* kinase activity (10). The protein products of the E1B oncogene, which cooperate with the E1A gene products and mediate transformation by adenovirus, do not fit into these models.

The E1B gene encodes two major proteins, the 19-kilodalton (19K) and 55K tumor antigens. Viral mutants defective for expression of either E1B protein are deficient for transformation, thereby establishing a role for both E1B proteins in the transformation process (4, 6). Neither E1B protein is homologous to other known oncoproteins, indicating that their function in transformation is by a novel mechanism. The function of the E1B 55K protein, however, may be related to its ability to bind the tumor suppressor gene product p53 (43).

The E1B 19K protein is associated with cytoplasmic and nuclear membranes and intermediate filaments (IFs) in the cytoplasm (vimentin) and the nuclear lamina (lamins) (37, 57, 57a). Until recently, a specific function of the 19K protein had not been established. We have discovered that when cells are transfected with plasmids encoding the 19K protein, the transiently expressed 19K protein associated with and dramatically altered the organization of IFs and the nuclear lamina (57a). This suggested that one cellular target for 19K function was the IF cytoskeleton and offered a possible

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mechanism by which the 19K protein functions in transformation and productive infection.

The aim of this investigation was to address the abilities of the 19K and 55K E1B proteins to cooperate with the E1A gene products in the transformation of primary cells. Vectors were constructed that were capable of expressing either the 19K or 55K E1B protein separately and were used in cotransfection experiments with a plasmid encoding the E1A gene. It was found, first, that both the 19K and 55K proteins can cooperate with the E1A proteins to produce transformed foci in primary rodent cells; second, that expression of the 19K protein was required for growth in semisolid media; and third, that transformed cells which express the 19K protein possess gross disruptions in the IF cytoskeleton. This last finding suggests that perturbations in the organization of IFs produced by the E1B 19K protein may lead to anchorage-independent growth and contribute to the transformed cell phenotype.

MATERIALS AND METHODS

Plasmid constructions. The pCMV19K plasmid was constructed by cloning E1B 19K protein-coding sequences from nucleotides 1704 to 2256 in front of the cytomegalovirus (CMV) promoter (11) followed by simian virus 40 small t intron and polyadenylation sequences (nucleotides 4713 to 2536 from the simian virus 40 genome) (57a). Expression from the overlapping E1B 55K reading frame was prevented by introducing a point mutation at nucleotide 2022 (C-to-T conversion) which produces a stop codon in the second codon of the 55K reading frame without affecting the amino acid sequence of the 19K protein (4). A plasmid expressing all of E1B from the CMV promoter, pCMVE1B, was constructed by insertion and substitution of wild-type viral DNA sequences of pE1 from nucleotides 1912 to 3931 into pCMV19K nucleotides 1912 to 2256. pCMVE1B encodes wild-type viral DNA sequences from nucleotides 1704 to 3931 and encompasses E1B 19K and 55K protein-coding sequences in their entirety. The plasmid expression vector for production of the E1B 55K protein, pCMV55K, was derived from pCMVE1B by deletion of 19K protein-coding sequences from nucleotides 1704 to 1912. A diagrammatic representation of the E1B plasmid expression vectors is presented (see Fig. 1). Plasmids encoding various segments of E1, pE1A (the E1A region), pE1B (the E1B region), and pE1 (E1A and E1B regions) were previously described (57a).

