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Specific disruption of intermediate filaments and the nuclear lamina by the 19-kDa product of the adenovirus E1B oncogene

(DNA tumor virus/transformation/cytoskeleton/vimentin/nuclear envelope)

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ABSTRACT **The 19-kDa protein encoded within the adenovirus E1B gene is essential for transformation by adenovirus and for proper regulation of viral early gene transcription. In order to investigate the biological function of the 19-kDa E1B protein, vectors were constructed to produce the 19-kDa protein in mammalian cells under the direction of heterologous promoters. Surprisingly, during transient expression, the E1B 19-kDa protein specifically associated with and disrupted the organization of intermediate filaments and the nuclear lamina, without disturbing the organization of other cytoskeletal networks. These results directly demonstrate an effect of a viral transforming protein on the cytoskeleton and suggest a role for intermediate filaments and the nuclear lamina in modulation of viral gene expression and the process of oncogenic transformation.**

The region of the adenovirus genome responsible for regulating gene expression in productively infected cells and for oncogenic transformation of primary cells is E1. E1 is composed of two distinct transcription units, designated E1A and E1B. E1A encodes nuclear phosphoproteins with the capacity to transactivate transcription of viral early promoters (reviewed in ref. 1), stimulate cellular DNA synthesis (2, 3), and immortalize primary cells (4, 5). The E1B gene is required for proper regulation of viral gene expression in productively infected cells (6–8) and for full manifestation of the transformed phenotype (reviewed in refs. 9, 10). There are two major proteins produced from the E1B transcription unit, the 19-kDa and 55-kDa tumor antigens (Fig. 4), both of which are involved in transformation (8, 11, 12). The E1B 55-kDa protein binds to the cellular tumor antigen p53 and presumably contributes to the transformation process by this association (13). But the E1B 19-kDa protein plays a major role in transformation, since coexpression of E1A and the E1B 19-kDa protein is sufficient for induction of foci in primary baby rat kidney cells and growth of these cells in long-term culture (49). Although Ha-*ras*, polyoma middle tumor antigen (5), and $pp60^{c\text{-}src}$ (14) can substitute for E1B in transformation assays, the 19-kDa protein shows no homology to known guanine nucleotide-binding proteins, protein kinases, cell surface receptors, or growth factors. Therefore, the involvement of the E1B 19-kDa protein in the transformation process likely occurs by a unique mechanism.

Mutational analysis of the E1B 19-kDa coding region revealed that viral mutants were not defective for virus replication in productively infected cells (15–20). These mutants, however, displayed pleiotropic phenotypes that made interpretation of the primary role of the E1B 19-kDa protein difficult (7). The phenotypes of E1B 19-kDa viral mutants included the degradation of host cell and viral DNA (deg phenotype), enhanced and unusual cytopathic effect (cyt phenotype), the formation of large plaques (lp phenotype), and a host range (hr) phenotype whereupon the mutant viruses replicate more efficiently than the wild-type virus. Genetic experiments demonstrated that (*i*) manifestation of the 19-kDa mutant phenotypes was dependent upon expression of the E1A proteins, suggesting an interaction between the E1A and E1B 19-kDa proteins (20); (*ii*) the 19-kDa protein functioned to protect DNA from degradation during infection; and (*iii*) it acted as a negative regulator of E1A-dependent viral early gene transcription (7). As the 19-kDa protein was found in the cytoplasm and the nuclear envelope lamina (21, 22), the effect of the 19-kDa protein on gene expression (7, 23–25) and DNA integrity was likely to be indirect, possibly by way of an effect on mRNA metabolism, chromatin and nuclear structure, or the function of E1A proteins (7).

Since determination of E1B 19-kDa protein function was refractory to standard genetic analysis, we decided to look for a biological function of the 19-kDa protein by using plasmid expression vectors to produce the 19-kDa protein in cells, outside the realm of a productive infection. One dramatic effect of 19-kDa protein expression was the disruption of intermediate filaments (IFs) and the nuclear lamina, thereby suggesting the means by which a cytoplasmic oncoprotein functions in the transformation process and during productive infection.

