JOURNAL OF VIROLOGY, Sept. 1996, p. 6323–6335 Vol. 70, No. 9
0022-538X/96/$04.00+0

Adenovirus Early Region 4 34-Kilodalton Protein Directs the Nuclear Localization of the Early Region 1B 55-Kilodalton Protein in Primate Cells

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Received 1 April 1996/Accepted 4 June 1996

The localization of the adenovirus type 5 34-kDa E4 and 55-kDa E1B proteins was determined in the absence of other adenovirus proteins. When expressed by transfection in human, monkey, hamster, rat, and mouse cell lines, the E1B protein was predominantly cytoplasmic and typically was excluded from the nucleus. When expressed by transfection, the E4 protein accumulated in the nucleus. Strikingly, when coexpressed by transfection in human, monkey, or baby hamster kidney cells, the E1B protein colocalized in the nucleus with the E4 protein. A complex of the E4 and E1B proteins was identified by coimmunoprecipitation in transfected HeLa cells. By contrast to the interaction observed in primate and baby hamster kidney cells, the E4 protein failed to direct the E1B protein to the nucleus in rat and mouse cell lines as well as CHO and V79 hamster cell lines. This failure of the E4 protein to direct the nuclear localization of the E1B protein in REF-52 rat cells was overcome by fusion with HeLa cells. Within 4 h of heterokaryon formation and with protein synthesis inhibited, a portion of the E4 protein present in the REF-52 nuclei migrated to the HeLa nuclei. Simultaneously, the previously cytoplasmic E1B protein colocalized with the E4 protein in both human and rat cell nuclei. These results suggest that a primate cell-specific factor mediates the functional interaction of the E1B and E4 proteins of adenovirus.

During a productive infection with human adenovirus type 2 (Ad2) or Ad5, proteins encoded by early region 1B (E1B) and early region 4 (E4) reprogram mRNA transport in the cell. At late times of infection, most of the newly synthesized cellular mRNA fails to appear in the cytoplasm whereas the newly synthesized viral mRNA is efficiently transported to the cytoplasm (reference 44 and references therein). In cells infected with a virus that cannot express the 55-kDa E1B protein (E1B-55 kDa) cellular mRNA is exported from the nucleus at near normal rates whereas viral mRNA fails to be transported (40, 53). A similar phenotype is observed in cells infected with a virus that cannot express the 34-kDa protein of E4 (E4-34 kDa) (28, 60, 61). Because the phenotype of viral mutants unable to express both proteins is no more severe than that of viruses unable to express either E1B-55 kDa or E4-34 kDa protein (9, 14), it has been proposed that a complex composed of the E4-34 kDa protein and the E1B-55 kDa protein is responsible for usurping the control of mRNA transport during an Ad infection. Consistent with this hypothesis, the E1B-55 kDa and E4-34 kDa proteins can be recovered as a complex in the infected cell (14, 63).

Prior work demonstrated that the E1B-55 kDa protein acts on viral transcripts at an intranuclear step after transcription and processing but before translocation across the nuclear membrane (40). Late viral transcripts with unused splice donor sites were more dependent on the E1B-55 kDa protein for efficient transport than fully spliced transcripts (39). The work of Ketner and associates has suggested that both the E4-34 kDa protein and the E4 ORF3 (open reading frame 3) protein enhance the stability of the viral RNA in the nucleus (8, 9, 60, 61). Additional studies have further suggested that the ORF3 protein and the 34-kDa (ORF6) protein of E4 express partially overlapping functions with respect to viral growth (30). In the absence of these proteins, viral DNA replication is delayed and aberrantly spliced forms of some late viral transcripts are found (10, 30). Recently, the E4 ORF3 protein has been shown to initiate a program of nuclear reorganization that favors viral DNA replication and transcription (17).

At late times of a productive infection with Ad2 or Ad5, a portion of the E1B-55 kDa protein is uniformly distributed in the cytoplasm and found in a perinuclear filamentous body adjacent to the nucleus. Most of the E1B-55 kDa protein is localized to the nucleus, where it is (i) diffusely distributed, (ii) concentrated in discrete structures appearing as spicules or flecks by light microscopy, and (iii) associated with the phase-dense centers of viral DNA replication and late transcription (4, 27, 51, 62). However, in cells infected with an E4-34 kDa-mutant virus, the E1B-55 kDa protein occurs primarily as nuclear spicules or flecks (27, 51). At the time of infection when cellular mRNA transport is blocked and late viral mRNA transport is maximal, the E4-34 kDa protein directs a portion of the E1B-55 kDa protein to the sites of late viral RNA synthesis and processing (51). From this observation, we proposed that the E1B-55 kDa–E4-34 kDa complex draws to the centers of viral RNA synthesis a limiting cellular factor necessary for efficient transport of nascent mRNA. At the sites of viral transcription, this factor would promote the export of transcripts derived from the viral chromosome whether of viral or cellular origin. By contrast to the distribution observed in infected cells, the E1B-55 kDa protein is largely excluded from the nucleus of Ad-transformed cells of human (26) or rodent origin (68, 73). In these cells, the E1B-55 kDa protein appears

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to be associated with p53 in a perinuclear, cytoplasmic body that contains centrosomal proteins and cyclin A (12, 13, 73, 74).

Here, we examine the interaction of the E1B-55 kDa protein and E4-34 kDa protein expressed by transfection. We show that the E1B-55 kDa protein is restricted to the cytoplasm in a subcellular distribution similar to that observed in transformed cells. The E4-34 kDa protein can overcome this restriction in human, monkey, and some hamster cells, in which it directs the nuclear localization of the E1B-55 kDa protein. The E4-34 kDa protein, which is itself nuclear in all cell types examined, is unable to change the cytoplasmic localization of the E1B-55 kDa protein in rat or mouse cells. However, the failure of the E4-34 kDa protein to direct the nuclear localization of the E1B-55 kDa protein in rat cells was overcome by fusion with HeLa cells. Shortly after heterokaryon formation, the once cytoplasmic E1B-55 kDa protein colocalized with the E4-34 kDa protein in both human and rat cell nuclei. These results suggest that the interaction of the E1B-55 kDa and E4-34 kDa proteins of Ad5 is mediated by a primate cell-specific factor or activity.

MATERIALS AND METHODS

Cells, plasmids, and viruses. HeLa (CCL2.2), 293 (26), and A549 cells were maintained in Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 10% newborn calf serum. CHO-K1 cells were maintained in Ham's F-12 medium supplemented with 0.1 mM nonessential amino acids and 10% fetal bovine serum (FBS). The Sp2/0-Ag14 myeloma line was maintained in RPMI with 10% FBS. All other cell lines identified in Table 1 (FS4, HEF, HeLa T4, HepG2, COS-1, CV-1, BS-C-1, Vero, BHK, V79-4, NIH 3T3, Sarcoma 180, 3T3-L1, CREF, REF-52, RFS, and tREFG1.0) were maintained in DMEM with 10% FBS.

DNA encoding the E1B-55 kDa and E4-34 kDa proteins was obtained by thermal cycle amplification of the wild-type (*dl*309) adenovirus DNA with Vent polymerase (New England Biolabs, Beverly, Mass.). The primers used for the amplification provided restriction sites that allowed the amplified E1B-55 kDa DNA to be cloned between the *Xba*I and *Sac*I sites of pGEM7z(+) (E1B-55 kDa primer pair, 5′-AGATGGAGCGAAGAAACCCATC-3′ plus 5′-AGAGCCCA TCACATTCTGACG-3′) and the E4 DNA to be cloned between the *Bam*HI and *Sal*I sites of pGEM3z(+) (E4-34 kDa primer pair, 5′-CCGGATCCATGACTA CGTCCGGCG-3′ plus 5′-CCGTCGACCGAAGGGGAAGTCCACGCC-3′) (Promega, Madison, Wis.). Both E1B and E4 genes were placed under the control of the T7 promoter in the pGEM vectors. The fidelity of the constructs was verified by partial sequence analysis. The E1B-55 kDa and E4-34 kDa open reading frames also were cloned into the expression vector pCMV-Neo-Bam (2). From these constructs (pCMV-34k and pCMV-55k), the Ad genes were expressed from the major immediate-early promoter of cytomegalovirus (CMV); the primary transcript also included an intron derived from human β-globin and polyadenylation signals from the simian virus 40 (SV40) late transcript.

The mutant adenoviruses used in this study included the E1B-55 kDa deletion mutant *dl*338 (53) and the E4-34 kDa (ORF6) deletion mutant *dl*355 (14, 28). The wild-type parent for these viruses, *dl*309, lacks a portion of the E3 region that has been shown to be dispensable for growth in tissue culture (32). The propagation and purification of these viruses by equilibrium density centrifugation as well as determination of virus titers by plaque assay on 293 cells have been described elsewhere (31). Cells were passaged 24 h before infection to achieve approximately 70% confluence at the time of infection.