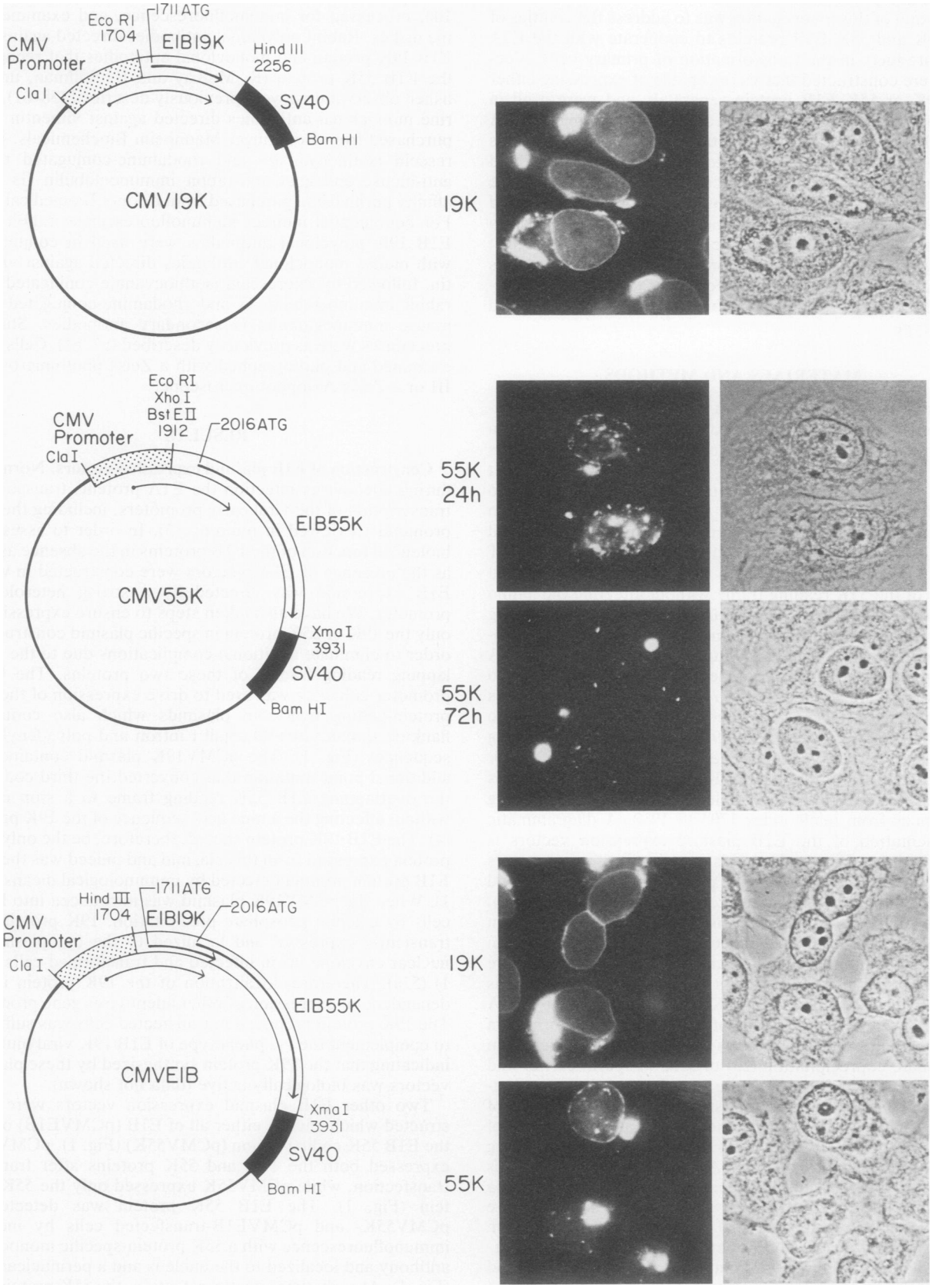
Cells and transfection procedures. BRK cells were grown in monolayer culture in Dulbecco modified Eagle medium with 5 or 10% fetal bovine serum. Primary BRK cells were prepared from baby rat kidneys of 6-day-old Fisher rats, as previously described (42). BRK cells transformed by E1A plus *ras* were a generous gift of E. Moran. Transformation and transient expression assays were performed by calcium phosphate coprecipitation (23) of salmon sperm carrier and plasmid DNAs. BRK cells were transfected 3 days postplating with 20 μg of DNA, comprising carrier DNA and 1 μg of each plasmid DNA, with the exception that transfections of pE1A plus pCMV19K plus T24 *ras* plasmids utilized 0.5 μg of pCMV19K and T24 *ras* DNAs. Growth in soft agar was assayed by suspending BRK cells in 0.4% agar-containing medium at a concentration of 5×10^5 cells per ml. Cells were then plated on a base layer of 0.8% agar-containing medium and evaluated for colony formation at 2 weeks postplating.

Antibodies and indirect immunofluorescence. HeLa and BRK cells were grown on glass cover slips and fixed with paraformaldehyde, followed by Triton X-100 extraction (61). BRK cells were also grown in tissue culture dishes, fixed with paraformaldehyde and permeabilized with Triton X-100, processed for immunofluorescence, and examined on the dishes. Rabbit polyclonal antibodies directed against the E1B 19K protein and monoclonal antibodies that recognize the E1B 55K protein (R. McKay and B. Stillman, unpublished observations) were previously described (58–61). Murine monoclonal antibodies directed against vimentin were purchased from Boehringer Mannheim Biochemicals. Fluorescein isothiocyanate- and rhodamine-conjugated rabbit anti-mouse and goat anti-rabbit immunoglobulin Gs were affinity purified and purchased from Cooper Biomedical, Inc. For double-label indirect immunofluorescence, rabbit anti-E1B 19K polyclonal antibodies were used in conjunction with mouse monoclonal antibodies directed against vimentin, followed by fluorescein isothiocyanate-conjugated anti-rabbit immunoglobulin G and rhodamine-conjugated anti-mouse immunoglobulin G secondary antibodies. Staining procedures were as previously described (57, 61). Cells were examined and photographed with a Zeiss photomicroscope III or a Zeiss Axiophot microscope.