MATERIALS AND METHODS

Plasmid Constructions. Advenovirus sequences from the left end of the viral genome (0–15.5 map units) encoding all of early region 1 (E1) (nucleotides 1–5778) were cloned into pBR322. This plasmid, designated pE1, was composed of wild-type viral DNA sequences from adenovirus 5 (nucleotides 1–1336) and adenovirus 2 (nucleotides 1337–5778) (Fig. 4). A plasmid encoding the E1A region, pE1A, was derived from pE1 by deletion of the E1B region between nucleotides 1769 (*Sac* I) and 3322 (*Bgl* II) (Fig. 4). A plasmid lacking E1A coding sequences but with an intact E1B gene, pE1B, was derived from pE1 by deletion of nucleotides 338 (*Ssp* I) to 1569 (*Hpa* I) (Fig. 4). Specific deletion of E1B 19-kDa coding sequences was accomplished by substituting a *Hpa* I to *Hin*dIII fragment of pE1 with viral DNA sequences from Ad5*dl*337, which contains a deletion of nucleotides 1769–1916 (18) (Fig. 4).

Expression of the E1B 19-kDa protein under the control of the mouse metallothionein promoter (pMT19K) was constructed by cloning a 1.4-kilobase (kb) fragment containing the promoter (E. Harlow, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) in front of E1B 19-kDa coding sequences from nucleotides 1704–2256. Expression from the overlapping E1B 55-kDa reading frame was prevented by a point mutation at nucleotide 2022 (cytosine to thymine conversion), which produces a stop codon in the second codon

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Abbreviations: SV40, simian virus 40; IF, intermediate filament; CMV, cytomegalovirus.

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of the 55-kDa reading frame without affecting the amino acid sequence of the 19-kDa protein (8). Promoter and E1B sequences were followed by simian virus 40 (SV40), small tumor antigen intron and poly(A) sequences, nucleotides 4713–2536 from the SV40 genome, and cloned into pUC (Fig. 1). Expression of the E1B 19-kDa protein directed by the cytomegalovirus promoter pCMV19K was accomplished by substituting a 576-base-pair (bp) fragment containing the CMV promoter and enhancer (ref. 26; C. Gorman, Genentech, San Francisco, CA) for the metallothionein promoter in pMT19K (Fig. 1).

Transfection Procedures. HeLa and COS cells were grown in monolayer culture in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and were transfected at 50% confluence. Transient expression assays were performed by calcium phosphate coprecipitation (27) followed by a glycerol shock at 4 hr after transfection.

Antibodies and Indirect Immunofluorescence. HeLa and COS cells were grown on glass coverslips and fixed with either methanol at −20°C (22) or paraformaldehyde followed by Triton X-100 extraction (28). The source of murine monoclonal antibodies was a follows: an anti-72-kDa heat shock protein antibody (29) was kindly provided by W. Welch (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY); an anti-lamin A and C (30) antibody was obtained from F. McKeon (University of California, San Francisco, CA); anti-rat cytokeratins 8 and 18 antibodies (31) were a generous gift of B. Lane (Imperial Cancer Research Fund, England); an anti-vimentin antibody was obtained from Boehringer Mannheim; an anti-tubulin antibody (32) was provided by S. Blose (Protein Data Bases, Huntington, NY); an anti-actin antibody was purchased from Amersham. Rabbit polyclonal antibodies directed against the E1B 19-kDa protein were previously described (7, 16). Fluorescein isothiocyanate- and rhodamine-conjugated rabbit anti-mouse and goat anti-rabbit IgGs were affinity purified and purchased from Cappel. Cells were examined and photographed with a Zeiss Axiophot microscope.

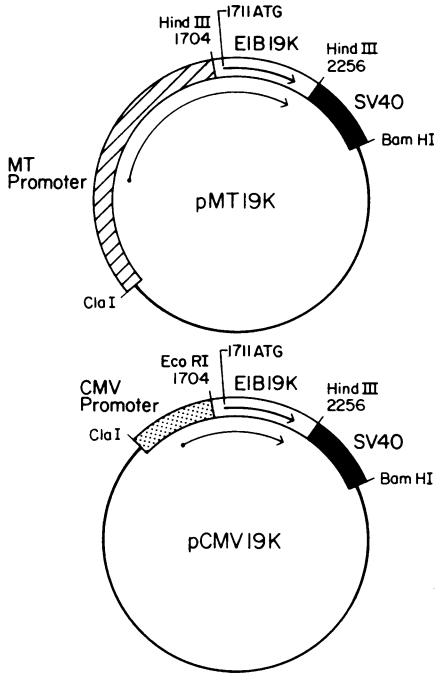

FIG. 1. E1B plasmid expression vectors. The E1B 19-kDa coding sequences were cloned either to direct expression from the metallothionein (MT) (pMT19K) or the cytomegalovirus (CMV) (pCMV19K) promoters. These plasmids also contained a stop codon to prevent expression from the overlapping E1B 55-kDa reading frame (see text).