The recombinant vaccinia virus used to express the T7 RNA polymerase, vTF7.3, was created by Fuerst et al. (22). A stock of this virus was prepared by sequential centrifugation to equilibrium in discontinuous and continuous sucrose gradients (1). The virus was stored as 100-μl aliquots in 10 mM Tris-Cl (pH 8.0) at −80°C. The titer of the virus (10^{10} PFU/ml) was determined by plaque assay using BS-C-1 cells (1). Before each use, the virus suspension was thawed and thoroughly vortexed.

Transfection. Cells transfected with the pCMV-34k and pCMV-55k constructs were passaged 24 h prior to transfection to achieve 25% confluence (1×10^4 to 2×10^4 cells per cm^2). Lipofectin (Life Technologies, Gaithersburg, Md.) was used to transfect 1 μg of total plasmid per 10^5 cells as recommended by the manufacturer. Maximal expression was obtained by transfection in the serum-free medium OptiMEM (Life Technologies), using a 3:1 (wt/wt) ratio of Lipofectin to DNA. The transfected cells were typically left in the presence of the DNA-liposome complex for 36 h before analysis.

Expression of the E1B-55 kDa and E4-34 kDa genes from the T7 promoter was achieved as follows. Cells passaged 24 h previously at 1×10^4 to 2×10^4 cells per cm^2 in a 35-mm-diameter dish (5×10^4 to 10^5 total cells in 2 ml) were infected with 5 PFU of vTF7.3 (22) per ml in 1 ml of OptiMEM. One to 2 μg of total plasmid was mixed with a threefold excess Lipofectin in 1 ml of OptiMEM. The DNA-Lipofectin mixture was added to the cells 30 min after the start of the infection, and the cells were returned to 37°C.

Immunoblotting and immunoprecipitation. Ad-infected cells were washed three times with phosphate-buffered saline (PBS), harvested by scraping, and resuspended at a concentration of 10^7 cells per ml of sodium dodecyl sulfate (SDS) sample buffer (100 mM Tris-Cl [pH 6.8], 5 mM EDTA, 1% SDS, 5% 2-mercaptoethanol). Proteins were denatured by heating to 95°C for 5 min, and the DNA was sheared by sonication. Material derived from 4×10^5 cells was separated by electrophoresis on a 10% polyacrylamide gel, using a 36:1 acrylamide/*N*,*N*′-methylenebisacrylamide ratio (Polysciences, Warrington, Pa.). After separation, the proteins were electrophoretically transferred to nitrocellulose paper (66), and the gel was stained to verify quantitative transfer. The nitrocellulose was blocked by overnight incubation at 4°C with 5% nonfat dry milk in Tris-buffered saline before addition of the primary antibody specific for the E1B-55 kDa protein, 2A6 (63).

The primary antibody was added as hybridoma tissue culture supernatant supplemented with 25 mM Tris-Cl (pH 8.0), 10% normal goat serum, and 0.05% Tween 20. After 3 h, the antibody was removed, and the blot was washed five times with Tris-buffered saline and then incubated with bacterial alkaline phosphatase-conjugated goat anti-mouse secondary antibody (Sigma Chemical Co., St. Louis, Mo.) at the recommended dilution for 60 min. Unbound antibody conjugate was removed, and the blot was washed as before. The antibody-antigen complex was visualized by the addition of 0.3 mg of 5-bromo-4-chloro-3-indolyl phosphate per ml and 0.15 mg of nitroblue tetrazolium per ml in 0.1 M Tris-Cl (pH 8.8)–0.1 M NaCl–10 mM $MgCl_2$ as described previously (11). The intensity of the specific signal was measured by reflection densitometry with a pdi model 325oe instrument and image quantitation software, version 2.6 (pdi, Huntington, N.Y.).

For immunoprecipitation assays, cells were labeled in cysteine and methionine-free DMEM for 30 min at 37°C. The medium was then removed and replaced with cysteine- and methionine-free DMEM containing 0.1 mCi of ^{35}S-labeled amino acids (Tran^{35}SLabel; ICN Biomedicals, Costa Mesa, Calif.) per ml from 10 through 14 h postinfection. Immunoprecipitations were performed as described previously (62). The E1B-55 kDa-specific antibody, 2A6, was described by Sarnow et al. (63), and the E4-specific monoclonal antibody, MAb 3, was described by Marton et al. (42). Immune complexes were separated by SDS-polyacrylamide gel electrophoresis (PAGE). The radioactive proteins were visualized by fluorography, using the method of Laskey and Mills (38).

Indirect immunofluorescence. Indirect immunofluorescence and photomicroscopy of whole cells was performed as previously described (51). Simultaneous double-label immunofluorescence was achieved with the rat monoclonal antibody 9C10 (Oncogene Science, Uniondale, N.Y.) (73), specific for the E1B-55 kDa protein, and the mouse monoclonal antibody MAb 3 (42), specific for the E4-34 kDa protein. The secondary antibodies (Jackson ImmunoResearch) were multiple-label-qualified goat antibodies conjugated to dichlorotriazinylamino fluorescein and lissamine rhodamine sulfonyl chloride. Samples were examined and photographed by epifluorescence with a Leitz Dialux 20 EB microscope.

PEG fusion. REF-52 cells (4×10^5) grown on 35-mm-diameter culture dishes were infected with vTF7.3 and transfected with plasmids to express the E1B-55 kDa and E4-34 kDa proteins as described above. Donor HeLa or REF-52 cells in a 10-cm-diameter culture dish were stained with 0.1 μg of bisbenzimide (Hoechst 33258; Sigma) per ml in PBS for 30 min at 37°C, washed, returned to normal growth conditions for 30 min, harvested by trypsinization, and resuspended in DMEM with 10% FBS. At 6 h postinfection, the virus-DNA mixture on the REF-52 cells was removed and replaced with 10^6 donor cells in 2 ml. The donor cells were allowed to attach for 1 h at 37°C. Heterokaryons were formed with polyethylene glycol 4000 (PEG; Polysciences) by the method of Davidson and Gerald (15). In brief, a 50% (vol/vol) mixture of molten PEG and DMEM was prepared and cooled to room temperature. At 7 h postinfection, the medium was thoroughly aspirated from the cocultures of REF-52 and donor cells, and 1 ml of 50% PEG solution was added. After 1 min, the PEG solution was removed and the cells were washed three times with DMEM, replacing the final wash with DMEM containing 10% FBS and 25 μg of cycloheximide (Sigma) per ml. The heterokaryons were returned to 37°C for an additional 4 to 5 h before processing for immunofluorescence as described above.

RESULTS

The E4-34 kDa protein increases the amount of E1B-55 kDa protein seen diffusely distributed through the nucleus during an Ad infection. Previous work suggested that the E4-34 kDa protein directs the E1B-55 kDa protein to the sites of viral DNA synthesis and viral RNA processing in the nucleus during an infection with wild-type Ad (51). Although quantitative differences in protein distribution are difficult to determine by indirect immunofluorescence, the results shown in Fig. 1A suggest that the E4-34 kDa protein also increases the amount

of E1B-55 kDa protein that appears diffusely distributed throughout the nucleus. HeLa cells infected with a wild-type virus (Fig. 1A, panel 1) appear to exhibit greater amounts of diffuse nuclear staining for the E1B-55 kDa protein than cells infected with an E4-mutant virus (Fig. 1A, panel 3). As noted previously (51), the E4-mutant virus-infected cells stained for the E1B-55 kDa protein (Fig. 1A, panel 3) appear to have a greater number of brightly stained flecks or spicules compared with the wild-type virus-infected cells (Fig. 1A, panel 1). These were evident as bright spots or lines of limited length in the nuclei. Furthermore, cells infected with the E4-mutant virus, *dl*355, frequently exhibit a greater amount of cytoplasmic staining for the E1B-55 kDa protein relative to wild-type-infected cells (arrowheads in Fig. 1A, panel 3). The specificity of the 2A6 antibody is evident by the absence of staining seen in cells infected with the E1B-55 kDa-mutant virus, *dl*338, seen in Fig. 1A, panel 5.

The greater intensity of E1B-55 kDa-specific stain in panel 1 (wild-type virus infected) than in panel 3 of Fig. 1 suggests that less E1B-55 kDa protein is synthesized in *dl*355-infected cells. However, the results seen in the immunoblot in Fig. 1B demonstrate that the same amount of E1B-55 kDa protein is synthesized by cells infected with either virus. The protein analyzed in Fig. 1B was obtained from cells infected in parallel with the cultures analyzed by immunofluorescence in Fig. 1A. Total cell protein from identical numbers of cells was separated by SDS-PAGE, transferred to a solid support, and visualized by antibodies specific for the E1B-55 kDa protein. Reflection scanning densitometry of the blot shown indicated that the amount of protein present in *dl*355-infected cells was no less than 95% of that obtained from an equivalent number of *dl*309-infected cells. The diminished immunofluorescent signal for the E1B-55 kDa protein in *dl*355-infected cells compared with *dl*309-infected cells may reflect the inaccessible nature of a portion of the E1B-55 kDa protein in the nuclei of *dl*355-infected cells. For example, a high local concentration of protein such as that found in the filamentous spicules may fail to yield a correspondingly bright signal because of antibody exclusion and saturation of the fluorescent signal recorded on film. Nonetheless, the E4-34 kDa protein increases the amount of diffusely distributed E1B-55 kDa protein seen in the infected cell nucleus. The ability of the E4-34 kDa protein to enhance the nuclear localization of the E1B-55 kDa protein was therefore directly tested by expression of the E4-34 kDa and E1B-55 kDa proteins outside the context of an Ad infection through transfection of cDNA expression vectors.