RESULTS

Construction of E1B plasmid expression vectors. Normally, during adenovirus infection the E1A proteins transactivate transcription of the viral early promoters, including the E1B promoter (reviewed in reference 5). In order to assess the biological function of the E1B proteins in the absence as well as the presence of E1A, vectors were constructed in which E1B expression was directed by a strong heterologous promoter. We have also taken steps to ensure expression of only the 19K or 55K protein in specific plasmid constructs in order to eliminate additional complications due to the overlapping reading frames of these two proteins. The CMV promoter-enhancer was used to drive expression of the 19K protein-coding region in plasmids which also contained flanking simian virus 40 small t intron and polyadenylation sequences (Fig. 1). The pCMV19K plasmid contained an additional point mutation that converted the third codon of the overlapping E1B 55K reading frame to a stop codon without affecting the amino acid sequence of the 19K protein (4). The E1B 19K protein should, therefore, be the only E1B protein expressed from this plasmid and indeed was the only E1B protein product detected by immunological means (Fig. 1). When the pCMV19K plasmid was introduced into HeLa cells by calcium phosphate precipitation, 19K protein was transiently expressed and localized to the cytoplasm and nuclear envelope, as in infected and transformed cells (Fig. 1) (57a). Therefore, localization of the 19K protein is not dependent on expression of other adenovirus gene products. The 19K protein produced in transfected cells was sufficient to complement the *cyt* phenotype of E1B 19K viral mutants, indicating that the 19K protein synthesized by these plasmid vectors was biologically active (data not shown).

Two other E1B plasmid expression vectors were constructed which encode either all of E1B (pCMVE1B) or just the E1B 55K coding region (pCMV55K) (Fig. 1). pCMVE1B expressed both the 19K and 55K proteins after transient transfection, while pCMV55K expressed only the 55K protein (Fig. 1). The E1B 55K protein was detected in pCMV55K- and pCMVE1B-transfected cells by indirect immunofluorescence with a 55K protein-specific monoclonal antibody and localized to the nucleus and a perinuclear spot (Fig. 1). At early times posttransfection, the 55K protein was predominantly nuclear but gradually accumulated in the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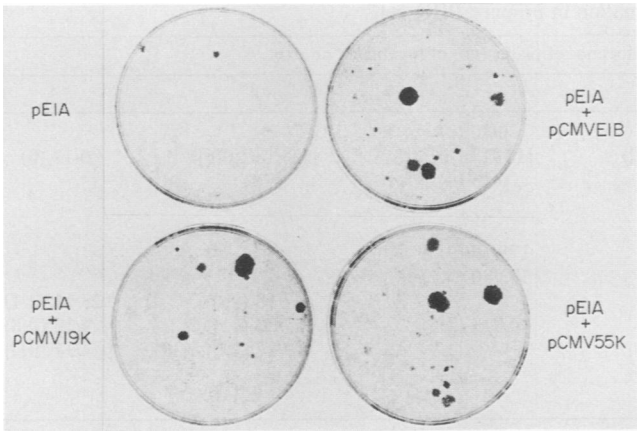

FIG. 2. Focus formation by E1A and E1B plasmids on primary BRK cells. Primary BRK cells were transfected with plasmids encoding E1A (pE1A), E1A and E1B (pE1A + pCMVE1B), E1A and the E1B 19K protein (pE1A + pCMV19K), and E1A and the E1B 55K protein (pE1A + pCMV55K). Plates were stained with Giemsa stain at 4 weeks posttransfection, and a representative plate of each plasmid combination from experiment 4 in Table 1 is shown.

perinuclear region with time (Fig. 1). Furthermore, the 55K protein was found to colocalize with p53 in a perinuclear spot (data not shown), resembling the localization of E1B 55K and p53 proteins in transformed cells (9, 64, 65).

Transformation of primary rodent cells with E1A and E1B. The abilities of the E1B 19K and 55K proteins to cooperate with E1A to transform primary BRK cells were determined by DNA-mediated gene transfer. BRK cells were transfected with plasmids encoding E1A alone (pE1A), E1A and E1B (pE1, pE1A plus pE1B, and pE1A plus pCMVE1B), E1A and the E1B 19K protein (pE1A plus pCMV19K), and E1A plus the E1B 55K protein (pE1A plus pCMV55K) (Fig. 1). At 3 to 4 weeks posttransfection, plates were fixed and stained with Giemsa stain, and the number of transformed foci was determined.

Transfection of BRK cells with DNA encoding E1A alone resulted in the appearance of a few small, not very dense, and often abortive foci that were difficult to establish in long-term culture (Fig. 2; indicated in brackets in Table 1) (42, 55, 66). Expression of E1B alone was insufficient to cause transformation, since transfection of BRK cells with pCMV19K, pCMV55K, and pCMVE1B did not result in foci formation (Table 1) (53), nor did transfection of cells with the T24 Ha-*ras* plasmid (data not shown) (42). Transfection of plasmids encoding E1A and E1B, on either the same or separate plasmids, resulted in the appearance of large numbers of dense foci (Table 1; Fig. 2). When E1A and E1B were encoded on the same plasmid (pE1), however, focus formation was greatly increased because of *cis* effects of E1B on the expression of E1A (29). Cooperation of E1A with E1B was not affected by expression of E1B from a strong promoter, since transfection of pE1A plus pE1B or pE1A plus pCMVE1B gave rise to foci at exactly the same frequency (Table 1).