Extraction Procedures. Cytoskeletal preparations were isolated from pCMV19K-transfected HeLa cells according to Capco *et al.* (33) with minor modifications (34). Briefly, cells plated on tissue culture dishes were extracted with 0.5% Triton X-100; this was followed by extraction with 250 mM ammonium sulfate and nuclease treatment (DNase I and RNase A). The remaining insoluble proteins were analyzed by polyacrylamide gel electrophoresis and Western blotting (22).

RESULTS

Construction of E1B 19-kDa Plasmid Expression Vectors. The E1B 19-kDa protein is a fairly abundant protein in adenovirus-infected and transformed cells. To reproduce these levels, the 19-kDa protein was expressed under the control of strong, heterologous promoters, the mouse metallothionein and the CMV promoter enhancer (Fig. 1). These plasmids, when introduced into human HeLa or simian COS cells by calcium phosphate precipitation, transiently express the E1B 19-kDa protein to levels comparable to those found in infected and transformed cells. The transiently expressed 19-kDa protein localized to the cytoplasm and nuclear envelope of transfected cells (see below), closely resembling its intracellular locale in transformed cells and in infected cells at intermediate times after infection (22).

Transient Expression of the E1B 19-kDa Protein Disrupts IFs and the Nuclear Lamina. Transfection of either HeLa or COS cells with the pCMV19K or pMT19K expression vector resulted in a subtle alteration in cell morphology. We therefore tested the possibility that expression of the E1B 19-kDa gene product could affect cell architecture. In normal HeLa cells or HeLa cells transfected with carrier DNA, the IFs existed as long filaments that extended from the vicinity of the nuclear envelope to the cell periphery (Fig. 2). Drastic alterations in the distribution of vimentin-containing IFs were observed in many of the cells that expressed the 19-kDa protein. Routinely, 10–50% of cells transfected with the pCMV19K plasmid expressed the 19-kDa and 12–50% of those had severe perturbations in the IF system at 48 hr after transfection. IF disruption was time dependent, occurring in eventually all 19-kDa-expressing cells by 72–96 hr. The IFs became detached from the nuclear envelope and the plasma membrane, formed large clusters in the cytoplasm, and often appeared fragmented (Fig. 2). The 19-kDa protein was often observed to colocalize with the disrupted IFs in the cytoplasm (Fig. 2).

Vimentin shares extensive amino acid and secondary structure homologies with the nuclear lamin proteins (30, 35). Furthermore, the 19-kDa protein copurifies with the lamina and has been localized there by immunoelectron microscopy (22). We therefore investigated the possibility that the 19-kDa protein might also disturb nuclear lamina structure in addition to cytoplasmic IFs. Lamin antibodies stain the nuclear envelope lamina in a uniform fashion, but 7% of 19-kDa protein-expressing cells at 48 hr after transfection possessed holes in the lamina (Fig. 2). The degree of lamina disruption increased with time and the level of 19-kDa expression and is underrepresented in Fig. 2 only because one focal plane is shown. As in the case of vimentin staining, the 19-kDa staining of the lamina was coincident with that of the lamins (Fig. 2).

Cytokeratins are another class of cytoplasmic IF-related proteins (36). In 19-kDa-expressing cells some disruption of the cytokeratin network was observed but it was not as pronounced as that found for the vimentin-type IFs (data not shown). Therefore, multiple classes of IFs are affected by 19-kDa expression, although vimentin and lamin networks were predominantly affected.