The E4-34 kDa protein directs the nuclear localization of the E1B-55 kDa protein when the two proteins are coexpressed in HeLa cells. cDNA expression vectors that direct expression of the E4-34 kDa and E1B-55 kDa proteins under control of the CMV immediate-early promoter were prepared and used to transfect HeLa cells. After transfection, the cells were processed for indirect immunofluorescence, and the localization of the E1B-55 kDa and E4-34 kDa proteins was determined by double-label immunofluorescence with a rat monoclonal antibody specific for the E1B-55 kDa protein, 9C10 (73), and a mouse monoclonal antibody specific for the E4-34 kDa protein, MAb 3 (42). The mouse antibody was visualized with a fluorescein-conjugated secondary antibody, and the rat antibody was visualized with a rhodamine-conjugated secondary antibody.

The E1B-55 kDa protein was found primarily in the cytoplasm when transiently expressed in HeLa cells by transfection. The cells shown in Fig. 2A are representative of those transfected with the E1B-55 kDa expression plasmid alone. The E1B-55 kDa protein was predominantly cytoplasmic in most cells and was typically excluded from the nucleus. Many cells also contained brightly stained bodies in a perinuclear position that resembled structures seen in Ad-infected HeLa cells (51) and Ad-transformed cells (4, 68, 73, 74). Typically fewer than 10% of the transfected cells exhibited strong nuclear staining for the E1B-55 kDa protein (data not shown).

The E4-34 kDa protein expressed from a cDNA expression vector was always in the nucleus, as seen in the representative cells shown in Fig. 2E. This result was expected, as the E4-34 kDa protein is fairly small (34 kDa) and is predicted to be basic (pI = 8.4). The E4-34 kDa protein was fairly uniformly distributed throughout the nucleoplasm and appeared to be excluded from the nucleoli, with little concentration about the nuclear membranes or cellular DNA. Similar staining patterns have been described for a number of nuclear matrix-associated proteins (e.g., reference 46) as well as other viral transactivators (e.g., reference 69).

When coexpressed, the E4-34 kDa protein directed the nuclear localization of a large portion of the E1B-55 kDa protein. The results shown in Fig. 2G demonstrate that in the presence of the E4-34 kDa protein, the E1B-55 kDa protein was primarily nuclear and that the nuclear staining patterns for the E4-34 kDa and E1B-55 kDa proteins coincided. In all experiments analyzed, more than 85% of the HeLa cells ($n > 500$) coexpressing the E4-34 kDa and E1B-55 kDa proteins demonstrated strong nuclear fluorescence for the E1B-55 kDa protein, as seen in Fig. 2G. At the level of resolution afforded by immunofluorescence microscopy, the localization of the E4-34 kDa protein was unaffected by coexpression with the E1B-55 kDa protein (compare Fig. 2E with Fig. 2H). Although a large portion of the staining for the E1B-55 kDa protein was seen in the nucleus, some E1B-55 kDa protein appeared to remain in the cytoplasm. In particular, the bright perinuclear or cytoplasmic bodies remained evident in the presence of the E4-34 kDa protein.

Expression of the E1B-55 kDa and E4-34 kDa proteins by cDNA expression vectors suggested that the E4-34 kDa protein enhances the nuclear localization of the E1B-55 kDa protein outside the context of an Ad infection. A simple explanation for the colocalization of the E1B-55 kDa and E4-34 kDa proteins is that the two proteins are physically associated in the nucleus of the transfected cell. Therefore, it should be possible to demonstrate formation of the E1B-55 kDa–E4-34 kDa complex in the transfected cells. However, the relatively low percentage of cells expressing the transfected constructs made detection of the complex by indirect immunoprecipitation difficult. While it may be possible to enrich for and purify the putative complex from the transfected cells by other means, the recombinant vaccinia virus expressing the bacteriophage T7 polymerase (22) provides an alternative means of expressing transfected constructs in a greater percentage of cells.

The recombinant vaccinia virus that expresses the bacteriophage T7 RNA polymerase (22) was used to express the E1B-55 kDa and E4-34 kDa proteins. HeLa cells were infected with the recombinant vaccinia virus to establish cytoplasmic expression of the T7 RNA polymerase. cDNA constructs with the E1B-55 kDa and E4-34 kDa genes under control of the T7 promoter were then transfected with the cationic liposome Lipofectin. The plasmids were transcribed in the cytoplasm, and the resulting RNA was readily translated in the vaccinia virus-infected cell. The cytoplasmic replication of vaccinia virus is evident by the extranuclear DNA stained by 4′,6-diamidino-2-phenylindole (DAPI) in Fig. 3C, F, and I. The localization of the proteins was determined by double-label immunofluorescence.