Cotransfection of pE1A plus pCMV19K plasmids greatly increased the frequency of focus formation over transfection of pE1A alone, resulting in about half the number of foci obtained with an intact E1B gene (Table 1). These foci grew rapidly and to a high density, resembling foci produced by E1A and an intact E1B gene and not those produced by transfection of E1A alone (Fig. 2). Interestingly, transfection of E1A plus Ha-*ras* plus E1B 19K protein-expressing plasmids gave more foci than E1A plus *ras* or E1A plus E1B 19K protein, suggesting that *ras* and the 19K protein exert an additive effect on transformation and act by different mechanisms. The E1B 55K protein also contributed to the transformation process by promoting the frequency of focus formation to half that of an intact E1B gene (Table 1). The resulting foci were large and dense, closely resembling foci produced by E1A and E1B (Fig. 2). Therefore, both E1B proteins contribute to the transformation process, since expression of either one along with E1A greatly promoted the formation of foci. The additive effects of the E1B 19K and 55K proteins on transformation, however, suggest that they function by independent pathways.

Morphologies of transformed cell lines. Individual foci arising from independent transfections of BRK cells with pE1A, pE1A plus pCMV19K, pE1A plus pCMV55K, and pE1A plus pE1B were cloned and expanded, and the resulting cell lines were passaged in culture for 3 to 6 months. All cell lines were checked by indirect immunofluorescence for expression of E1A and E1B 19K and 55K proteins (data not shown). The level of expression of E1B proteins in the transformed cell lines was apparently unrelated to whether E1B was expressed from the E1B or the CMV promoter, since lines derived from pCMV constructs did not overexpress E1B proteins relative to cell lines derived from pE1A plus pE1B.

We were only rarely successful in obtaining pE1A-derived cell lines, a difficulty probably related to their slower growth rate. E1A-immortalized cells were very flat, tended to grow in parallel orientation, and often resembled the primary BRK cells (Fig. 3A) (6, 26, 46, 47, 66). In contrast, foci derived from cotransfection of pE1A with the pCMV19K, pCMV55K, pE1B, or pCMVE1B plasmids were readily established into continuous cell lines. Furthermore, the cells that expressed E1B gene products possessed very transformed morphologies (Fig. 3). Subtle differences, however, existed in the morphologies of the cell lines, depending on whether they expressed the E1B 19K or 55K protein. Cell lines expressing E1A plus E1B 19K protein were more refractile and spindle shaped, tended to form colonies with sharp edges, and had the capacity to grow to a 10-fold-higher density than an E1A-immortalized cell line (Fig. 3C and D). Cell lines expressing the E1A plus E1B 55K protein also grew to high densities, but they had an unusual shape in that they were somewhat cuboidal (Fig. 3E and F). The morphologies of the cell lines transformed by E1A plus E1B seemed to reflect contributions of both E1B proteins (Fig. 3B). Therefore, either E1B protein was capable of promoting focus formation, producing morphological changes associ-

FIG. 1. E1B plasmid expression vectors. The E1B 19K protein-coding sequences were cloned to direct expression from the CMV (pCMV19K) promoter. The pCMV19K plasmid also contains a stop codon to prevent expression from the overlapping reading frame encoding the E1B 55K protein (see text). CMV expression vectors encoding the E1B 55K protein (pCMV55K) and E1B (pCMVE1B) contained adenovirus sequences between nucleotides 1912 and 3931, and 1704 and 3931, respectively. A detailed account of the constructions is presented in Materials and Methods. Micrographs on the right demonstrate detection of the 19K and 55K proteins after transfection of HeLa cells with the indicated plasmid DNAs. Indirect immunofluorescence was performed at 48 h posttransfection. Magnification, ×985.

TABLE 1. Induction of focus formation in primary BRK cells

DNA	No. of foci/no. of plates (no. of foci/plate)[a] in expt:				
	1	2	3	4	5
Carrier			0/20 (0)		
pE1A	[10]/12 ([0.8])	[8]/12 ([0.7])	[12]/17 ([0.7])	[11]/18 ([0.6])	0/12 (0)
pCMV19K			0/18 (0)		
pCMVE1B		0/11 (0)			
pCMV55K	0/12 (0)				
pE1			179/19 (9.5)		
pE1A + pE1B	83/10 (8.3)	38/12 (3.2)	102/20 (5.1)		
pE1A + pCMVE1B		47/12 (3.9)		47/18 (2.6)	23/11 (2.1)
pE1A + pCMV19K	61/12 (5.1)		47/19 (2.5)	27/20 (1.4)	9/12 (0.8)
pE1A + pCMV55K				29/17 (1.6)	4/11 (0.4)
pE1A + Ha-*ras*	61/11 (5.5)				
pE1A + Ha-*ras* + pCMV19K	88/12 (7.3)				

[a] Brackets indicate that foci were small, not very dense, and often abortive.

ated with the transformed phenotype, and allowing the cells to grow rapidly and to a high density.

Growth of transformed cell lines in soft agar. BRK cell lines were plated in agar in order to assay anchorage-independent growth. An E1A-immortalized cell line was completely unable to grow in agar, where cells transformed by both E1A and E1B did grow (Table 2). Expression of the E1B 19K protein along with E1A was sufficient to permit growth in agar, forming macroscopic colonies within 1 to 2 weeks, whereas expression of E1A and the E1B 55K protein was not (Table 2).