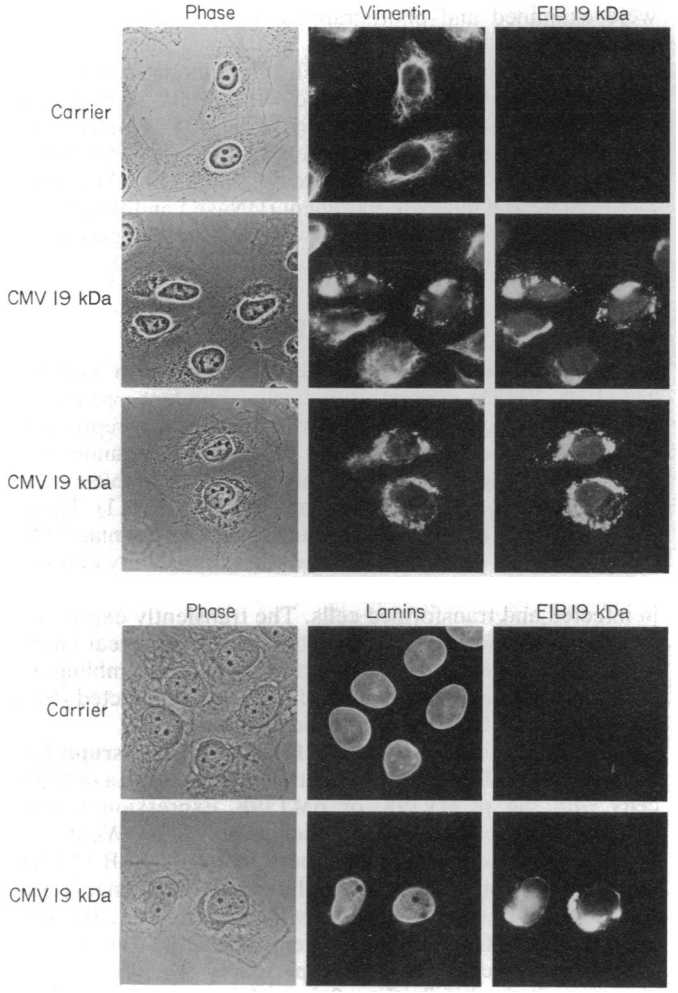

FIG. 2. Disruption of IFs and the lamina by 19-kDa expression in transient assays. HeLa cells were transfected either with carrier DNA or with pCMV19K plasmid DNA and at 48 hr after transfection cells were fixed in either paraformaldehyde (upper panels) or methanol (lower panels). Double-label indirect immunofluorescence was performed with a rabbit polyclonal antibody directed against the E1B 19-kDa protein in conjunction with murine monoclonal antibodies specific for vimentin (upper panels) or lamins (lower panels). The phase-contrast and immunofluorescent staining for vimentin or lamins and the E1B 19-kDa protein are shown for the same cells in each horizontal row. (×550.)

E1B 19-kDa Expression Does Not Affect Tubulin and Actin Networks. To establish whether the 19-kDa protein specifically altered IF-related structures or had a global effect on the cytoskeleton, tubulin and actin distributions were examined in cells transiently expressing the 19-kDa protein. In COS cells 19-kDa expression resulted in massive fragmentation of IFs (Fig. 3). Aggregation of vimentin staining was observed in the perinuclear region coincident with 19-kDa staining. In contrast to the disruption of IFs, microtubules (Fig. 3) and microfilaments (data not shown) were completely unaffected. The lack of severe alterations in cell morphology caused by IF disruption is likely due to specificity of the 19-kDa protein for the IF system and maintenance of the tubulin and actin cytoskeletal frameworks.

Disruption of IFs and Lamin Organization Is Specific for Cells Expressing the E1B 19-kDa Protein. The organization of IFs and lamina was examined by indirect immunofluorescence in HeLa and COS cells transfected with a series of E1 plasmids, encoding E1A and E1B proteins under control of their own promoters (Fig. 4). Transfection of a plasmid encoding all of early region 1 (pE1) caused IF and lamina disruption comparable to that of pCMV19K (data not shown). In fact, the level of 19-kDa expression in pE1-transfected cells was equivalent to pCMV19K-transfected cells, presumably due to trans-activation of the E1B promoter by E1A. No IF and lamina disruption was observed after transfection of p*dl*337 (Fig. 4), which encoded E1 but in addition contained a deletion with the 19-kDa gene (data not shown). IF and lamina disruption did occur in pE1B-transfected cells, where E1B expression is directed by the E1B promoter (Fig. 4), although reduced level of 19-kDa expression in pE1B resulted in less frequent and less severe effects on IFs and the lamina (data not shown). High-level expression of the 19-kDa protein was clearly not required for IF and lamina disruption, although it hastened its occurrence. Furthermore, as 19-kDa expression did not induce expression of the 72-kDa heat shock protein (data not shown), IF and lamina disruption did not merely represent a manifestation of the stress response due to expression of a viral protein in mammalian cells. Perturbation of IFs and the lamina was not a transfection artifact, nor was it due to overexpression of proteins in general. Rather, IF and lamina disruption was a specific consequence of expression of the E1B 19-kDa protein.