The intracellular localization of the E1B-55 kDa and E4-34

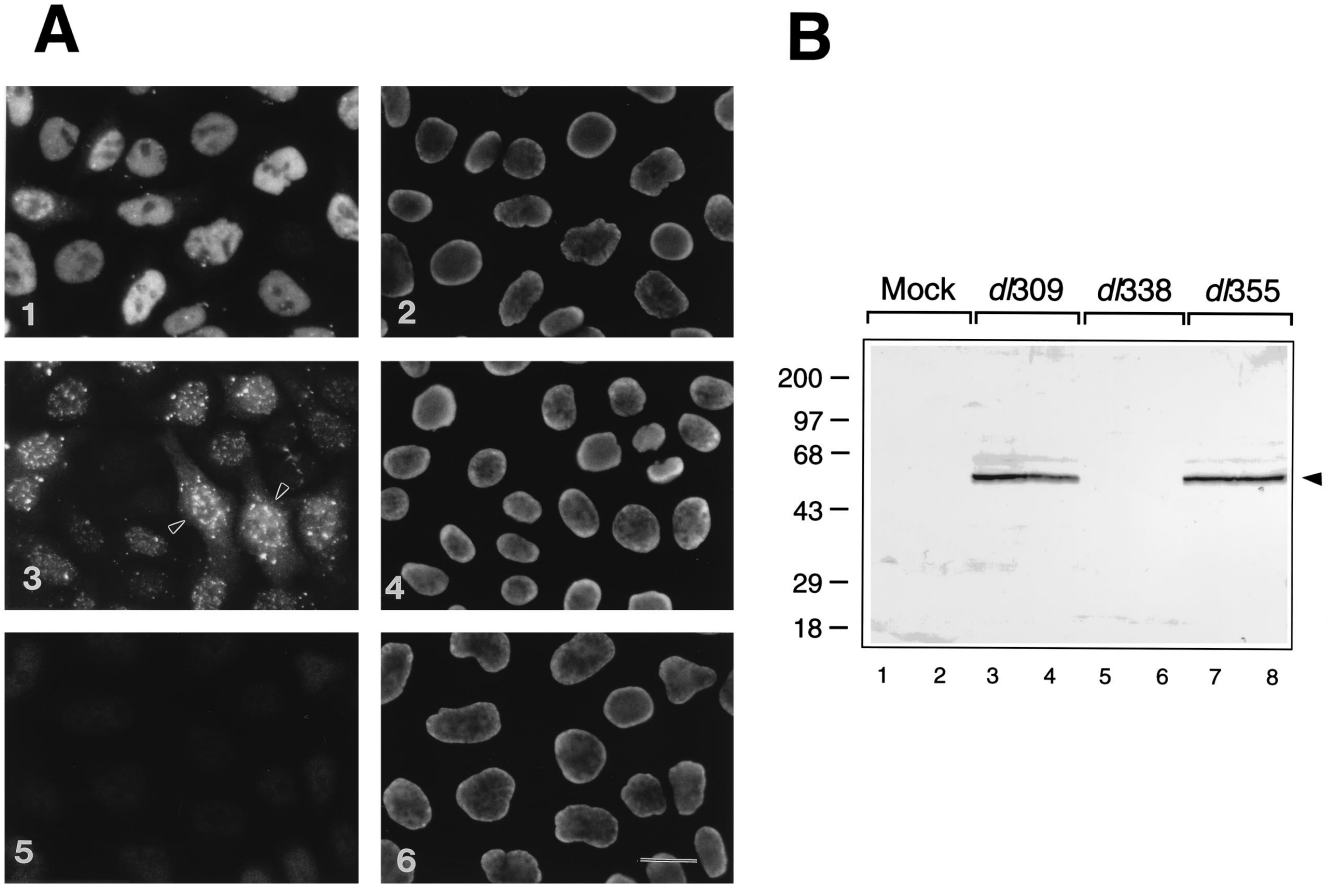


FIG. 1. The diffuse nuclear form of the E1B-55 kDa protein is less apparent in HeLa cells infected with the E4-34 kDa-mutant virus than in cells infected with a wild-type Ad. HeLa cells were infected with either wild-type (*dl*309), E4-34 kDa-mutant (*dl*355), or E1B-55 kDa-mutant (*dl*338) virus at a multiplicity of 20, and the infection was allowed to proceed for 14 h. The distribution of the E1B-55 kDa protein in the infected cells is shown by immunofluorescence in panel A, and the relative amount of the E1B-55 kDa protein present in the infected cells is shown by immunoblotting in panel B. (A) Indirect immunofluorescent staining for the E1B-55 kDa protein (panels 1, 3, and 5) and the same field with DAPI staining to visualize the nucleus (panels 2, 4, and 6). 1 and 2, wild-type-infected cells; 3 and 4, E4-34 kDa-mutant-infected cells; 5 and 6, E1B-55 kDa-mutant-infected cells. Arrowheads in panel 3 identify cells showing strong cytoplasmic staining for the E1B-55 kDa protein. Bar = 20 μm. (B) Total cellular protein was obtained 14 h postinfection from cells infected with the wild-type, E1B mutants, and E4-mutant viruses, separated by SDS-PAGE, and transferred to a solid support. The E1B-55 kDa protein present in the cellular extract was visualized in with monoclonal antibody 2A6. Extracts from two independent infections were analyzed as follows: lanes 1 and 2, mock; lanes 3 and 4, wild type; lanes 5 and 6, E1B-55 kDa mutant; lanes 7 and 8, E4-34 kDa mutant. The migration and masses (in kilodaltons) of molecular weight standards are indicated on the left. Reflected-light scanning densitometry of the blot indicated that the amount of E1B-55 kDa-specific signal in lanes 7 and 8 (E4 mutant) was no less than 95% of the signal in the wild-type-infected cells (lanes 3 and 4).

kDa proteins expressed by the vaccinia virus/T7 system was the same as that observed for the proteins expressed from the intrinsically active CMV promoter expression vectors. In addition, the amount of protein synthesized under direction of the T7 promoter and vaccinia virus T7 system appeared to be greater than that observed in cells transfected with the CMV-driven construct as well as Ad-infected cells (data not shown). As seen in the representative results of Fig. 3A, the E1B-55 kDa protein expressed alone with the vaccinia virus/T7 system was strictly cytoplasmic ($>97\%, n > 500$). Most of the E1B-55 kDa protein appeared to be distributed throughout the cytoplasm and excluded from the nucleus, and some of appeared in brightly stained, perinuclear or cytoplasmic bodies.

The localization of the E4-34 kDa protein expressed by the vaccinia virus/T7 system (Fig. 3E) was identical to that observed in cells transfected with the intrinsically active expression vectors (Fig. 2E). In cells exhibiting very bright nuclear fluorescence, a distinct cytoplasmic staining for the E4-34 kDa protein was also observed. The cells shown in Fig. 3E illustrate the degree of cytoplasmic staining associated with a strong nuclear signal. Nonetheless, in all cases, the predominant localization for the E4-34 kDa protein expressed by this method was the nucleus.

When coexpressed with the E1B-55 kDa protein in the vaccinia virus/T7 system, the E4-34 kDa protein again directed the nuclear localization of the E1B-55 kDa protein (Fig. 3G). As noted for cells expressing the E1B-55 kDa protein from an Ad infection or from the intrinsically active promoters, a portion of the E1B-55 kDa protein occurs in discrete, cytoplasmic bodies (Fig. 3G). Within the nucleus, staining for the E4-34 kDa and E1B-55 kDa protein appeared to coincide (compare Fig. 3G with Fig. 3H). As before, the strong coincidence of E4- and E1B-55 kDa-specific fluorescence suggested that the two proteins may be associated in the cell. Because of the high level of expression achieved with the vaccinia virus/T7 system as well as because of the high percentage of positively expressing cells (up to 90% in many experiments), these cells were analyzed to determine if the colocalization of the two Ad proteins correlated with their the physical association.

Some of the E4-34 kDa protein associates with the E1B-55 kDa protein when the proteins are coexpressed in HeLa cells. Cells were infected with the recombinant vaccinia virus vTF7.3

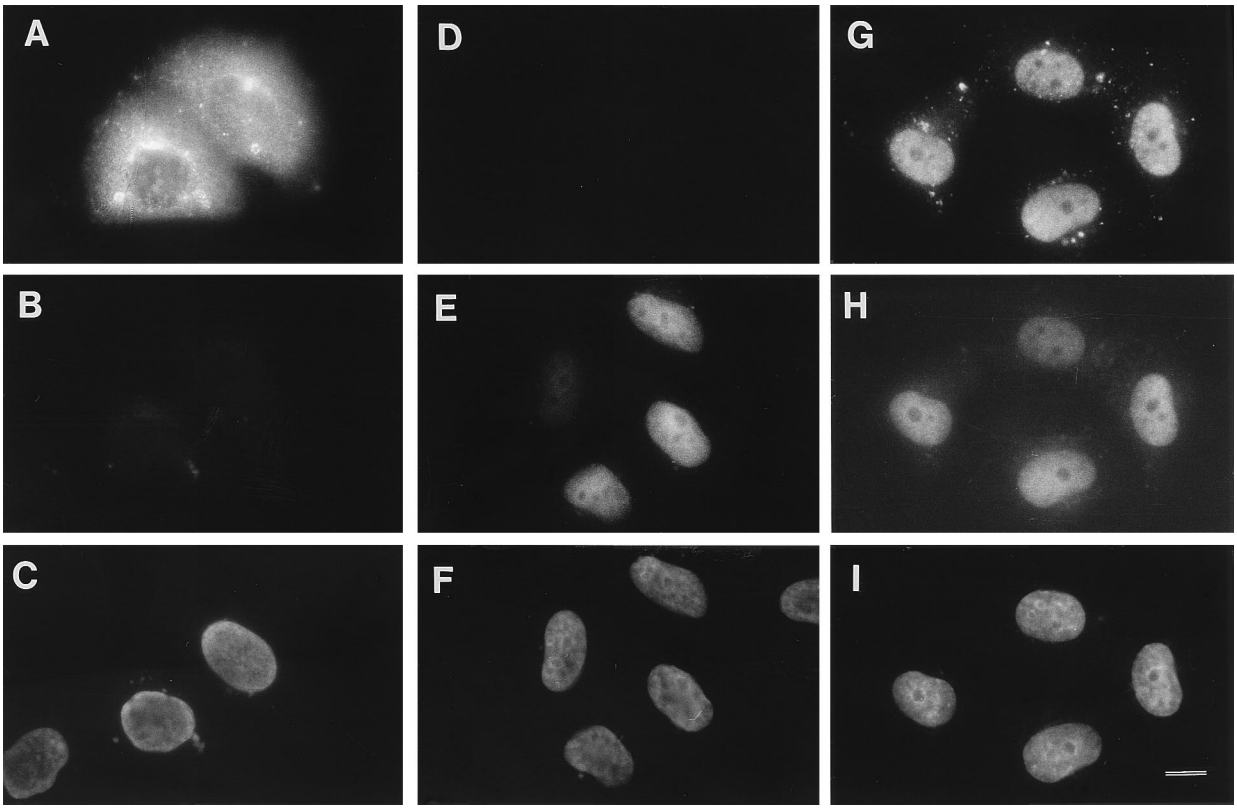


FIG. 2. The E4-34 kDa protein directs the nuclear localization of the E1B-55 kDa protein following transfection of cDNAs in uninfected HeLa cells. HeLa cells were transfected with the appropriate cDNA under the control of the CMV immediate-early promoter to express the E1B-55 kDa protein (A to C), the E4-34 kDa protein (D to F), or both proteins (G to I). The plasmid DNA was introduced with the cationic liposome Lipofectin, and expression of the protein was analyzed by double-label immunofluorescence 32 h following transfection. Representative cells are shown. (A, D, and G) Staining for the E1B-55 kDa protein with rat monoclonal antibody 9C10; (B, E, and H) staining for the E4-34 kDa protein with mouse monoclonal antibody MAb 3; (C, F, and I) DNA visualized with DAPI. Bar = 10 μm.

and transfected with constructs to express the E1B-55 kDa and E4-34 kDa proteins. Protein was radioactively labeled and analyzed by immunoprecipitation. By using the conditions previously shown to preserve the interaction between the E1B-55 kDa and E4-34 kDa proteins (62), a large number of nonspecific proteins were immunoprecipitated by the E1B-55 kDa-specific antibody 2A6 from the vaccinia virus-infected cells (Fig. 4, lanes 4 and 9). As expected, the E1B-55 kDa-specific antibody immunoprecipitated the E1B-55 kDa protein from all lysates containing that protein (Fig. 4, lanes 1, 3, and 5). Similarly, the E4-specific antibody MAb 3 immunoprecipitated the E4-34 kDa protein (Fig. 4, lane 7). The E4-specific antibody was not able to coimmunoprecipitate the E1B protein (data not shown). However, the E1B-55 kDa-specific antibody precipitated the E1B-55 kDa–E4-34 kDa complex from the vaccinia virus-infected cells transfected with both E1B-55 kDa and E4-34 kDa plasmids (Fig. 4, lane 3).