Disruption of IFs in transformed BRK cell lines. The arrangement of IFs was investigated in BRK cell lines that were immortalized by E1A alone or transformed by E1A plus E1B, E1A plus pCMV19K, E1A plus pCMV55K, or E1A plus *ras*. As expected, the cell lines which expressed the E1B 19K protein displayed perturbations in the arrangement of IFs, whereas in lines that did not express the 19K protein, the arrangement of IFs appeared normal. What was surprising, however, was the degree of IF disruption. In primary BRK cells, BRK cells immortalized by E1A, and BRK cells transformed by E1A plus pCMV55K or E1A plus *ras*, the IFs appeared as continuous filaments radiating out from the nuclear envelope to the cell perimeter (Fig. 4A and B; Table 3). IF disruption was observed in BRK cells transformed by pE1A plus pCMV19K or pE1A plus pE1B plasmids (Fig. 4; Table 3). The types of IF distributions in these cell lines fell into four categories: cells in which the IFs appeared normal and staining of the 19K protein was faint; cells in which the IFs appeared short and very disorganized, with 19K protein staining evident in areas of the most pronounced disorganization (Fig. 4C and D); cells in which vimentin appeared in large perinuclear aggregates coincident with the localization of the 19K protein (Fig. 4E and F); and cells which appeared predominantly devoid of vimentin staining (Fig. 4G and H). Whether the absence of vimentin staining represents the total lack of vimentin IFs or is a consequence of epitope masking is not yet known.

The level of IF disruption varied from cell line to cell line and correlated with the level of expression of the 19K protein in the particular cell line and in the individual cell. Most of the cell lines transformed by E1A plus E1B, however, showed extreme levels of IF disruption and were composed of a mixture of cells showing the disorganized, aggregated, or no vimentin staining patterns. The reason for heterogeneity in the levels of 19K protein and IF disruption within individual cells is unclear and perhaps reflects cell cycle regulation of 19K expression or the amount of insoluble 19K protein bound to IF structures. The severity of IF and lamina disruption increased with greater levels of 19K protein expression. Low levels of 19K protein expression from the metallothionine promoter under uninduced conditions, for example, are insufficient to disrupt IFs (data not shown). The 19K protein is found in abundant quantities relative to some other viral early proteins in infected and transformed cells, although its level probably only approaches one-fifth that of vimentin, which is one of the most abundant cellular proteins (data not shown). Furthermore, only about 20 to 25% of the 19K protein is insoluble in transfected (57a) and transformed (data not shown) cells, although the insoluble 19K protein increases somewhat in infected cells at late times, when all the 19K protein is in the nuclear envelope and lamina (57). This is in sharp contrast to vimentin and lamins, which are predominantly insoluble. We can only conclude that low or substoichiometric ratios of 19K protein to IF proteins are sufficient for IF disruption. Whether the 19K protein binds directly to either vimentin or lamins is currently under investigation.

Despite the disruption of the vimentin network in BRK cell lines transformed by E1A and E1B, tubulin and actin distributions appeared normal (data not shown). As we were unable to detect cytokeratins 8 and 18 in the transformed BRK cells, although these cytokeratins are expressed in primary BRK cells and E1A-immortalized BRK cells (15, 40), the effect of the 19K protein on the cytokeratin network of BRK cells is unknown. The absence of cytokeratins in cells transformed by E1A plus E1B is not a reflection of E1B 19K expression, however, since these same cytokeratins were undetectable in the transformed cell lines expressing the E1A and E1B 55K proteins. Furthermore, the lamina of 19K protein-expressing transformed cell lines remained more or less intact, although small discontinuities in lamin staining could occasionally be observed (data not shown). The organization of the lamina in these transformed cells is currently under detailed investigation. In conclusion, BRK cells transformed by adenovirus DNA sequences displayed gross perturbations in the vimentin IF network, and this result directly correlated with expression of the E1B 19K protein. Furthermore, IF disruption is not necessarily obligatory for transformation, since cells transformed by E1A plus E1B 55K protein or E1A plus *ras* cells did not possess disrupted IFs.

DISCUSSION

Function of the E1B proteins in transformation. Insight into the specific contribution of E1B to the transformation pro-