Cofractionation of the 19-kDa Protein with IFs. Colocalization of the majority of the 19-kDa protein in the cytoplasm with vimentin filaments in transient expression assays suggested that the 19-kDa protein may interact directly with IFs, as it does with the nuclear lamina (22). pCMV19K-transfected HeLa cells were extracted with non-ionic detergent, followed by high salt and nuclease treatments according to Capco *et al.* (33). This procedure preserves insoluble IFs and the lamina cytoskeleton (Fig. 5), while extracting most of the cellular proteins (data not shown). The 19-kDa protein cofractionated with the insoluble cytoskeleton in a similar fashion to that of vimentin and lamins A and C (Fig. 5). Furthermore, colocalization of the 19-kDa protein and vimentin was preserved in isolated cytoskeletons (data not shown).

DISCUSSION

The adenovirus E1B 19-kDa transforming protein functions to specifically disrupt IFs and the nuclear lamina of transfected cells. To our knowledge, there has been no previous demonstration of a non-IF protein other than an antibody (38, 39) that is capable of specifically altering the organization of IFs and the lamina *in vivo*. We have since observed IF and lamina disruption in adenovirus-infected (unpublished data) and transformed cells (49), which indicates that reorganization of IFs is a normal consequence of 19-kDa expression and that it is biologically relevant to the adenovirus life cycle. We can now begin to address (*i*) how the 19-kDa protein disrupts IFs and the lamina and (*ii*) why disruption of these structures could prevent the occurrence of the pleiotropic phenotypes in E1B mutant infected cells and promote oncogenic transformation.

Mechanism of IF and Lamina Disruption by the 19-kDa Protein. Cytoplasmic IF proteins and the nuclear lamin proteins are members of the same multigene family, sharing extensive amino acid and secondary structure homologies (30, 35). They also share the ability to assemble into insoluble filamentous networks in the cytoplasm and the nucleus, respectively (40–42). It was unusual and unexpected that expression of a viral transforming protein was capable of specifically disrupting structures that are resistant to dissolution by extreme regimens of nondenaturing detergents and high salt extractions. We favor a direct mechanism for 19-kDa-mediated IF and lamina disruption for the simple reason that the 19-kDa protein was found to colocalize and cofractionate with IFs and the lamina. This implies that there is a physical interaction between IF-related proteins and the E1B 19-kDa protein.

We have not detected any proteolytic cleavage or altered phosphorylation states of either vimentin or lamins resulting