It seems probable that the E4-34 kDa protein seen in Fig. 4, lane 3, was associated with the E1B-55 kDa protein because the E1B-55 kDa-specific antibody did not immunoprecipitate the E4-34 kDa protein from cell lysates that contained only the E4-34 kDa protein (Fig. 4, lane 2). The complex of E1B-55 kDa and E4-34 kDa proteins identified in Fig. 4, lane 3, could have formed in the buffer upon lysis of the cells and may not reflect an association formed in vivo. This possibility seems unlikely because of the results seen in Fig. 4, lane 5, representing an assay in which cells were transfected with the E1B-55 kDa- and E4-34 kDa-expressing constructs and mixed prior to lysis to determine if proteins synthesized in separate cells can form the complex in the extract. When this mixture was immunoprecipitated with antibodies to the E1B-55 kDa protein, no E4-34 kDa protein in excess of that which nonspecifically binds antibody 2A6 was precipitated (compare lanes 2 and 5). Thus, it seems likely that the complex of the E1B-55 kDa and E4-34 kDa proteins formed in the cell.

The relatively small fraction of E4-34 kDa protein indirectly precipitated with the E1B-55 kDa-specific antibody suggests that only a small portion of the E4-34 kDa protein can be recovered as a complex with the E1B-55 kDa protein. On the other hand, the morphological data in Fig. 2 and 3 suggest that most of the E1B-55 kDa protein shows a dramatic change in localization upon coexpression of the E4-34 kDa protein. The reason for this apparent discrepancy is not known but could reflect, for example, the weak nature of the E1B-55 kDa–E4-34 kDa interaction or indicate that once resident in the nucleus, the E1B-55 kDa and E4-34 kDa proteins no longer remain associated.

The E4-34 kDa protein fails to direct the nuclear localization of the E1B-55 kDa protein in REF-52 cells. The ability of the E4-34 kDa protein to direct the nuclear localization of the E1B-55 kDa protein also was examined in the established, nontransformed rat cell line REF-52 (20), using the vaccinia virus/T7 expression system. When the proteins were expressed separately, the localization of the E1B-55 kDa and E4-34 kDa proteins was identical to that observed in HeLa cells. The E1B-55 kDa protein was restricted to the cytoplasm, as seen in a representative cell shown in Fig. 5A. Also as observed in HeLa cells, the E4-34 kDa protein was primarily localized to

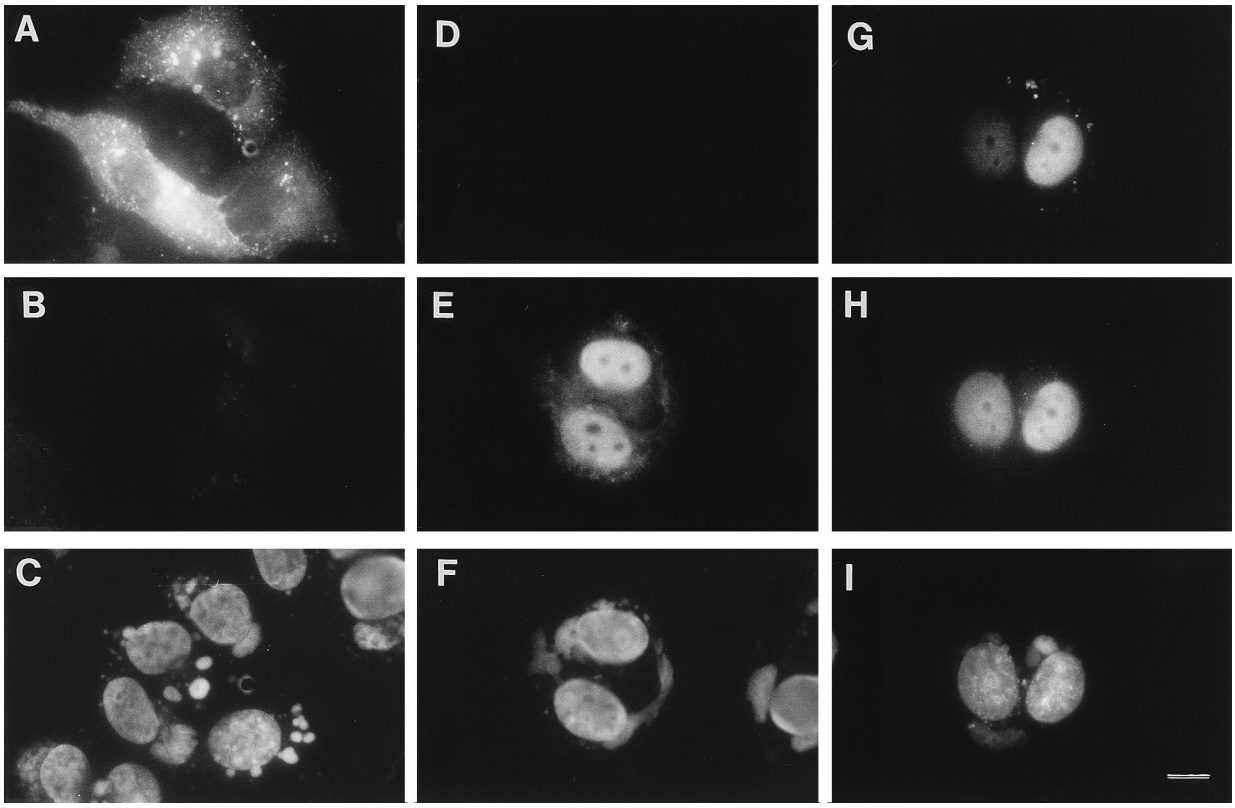


FIG. 3. The E4-34 kDa protein directs the nuclear localization of the E1B-55 kDa protein when expressed with the vaccinia virus/T7 system in human cells. HeLa cells were infected with the recombinant vaccinia virus vTF7.3 to establish expression of the T7 RNA polymerase and then transfected with cDNA under control of the T7 promoter to express the E1B-55 kDa protein (A to C), the E4-34 kDa protein (D to F), or both proteins (G to I). Simultaneous double-label immunofluorescence was performed at 13.5 h posttransfection, and representative cells are shown. (A, D, and G) Staining for the E1B-55 kDa protein with rat monoclonal antibody 9C10; (B, E, and H) staining for the E4-34 kDa protein with mouse monoclonal antibody MAb 3; (C, F, and I) DNA visualized with DAPI. DAPI staining apparent outside the nucleus (C, F, and I) reflects the cytoplasmic replication of the vaccinia virus DNA. Bar = 10 μm.

the REF-52 nucleus, as seen in Fig. 5E. Again, when a very high level of expression of the E4-34 kDa protein was observed, some E4 staining was observed in the cytoplasm (data not shown). However, when the two Ad proteins were coexpressed in the REF-52 cells, the E4-34 kDa protein failed to affect the localization of the E1B-55 kDa protein (Fig. 5G and 5H). Whether coexpressed or expressed separately, the E1B-55 kDa protein was restricted to the cytoplasm whereas the E4-34 kDa protein was found in the nuclei in REF-52 cells. Whether the E1B-55 kDa and E4-34 kDa proteins were transiently expressed with the vaccinia virus/T7 system, transiently expressed from intrinsically active promoters, or constitutively expressed from stably integrated transgenes, the E4-34 kDa protein failed to affect the localization of the E1B-55 kDa protein in these cells (data not shown).

The E4-34 kDa protein may have failed to direct the nuclear localization of the E1B-55 kDa protein for many reasons, including differences in cell type or species. Both of these potential contributions to the interaction of the E1B-55 kDa and E4-34 kDa proteins were examined further, using a variety of established cell lines.

The E4-34 kDa protein fails to direct the nuclear localization of the E1B-55 kDa protein in cell that are not permissive for Ad replication. The vaccinia virus/T7 system was used to express the E1B-55 kDa and E4-34 kDa proteins in primary and established human, monkey, hamster, rat, and mouse cell lines. The results in Table 1 reflect two or more independent experiments using cells from a different passage for each experiment. The localization of the E1B-55 kDa protein was classified as nuclear when the intensity of staining for the E1B-55 kDa protein in the nucleus was greater than that observed in the cytoplasm. The protein was considered to have a cytoplasmic distribution when staining was excluded from the nucleus or was greater in the cytoplasm than in the nucleus. In 293 cells, the endogenous E1B-55 kDa protein was uniformly distributed between the cytoplasm and nucleus; this distribution was categorized as cytoplasmic, in contrast to the strong nuclear localization for the E1B-55 kDa protein seen upon expression of the E4-34 kDa protein.