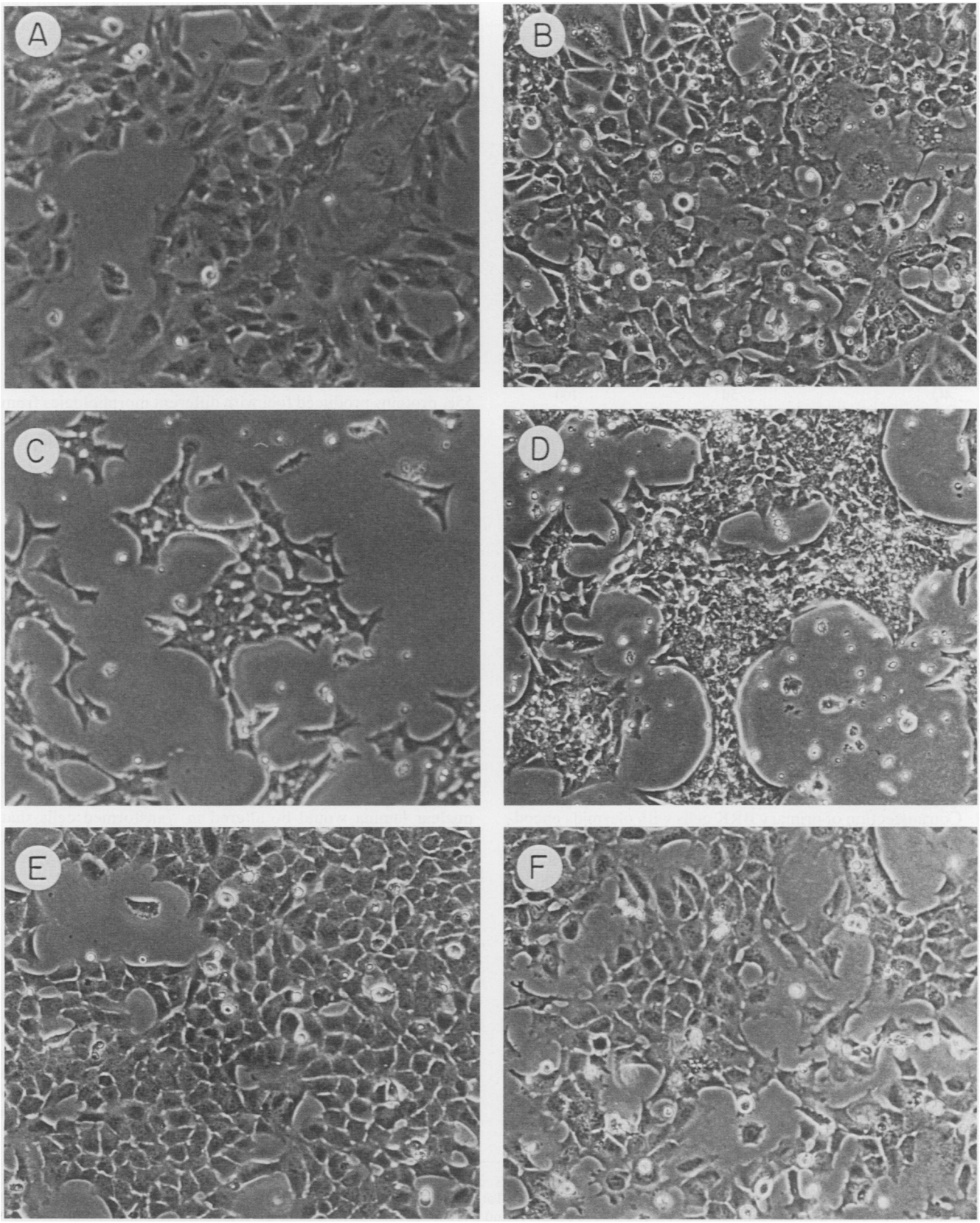

FIG. 3. Morphologies of transformed cells differentially expressing E1A and E1B gene products. Established cell lines derived from foci of plasmid DNA-transfected BRK cells were plated and photographed at subconfluent density. (A) E1A-1 (pE1A derived); (b) 4Q (pE1A and pE1B derived); (C and D) 3K and 17K (pE1A and pCMV19K derived), respectively; (E and F) 55A and 55C (pE1A and pCMV55K derived), respectively. Magnification, ×25.

TABLE 2. Growth of transformed cell lines in semisolid media

Transforming agent(s) and cell line	% Growth	
	Expt 1	Expt 2
E1A		
E1A-1	<0.0002	<0.0002
E1A + E1B 19K		
3K	100	
17K	5	10
19A		90
E1A + E1B 55K		
55A		<0.0002
55B		<0.0002
55C		<0.0002
E1A + E1B		
4D	0.5	
4E	20	
4P	50	
4Q	50	100

cess has been provided by comparison of cell lines immortalized by E1A alone versus those transformed by E1A and E1B. The E1B gene products promote focus formation (6, 26, 46), alter morphology (6, 26), hasten growth rates (26, 55), prevent contact inhibition, and promote anchorage independence and tumorigenicity (7, 19, 38, 51, 56). How E1B accomplishes this is of great interest. The function of the individual E1B proteins in the transformation process has, however, been difficult to determine because of a number of factors: the overlapping reading frames of the 19K and 55K proteins (4, 35, 41, 55, 64), the *cis* effect of the E1B region on E1A transcription (29), the employment of different assay systems for examining transformation (28, 44), and the method of introduction of E1 sequences (6). By ensuring the individual expression of E1B proteins, encoding them on separate plasmids, and performing transformation assays by DNA transfection of primary cells, we have taken steps to minimize these complications.