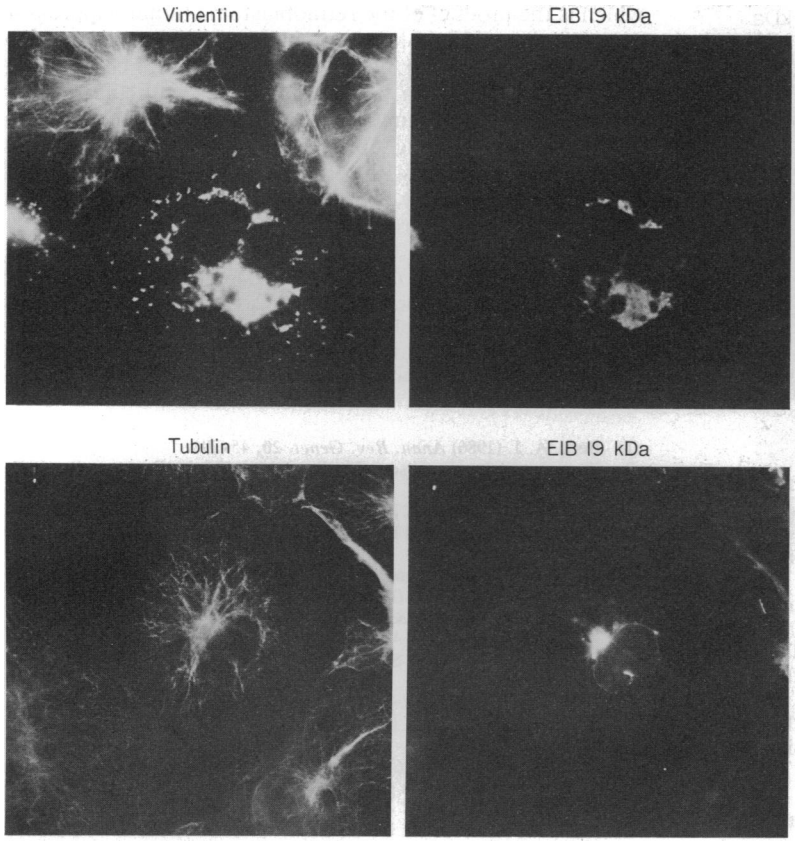

FIG. 3. Effect of 19-kDa expression on the distribution of microtubules. COS cells were transfected with pCMV19K plasmid DNA and fixed with methanol at 48 hr after transfection. Double-label indirect immunofluorescence was carried out with antibodies directed against vimentin and the E1B 19-kDa protein (upper panels) or tubulin and the E1B 19-kDa protein (lower panels). Note that the same set of cells is shown in each horizontal row. (×550.)

from 19-kDa expression, two mechanisms by which IF structure is thought to be regulated (reviewed in refs. 36, 43, 44). Thus, IF and lamina disruption by the 19-kDa protein must occur by a unique mechanism. As the 19-kDa protein was found associated with IFs and the lamina in a detergent- and salt-resistant form, the 19-kDa protein may bind to IFs and the lamina and cause their disassembly.

Implications for IF and Lamina Function. The function of either IFs or the lamina has not been determined (reviewed in refs. 45, 46). IF disruption by the 19-kDa protein does not cause gross alterations in cell morphology in transient expression assays, suggesting that IFs are not a dominant factor governing cell shape. However, expression of the 19-kDa protein in infected cells is required to prevent the occurrence of abnormal cytopathic effect (7, 12, 15, 18). This disruption of IFs may be necessary to modify cell morphology and thereby prevent occurrence of the cyt phenotype and premature death of the host cell. In adenovirus-transformed

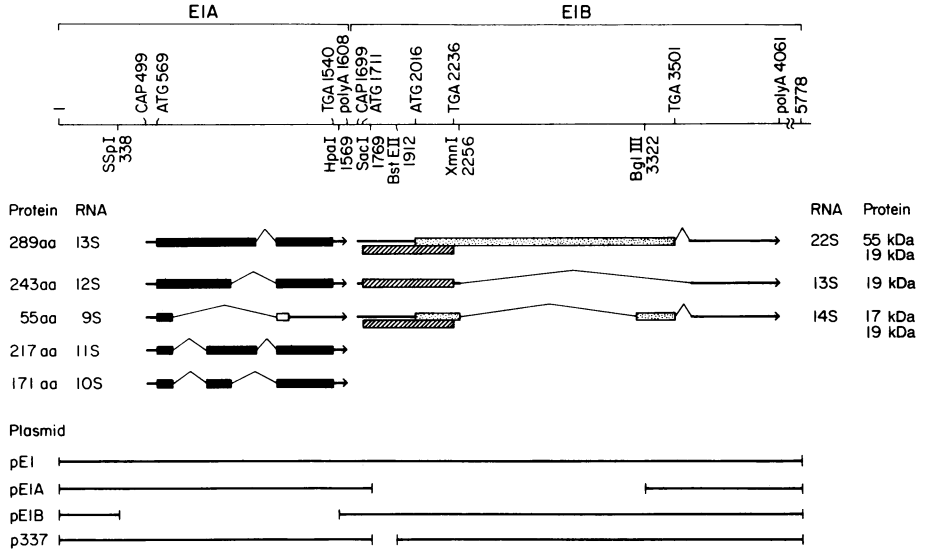

FIG. 4. Schematic representation of E1 transcripts, polypeptides, restriction sites, and plasmids. (*Upper*) Numbers represent nucleotides 1–5778 of the adenovirus genome; relevant restriction sites are marked, and the locations of transcripts and coding regions are indicated. Arrows indicate differentially spliced mRNAs, with carets marking introns removed by alternative splicing. Boxes indicate protein coding regions: solid boxes, protein products related to the E1A 13S gene product; striped boxes, the E1B 19-kDa polypeptide; stippled boxes, protein products related to the E1B 55-kDa polypeptide. A more comprehensive description of E1B 55-kDa related polypeptides can be found in Lewis and Anderson (37). aa, amino acids. (*Lower*) Schematic representation of E1 plasmids: solid lines, sequences of adenovirus DNA carried by the plasmids; open spaces, regions encompassing deletions.