In all but one cell line (COS-1), more than 75% of successfully transfected cells displayed a clearly cytoplasmic localization for the E1B-55 kDa protein when expressed in the absence of the E4-34 kDa protein. When expressed alone in COS-1 cells, the E1B-55 kDa protein was nuclear in approximately 50% of the cells and cytoplasmic in 50% of the cells. The reason for the nuclear localization of the E1B-55 kDa protein in some COS-1 cells is not known. It seems likely that the SV40 large tumor antigen is responsible for this effect, as COS-1 cells differ from CV-1 cells by constitutively expressing the SV40 large tumor antigen (24).

The ability of the E4-34 kDa protein to direct nuclear localization of the E1B-55 kDa protein varied among cell lines. As in HeLa and REF-52 cells, the localization of the E4-34 kDa protein (nucleus) was not affected by the E1B-55 kDa protein under any circumstance. An intriguing trend is apparent among the cells surveyed; the E4-34 kDa protein directed nuclear

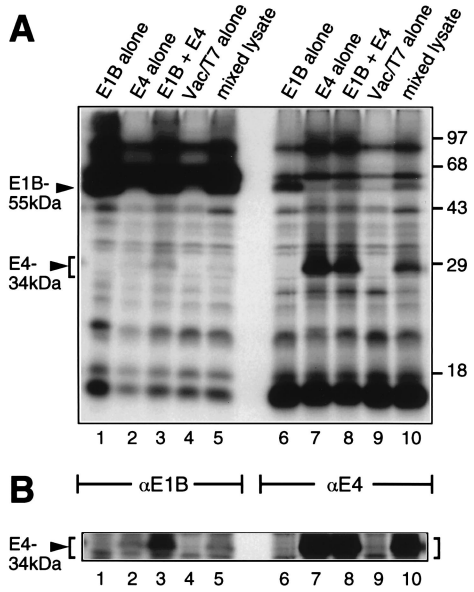


FIG. 4. A portion of the E4-34 kDa protein associates with the E1B-55 kDa protein when the proteins are coexpressed with the vaccinia virus/T7 expression system in HeLa cells. Expression of the T7 RNA polymerase was established by infecting HeLa cells with the recombinant vaccinia virus (Vac) vTF7.3. The cells were transfected 0.5 h postinfection with a vector control (lanes 4 and 9) or the appropriate cDNA plasmid under control of the T7 promoter to express the E1B-55 kDa protein (lanes 1 and 6), the E4-34 kDa protein (lanes 2 and 7), or both proteins (lanes 3 and 8). Material analyzed in lanes 5 and 10 was immunoprecipitated from a mixture of cells that were transfected separately with cDNAs for the E1B-55 kDa and E4-34 kDa proteins before lysis. Proteins were labeled with [^{35}S]methionine between 10 and 14 h postinfection, and cell lysates were immunoprecipitated with antibody specific for the E1B-55 kDa protein (2A6; lanes 1 to 5) and for the E4-34 kDa protein (MAb 3; lanes 6 to 10). Identical amounts of trichloroacetic acid-precipitable radioactivity (2×10^7 cpm) were used in the immunoprecipitation reactions for lanes 1 to 4 and 6 to 9. Approximately twice the amount of radioactivity was used in the immunoprecipitations shown in lanes 5 and 10 (to ensure equivalent amounts of radioactive E1B-55 kDa and E4-34 kDa protein present in each immunoprecipitation). The positions of the E1B-55 kDa and E4-34 kDa proteins are indicated at the left. The migration and masses (in kilodaltons) of molecular weight standards are indicated on the right. An eightfold overexposure of the region of the fluorogram containing the E4-34 kDa protein indicated by brackets in panel A is shown in panel B.

localization in all cell lines that could support the replication of Ad2 or Ad5. These included all human and monkey cell lines tested as well as one of three hamster cell lines. By contrast, the E4-34 kDa protein could not direct the nuclear localization of the E1B-55 kDa protein in any of the rat- or mouse-derived cell lines. Interestingly, the hamster cell line that was permissive for the interaction of the E1B-55 kDa and E4-34 kDa proteins, BHK-21, was reported to support the replication of group C Ad (16, 59). On the basis of these limited findings, it appears that the E4-34 kDa protein can direct the nuclear localization of the E1B-55 kDa protein only in cells that are permissive for the replication of group C Ad.

A HeLa cell-specific factor allows the E4-34 kDa protein to direct the nuclear localization of E1B-55 kDa protein in REF-52 heterokaryons. The ability of the E4-34 kDa protein to direct the nuclear localization of the E1B-55 kDa protein in human and monkey cells but not in mouse and rat cell lines may reflect an inhibitory environment in rodent cells or a permissive environment in primate cells. The experiment shown in Fig. 6 suggests that HeLa cells contain a factor or activity that permits the E4-34 kDa protein to direct the nuclear localization of the E1B-55 kDa protein. In this experiment, the E1B-55 kDa and E4-34 kDa proteins were expressed in REF-52 cells with the vaccinia virus/T7 system. Untransfected, uninfected donor HeLa cells (or REF-52 cells as a control) were fused to the adherent cells with PEG, and the heterokaryons were maintained in the presence of cycloheximide to inhibit new protein synthesis. Donor cell nuclei were distinguished by prelabeling the donor cells with the vital DNA dye bisbenzimide (29). In addition, REF-52 nuclei were characteristically smaller than HeLa nuclei and displayed fewer (one or two, versus three to four in the HeLa nucleus) and smaller nucleoli. Figure 6A is a montage of phase-contrast micrographs of REF-52 cells that were fused to infected and transfected REF-52 cells to illustrate the characteristic morphology of REF-52 nuclei; Fig. 6B is a similar montage of HeLa cells that were plated and exposed to PEG.

Figures 6C through H present the analyses of two groups of cells demonstrating that fusion of REF-52 cells with other REF-52 cells does not cause the nuclear localization of the E1B-55 kDa protein. The donor nuclei in these heterokaryons can be distinguished by their smaller size and dense appearance when examined by phase-contrast microscopy (i.e., were phase dense). The recipient nuclei appear swollen, phase lucid, and devoid of any visible nucleoli. Staining for the E4-34 kDa protein in these cells is shown in the Fig. 6D and G. The E4-34 kDa protein is predominantly nuclear in Fig. 6D and enriched in the nuclei in Fig. 6G. Note that both donor and recipient nuclei contain the E4-34 kDa protein. Therefore, the E4-34 kDa protein that was in the recipient nucleus (Fig. 5E) left the nucleus and entered the donor nuclei in the 4 h following heterokaryon formation. Such behavior, originally described for nuclear proteins of amoebae (25), has since been described for both nuclear and nucleolar proteins (reviewed in references 50 and 64). The distribution of E4-34 kDa protein seen in Fig. 6G was frequently observed after heterokaryon formation. In this heterokaryon, the E4-34 kDa protein is present in the nucleus as well as the cytoplasm. The percentage of heterokaryons showing a strong cytoplasmic localization for the E4-34 kDa protein varied from 10 to 40% in independent experiments.

The E1B-55 kDa protein remained cytoplasmic in the REF-52:REF-52 heterokaryons. By contrast to the predominantly nuclear localization of the E4-34 kDa protein, staining for the E1B-55 kDa protein seen in Fig. 6E and H is excluded from the nuclei. The rim-staining pattern about the donor nuclei in Fig. 6H suggests that the E1B-55 kDa protein is not immobilized in the cytoplasm but can migrate at least to the nuclear membrane. Similar staining patterns have been reported for nuclear proteins that were targeted to the nucleus but failed to translocate through the nuclear membrane (48).

Fusion with HeLa cells enabled the E4-34 kDa protein and E1B-55 kDa protein present in the REF-52 cells to enter both human and rat cell nuclei. Representative examples are shown in Fig. 6I through N, which again present the analysis of two groups of cells. The heterokaryon seen in Fig. 6I appears to be formed by the fusion of two HeLa cells and one REF-52 cell. The three nuclei identified in the phase-contrast micrograph of Fig. 6I are stained for the E4-34 kDa protein in Fig. 6J. Staining for the E1B-55 kDa protein (Fig. 6K) is observed in the nuclei with essentially the same distribution as the E4-34 kDa protein. A similar result is evident in the heterokaryon shown in Fig. 6L. This heterokaryon appears to have resulted from the fusion of at least four HeLa cells with four REF-52 cells. Again, staining for the E4-34 kDa protein (Fig. 6M) appears coincident with staining for the E1B-55 kDa protein (Fig. 6N). The staining for both the E1B-55 kDa and E4-34 kDa proteins in this heterokaryon appears to be brighter in the HeLa nuclei.