Cotransfection of primary BRK cells with plasmids encoding E1A and those which differentially express the E1B proteins has unambiguously demonstrated that expression of either the 19K or 55K E1B gene product is sufficient for cooperation with the E1A gene products and transformation of primary rodent cells, albeit at half the efficiency of an intact E1B gene. As the effects of the 19K and either the 55K or Ha-*ras* gene products were additive, they very likely function to transform cells by independent mechanisms. These foci expressing E1A plus E1B 19K proteins or E1A plus E1B 55K proteins were readily expanded into continuous cell lines that displayed various aspects of the transformed cell phenotype. Possessing cell lines that differentially express E1B proteins has enabled us to probe the involvement of individual E1B proteins in transformation. E1A and E1B 19K and E1A and E1B 55K protein-expressing cell lines are clearly different from E1A-immortalized cell lines in that they have more transformed morphologies and grow rapidly and to high densities. We expected, however, that like viral mutant-transformed cells, these cell lines might not display all the properties of completely transformed cells.

The introduction of mutations into either the E1B 19K or 55K protein-coding regions caused a reduction in the frequency of transformation, demonstrating contribution by both E1B proteins in the transformation process (1, 4, 6, 14, 30, 38, 51, 52). Viruses expressing mutant E1B 19K proteins produced foci that were deficient for growth in soft agar and low-calcium medium and were not tumorigenic, suggesting a role for the 19K protein in anchorage-independent growth and tumor formation (6, 19, 38, 50, 51). Comparison of our cell lines expressing the E1A and E1B 19K proteins or the E1A and E1B 55K proteins supported these conclusions, since the E1A and E1B 19K protein-expressing cell lines grew in soft agar, in which the E1A and E1B 55K protein-expressing lines did not. Viruses expressing mutant E1B 55K proteins produced foci with different morphologies from those arising from infection with the wild-type virus, implicating the 55K protein in contributing to morphological aspects of transformation (6). Furthermore, the morphologies of cell lines transformed by E1A plus E1B seemed to reflect a combinatorial effect of both E1B proteins.

Possible role for IF disruption in transformation. Recently, we have discovered a clue as to the function of the E1B 19K protein. When the 19K protein is expressed in cells, either transiently or during viral infection, it becomes complexed with vimentin filaments and the nuclear lamina, causing their disruption (57a). Thus, IF disruption identified a biological activity associated with the 19K protein and provided one possible mechanism by which the biological effects of the 19K protein in infected and transformed cells might be mediated. These results also demonstrated that the E1B 19K protein possesses biological activity independent of E1A and gave credence to the idea that E1B gene products do more than augment the levels of E1A proteins, thereby promoting transformation, as some data might indicate (44, 54). This raised the possibility that the organization of IFs and/or the nuclear lamina would be altered in transformed cells that express the 19K protein, which was indeed the case (Fig. 4). We anticipate that altered organization of IFs may contribute to the ability of the 19K protein to promote focus formation by E1A and growth of transformed cells in semisolid media. That is, a well-organized IF cytoskeleton may slow cell growth. This possibility is intriguing in light of the fact that expression of IF proteins is developmentally regulated and is often associated with differentiation (49).

Morphological alterations associated with the transformed cell phenotype have long implicated a role for the actin cytoskeleton in transformation. Changes in the organization of the actomyosin network were first observed in simian virus 40-transformed cells (36, 39). $pp60^{v\text{-}src}$ has been shown to phosphorylate a number of cytoskeletal proteins, which likely contributes to disruption of stress fiber organization

FIG. 4. Distribution of IFs in transformed BRK cell lines. BRK cell lines E1A-A (pE1A immortalized), 55A (pE1A plus pCMV55K transformed), and 4D and 4Q (pE1A plus pE1B transformed) (Table 3) were fixed with paraformaldehyde and stained for indirect immunofluorescence with antivimentin (E1A-1 and 55A in panels A and B, respectively) or for double-label indirect immunofluorescence with antivimentin (C, E, and G) and E1B 19K protein (D, F, and H)-specific antibodies (4D and 4Q). E1A-A (A) and 55A (B) cell lines did not express the E1B 19K protein, and therefore only the antivimentin staining is shown. In panels C to H, each pair is the same field of cells and is representative of typical abnormal vimentin distributions observed in the 4D and 4Q cell lines; (C) disorganized; (E) aggregated; (G) no vimentin staining (see text). Magnification ×985 (A, B, and E to H) or ×1,564 (C and D).