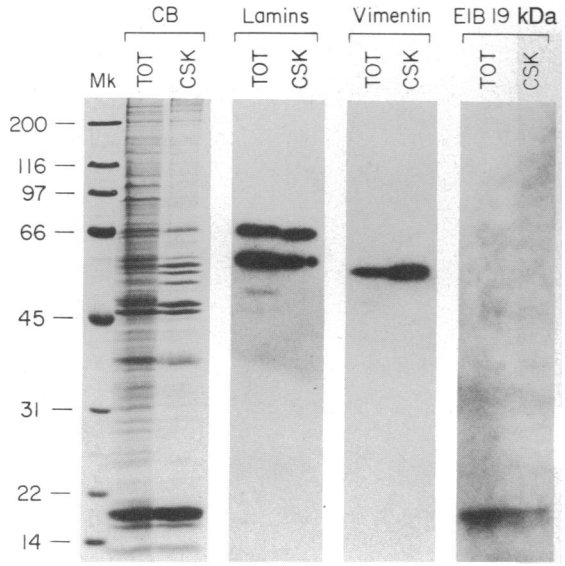

FIG. 5. Identification of the E1B 19-kDa protein as a component of the vimentin cytoskeleton. Total cellular protein (TOT) or cytoskeletal preparations (CSK) from pCMV19K-transfected HeLa cells were subjected to polyacrylamide gel electrophoresis. Fifteen percent gels were either stained with Coomassie blue (CB) or analyzed by Western blotting for lamins A and C, vimentin, or the E1B 19-kDa protein, as indicated. Molecular weight markers (Mk) are indicated in kDa.

cells, detachment of IFs from the plasma membrane may alter the organization of the cell surface and subsequently affect cell–cell attachment and growth regulation. This might then promote anchorage-independent growth and tumorigenicity. Alternatively, given that Ha-*ras* (5), pp60$^{c\text{-}src}$ (14), and the E1B 19-kDa protein (49) can all cooperate with E1A in a transformation assay, an involvement of the 19-kDa protein at some point in a signal transduction pathway might be expected. Perturbation of IFs by the 19-kDa protein could alter the organization of the plasma membrane or cytoplasm in such a way as to affect signal transduction from the cell surface to the nucleus. Possessing cells in which the IFs are disrupted renders this hypothesis testable.

The lamin proteins are thought to be ubiquitous components of eukaryotic cells and are probably essential for maintenance of nuclear chromatin structure (reviewed in refs. 44, 46) and nuclear assembly following mitosis (39, 47). Lamina disruption by the 19-kDa protein could cause alterations in nuclear structure and subsequently alter gene expression or growth regulation and thus promote transformation. During productive infection, expression of the E1B 19-kDa protein prevents the occurrence of DNA degradation and accelerated early gene expression (7). Perturbation of the organization of the lamina caused by the 19-kDa protein may alter nuclear or chromatin structure and thereby prevent DNA degradation and elevated viral gene expression. Studies with adenovirus E1B 19-kDa viral mutants have demonstrated that correct localization of the 19-kDa protein to the nuclear envelope is required to prevent the degradation of host cell DNA (22). Given that the 19-kDa protein modifies lamina organization, physical association of the 19-kDa protein with the lamina may be required to alter the lamina and maintain the integrity of cellular DNA during adenovirus infection.

The findings presented here address not only the function of an adenovirus-transforming protein but also the function of those cellular components with which it interacts. The E1A proteins associate with a number of cellular proteins, one of which is the product of the retinoblastoma tumor suppressor gene (48). The E1B 55-kDa protein has also been observed to associate with another tumor suppressor gene, cellular p53 (13). The E1B 19-kDa protein associates with the nuclear lamina and IFs and functions to interfere with their organization. It is becoming apparent that association of viral transforming proteins with cellular components and subverting their function may represent the mechanism by which DNA tumor viruses promote oncogenic transformation.

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