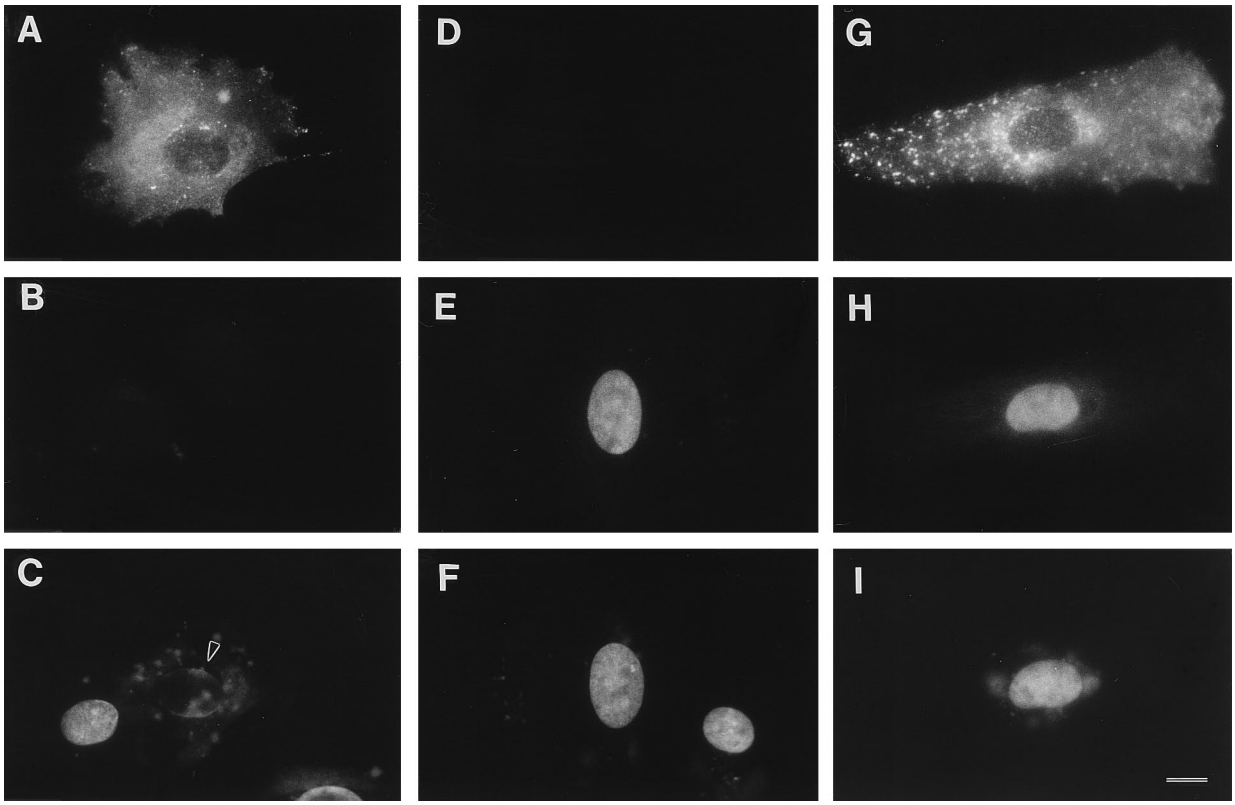


FIG. 5. The E1B-55 kDa protein remains cytoplasmic in the presence of the E4-34 kDa protein expressed with the vaccinia virus/T7 system in rat cells. Expression of the E4-34 kDa and E1B-55 kDa proteins in the rat cell line REF-52 was achieved with the vaccinia virus/T7 system as described in the legend to Fig. 3. Cells were transfected with cDNA to express the E1B-55 kDa protein (A to C), the E4-34 kDa protein (D to F), or both proteins (G to I). Simultaneous double-label immunofluorescence was performed at 11.5 h posttransfection, and representative cells are shown. (A, D, and G) The E1B-55 kDa protein visualized with the rat monoclonal antibody 9C10; (B, E, and H) the E4-34 kDa protein visualized with the mouse monoclonal antibody MAb 3; (C, F, and I) DNA stained with DAPI. The arrowhead in panel C indicates the nucleus of the cell visualized in panel A. The weak DAPI staining observed in panel C was infrequently observed for unknown reasons. DAPI staining apparent outside the nucleus (C, F, and I) reflects the cytoplasmic replication of the vaccinia virus DNA. Bar = 10 μm.

The reason for this is not understood, although this difference was observed in most ($>\approx$75%) of the heterokaryons of REF-52 and HeLa cells.

The translocation of the E1B-55 kDa protein expressed in REF-52 cells to the nucleus upon fusion with HeLa cells suggests that HeLa cells contribute a factor or property that permits the Ad E1B-55 kDa and E4-34 kDa proteins to productively interact.

DISCUSSION

In this study, the intracellular localization of the Ad E1B-55 kDa and E4-34 kDa proteins was determined in the absence of other adenovirus proteins. When expressed by transfection in a variety of cell lines, the E4-34 kDa protein was in the nucleus whereas the E1B-55 kDa protein was found predominantly in the cytoplasm.

The E1B-55 kDa protein lacks a nuclear localization signal typical of the SV40 large T antigen or a bipartite nuclear localization signal common to most nuclear proteins (reviewed in reference 6). Consequently, it would not be expected to concentrate in the nucleus. However, because proteins of this size can enter the nucleus by passive means (47), a fraction of the E1B-55 kDa protein present in the cell might be expected to accumulate in the nucleus. Nevertheless, in nearly all cell types examined, the E1B-55 kDa protein was restricted to the cytoplasm. This behavior is characteristic of an increasing number of proteins that are retained in a specific subcellular compartment and are not free to diffuse (reference 64 and references therein; 55). We have not identified the signals present in the E1B-55 kDa protein that could specify cytoplasmic retention, such as an ankyrin-type motif (7). In addition, unpublished results suggest that this cytoplasmic retention is not due to an interaction with p53; when human p53 is overexpressed by transfection with the E1B-55 kDa protein in either rodent or human cells, the two proteins colocalize in the nucleus. The E1B-55 kDa protein was also reported to be cytoplasmic when expressed in yeast cells (41). These investigators relieved the cytoplasmic restriction on the E1B-55 kDa protein in yeast cells by appending the SV40 large T antigen nuclear localization signal to the protein. Evidence for an active retention in the cytoplasm comes from ongoing work examining the localization of mutant E1B-55 kDa proteins. Most alterations to the E1B-55 kDa protein result in the loss of cytoplasmic restriction, causing the mutant protein to partition between the nucleus and the cytoplasm (30a). Furthermore, in Ad-transformed cells, the E1B-55 kDa protein also appears primarily in the cytoplasm, often in the perinuclear cytoplasmic body associated with p53 (4, 68, 73, 74).

The finding that the E1B-55 kDa protein is restricted to the cytoplasm after transfection contrasts with the distribution observed after infection with Ad. The E1B-55 kDa protein was not excluded from the nucleus in cells infected with a mutant virus unable to direct the synthesis of the E4-34 kDa protein (51). The E1B-55 kDa protein evidently enters the nucleus by

TABLE 1. E4-34 kDa protein-directed nuclear localization of the E1B-55 kDa protein in human and nonhuman cells[a]

Cell line[b]		E1B-55 kDa localization[c]	
Species and designation	Description	Alone[d]	With E4[e]
Human			
A549	Lung carcinoma, CCL185	Cyto	Nuc
FS4	Foreskin fibroblast	Cyto	Nuc
HEF	Embryo fibroblast	Cyto	Nuc
HeLa	Cervical carcinoma, CCL2	Cyto	Nuc
HeLa T4	HeLa with constitutive CD4 expression	Cyto	Nuc
HepG2	Hepatocellular carcinoma, HB 8065	Cyto	Nuc
293	Embryo kidney, Ad5 E1 transformed	Cyto	Nuc
Monkey			
BS-C-1	African green monkey	Cyto	Nuc
COS-1	SV40-transformed CV-1, CRL 1650	Nuc + Cyto[f]	Nuc
CV-1	African green monkey kidney, CCL70	Cyto	Nuc
Vero	African green monkey kidney	Cyto	Nuc
Hamster			
BHK	Baby hamster kidney	Cyto	Nuc
V79-4	Chinese hamster lung, CCL93	Cyto	Cyto
CHO-K1	Chinese hamster ovary, CCL61	Cyto	Cyto
Mouse			
NIH 3T3	NIH Swiss mouse embryo fibroblast	Cyto	Cyto
Sarcoma 180	Swiss Webster sarcoma, TIB 66	Cyto	Cyto
Sp2/0-Ag14	BALB/c myeloma, CRL 1581	Cyto	Cyto
3T3-L1	Swiss embryo preadipocyte, CL 173	Cyto	Cyto
Rat			
CREF	Embryo fibroblast	Cyto	Cyto
REF-52	Embryo fibroblast	Cyto	Cyto
RFS	Fibrosarcoma	Cyto	Cyto
tREFG1.0	Embryo fibroblast, herpes simplex virus type 2 transformed	Cyto	Cyto

[a] Localization of the E4-34 kDa and E1B-55 kDa proteins was determined by double-label immunofluorescence following expression of the proteins with the recombinant vaccinia virus/T7 system. In all cases, the E4-34 kDa protein was restricted to the nucleus as seen in Fig. 2, 3, and 5. The localizations were repeated in at least two independent experiments for all cell lines. All cell lines except 293 cells were successfully transfected with 1 μg of DNA per 2×10^5 cells and could be analyzed between 8 and 12 h postinfection. Because the 293 cell line constitutively expresses the E1B-55 kDa protein, only the E4-34 kDa protein was expressed by transfection.