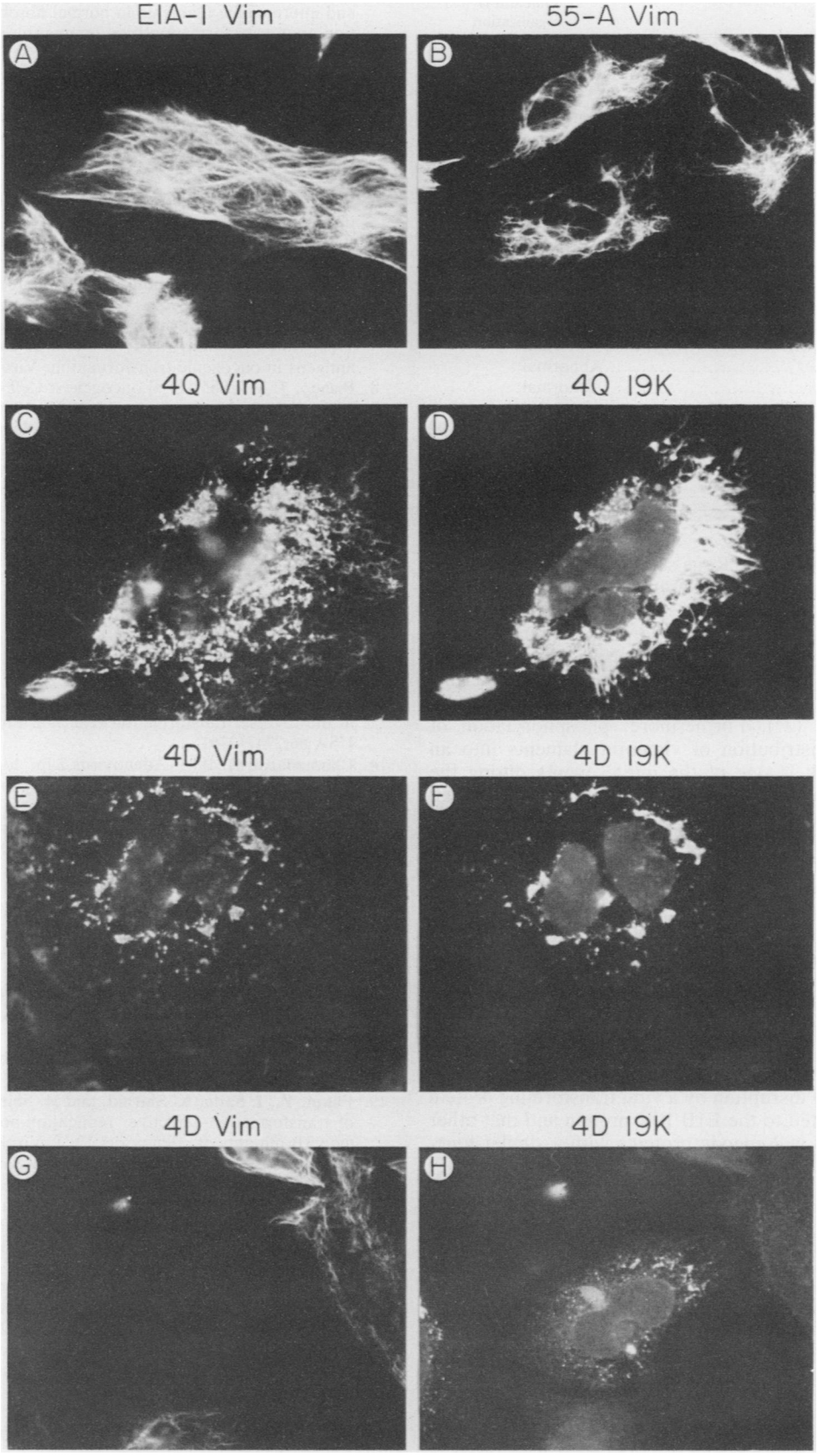
E1A-1 Vim
55-A Vim
A
B
4Q Vim
4Q 19K
C
D
4D Vim
4D 19K
E
F
4D Vim
4D 19K
G
H

TABLE 3. Disruption of IFs in BRK cell lines

Cell line	Vimentin IF organization
Primary BRK	Normal
pE1A-immortalized E1A-1	Normal
pE1A- and T24 Ha-*ras*-transformed E1A-RAS1	Normal
pE1A- and pCMV55K-transformed	
55A	Normal
55B	Normal
55C	Normal
pE1A- and pCMV19K-transformed	
3K	Abnormal + normal
19A	Abnormal + normal
17K	Abnormal + normal
pE1A- and PE1B-transformed	
4A	Abnormal + normal
4D	Abnormal
4E	Abnormal
4L	Abnormal
4P	Abnormal
4Q	Abnormal
4Z	Abnormal
19G	Abnormal + normal
E1AE1BB	Abnormal

and altered morphology of Rous sarcoma virus-transformed cells (see reference 22 and references therein). Yet other transformed cells have altered expression of cytoskeletal proteins (16, 21, 25, 32, 34). Altered organization of IFs in transformed cells has also been reported. Rous sarcoma virus-transformed cells, for example, display subtle perturbations in IF organization (2), as do IFs in other virally transformed cells (27). Furthermore, phosphorylation of vimentin and redistribution of vimentin filaments into an organized network is one of the initial events during the process of reverse transformation induced by cyclic AMP (13). How IF disruption may promote transformation is not certain but may be related to modulation of signal transduction, cell-cell interaction, or chromatin structure.

In E1-transformed BRK cells, it is the organization of IFs and not the microtubule and actin cytoskeletal frameworks that is drastically affected. This is the first demonstration of a viral transforming protein able to specifically affect IF organization. Interestingly, a transforming protein encoded by Epstein-Barr virus, the latent infection membrane protein, has been reported to colocalize and cofractionate with IFs and has been proposed to be responsible for altered IF organization in Epstein-Barr virus-transformed cells (33). It is probable that IF disruption by a viral transforming protein will not be restricted to the E1B 19K protein and that other oncogenic viruses will encode proteins with a similar function.

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