[b] Where possible, the American Type Culture Collection designation for the cell line is indicated.

[c] An immunofluorescent signal that was excluded from the nucleus or that was uniformly bright between the nucleus and cytoplasm was scored as Cyto. An immunofluorescent signal that was stronger in the nucleus than the cytoplasm, was scored as Nuc. In all cases except the COS-1 cells transfected with the E1B-55 kDa construct alone (see footnote [f]), more than 90% of the positive cells demonstrated the designated pattern.

[d] The E1B-55 kDa protein was expressed alone by transfection of 1 μg of the cDNA with 1 μg of the vector control.

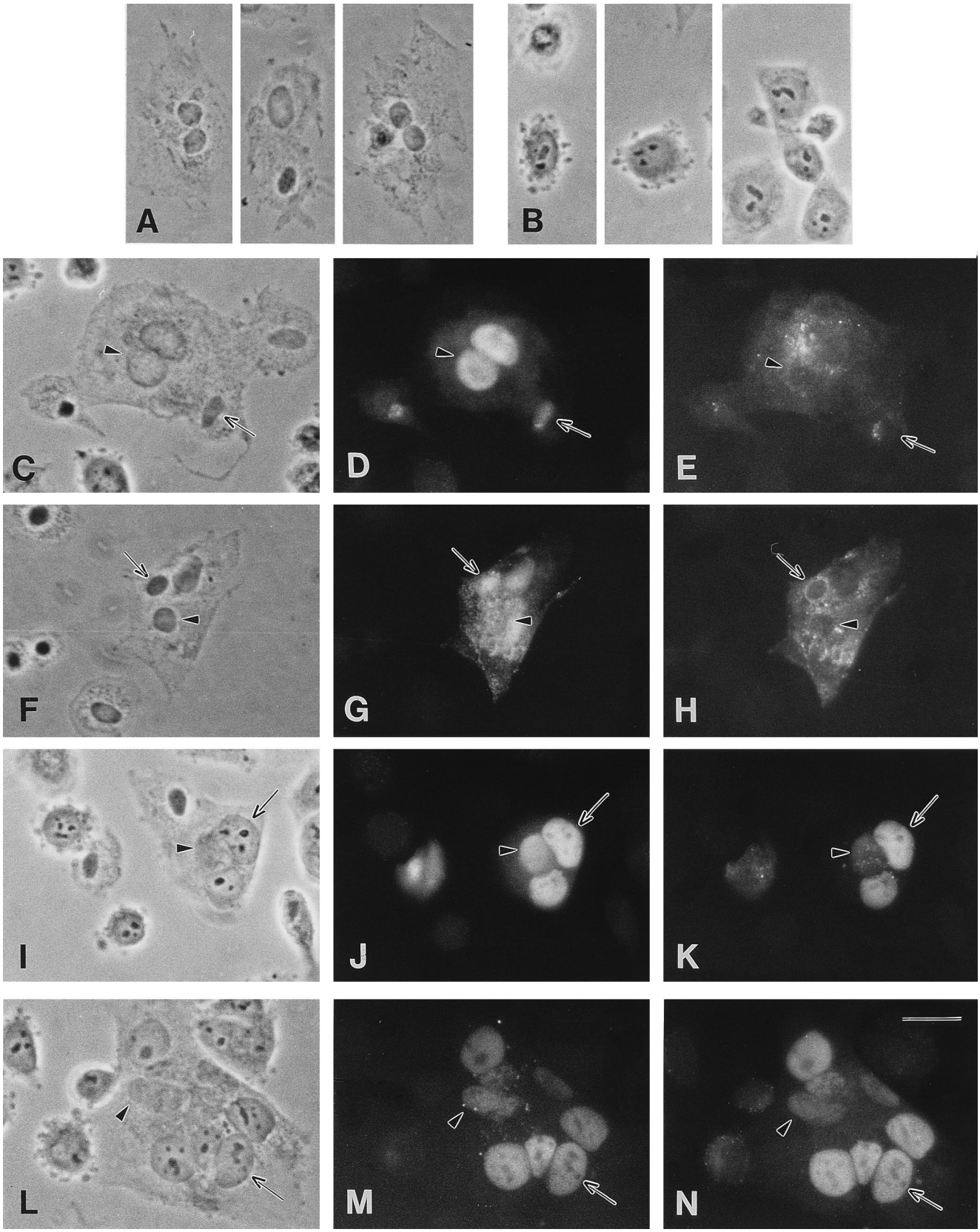
[e] The E1B-55 kDa protein was coexpressed with the E4-34 kDa protein by cotransfection of 1 μg of each of the appropriate plasmid cDNAs.

[f] COS-1 cells transfected with the E1B-55 kDa construct alone contained approximately equal numbers of cells with a distinctly cytoplasmic staining pattern and with a strong nuclear staining pattern.

an E4-independent pathway during an Ad infection. The 13-kDa product of ORF3 in the E4 region can partially complement the function of the 34-kDa product (8, 10, 30), but the ORF3 protein failed to direct nuclear localization of the E1B-55 kDa protein following cotransfection (unpublished results).

The E1B-55 kDa protein was cytoplasmic when expressed by transfection in the African green monkey-derived cell lines BS-C-1, CV-1, and Vero (Table 1). By contrast, the E1B-55 kDa protein was nuclear in approximately half of the transfected COS-1 cells and was excluded from the nucleus in the remaining transfected COS-1 cells (Table 1). In addition, the only Ad-transformed cell line reported to show a predominantly nuclear localization for the E1B-55 kDa protein was the ND4-transformed hamster cell line (63). Both COS-1 and ND cells express the SV40 large T antigen (24, 58). The presence of large T antigen is the only known difference between COS-1 cells, in which the E1B protein can be found in the nucleus, and CV-1 cells, in which it is cytoplasmic. The mechanism by which large T antigen might influence the cellular location of the E1B protein remains unclear. The observation that only half of the transfected COS-1 cells contained E1B-55 kDa protein in the nucleus could reflect a cell cycle dependence on the nuclear localization of the shared cellular target. In this regard, several nuclear proteins, including p53, have been shown to enter the nucleus in a cell cycle-dependent manner (e.g., reference 19 and 54).

The work reported here shows that the adenovirus E4-34 kDa protein can direct the nuclear localization on the E1B-55 kDa protein in primate cells (Fig. 2 and 3; Table 1). When the E1B-55 kDa and E4-34 kDa proteins were coexpressed by transfection, the E1B-55 kDa protein colocalized with the E4-34 kDa protein in the nuclei of seven human cell lines and four monkey cell lines. In addition, the E4-34 kDa protein directed the nuclear localization of the E1B-55 kDa protein in one (BHK) of three hamster cell lines. The mechanism by which the E4-34 kDa protein changes the intracellular localization of the E1B-55 kDa protein remains to be determined. Perhaps the colocalization of the two proteins reflects the physical association of the proteins. In wild-type Ad-infected cells, approximately 50% of the E4-34 kDa protein was recovered in association with the E1B-55 kDa protein (14). A portion of the E4-34 kDa protein also coprecipitated with the E1B-55 kDa protein in cotransfected HeLa cells (Fig. 4). However, in the immunoprecipitation shown in Fig. 4, less than 15% of the E4-34 kDa protein coprecipitated with the E1B-55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kDa protein from the vaccinia virus-infected, transfected cells. This result differs from the morphological data in Fig. 2 and 3, which suggest that the localization of most of the E1B-55 kDa protein was dramatically changed upon coexpression of the E4-34 kDa protein. The reason for the apparent discrepancy between the extensive nuclear colocalization and limited physical association is not known but could, for example, reflect the weak nature of the E1B-55 kDa–E4-34 kDa complex or indicate that once resident in the nucleus, the E1B-55 kDa and E4-34 kDa proteins no longer remain associated. Nonetheless, both the immunoprecipitation assay and morphological assay clearly reveal an interaction between the E4-34 kDa and E1B-55 kDa proteins.

Other Ad proteins as well as proteins expressed by influenza virus type A and herpes simplex virus type 1 viruses direct the nuclear localization of specific viral proteins. The Ad DNA polymerase shows increased nuclear localization in monkey CV-1 cells when coexpressed with the preterminal protein (75). Coexpression of either the PB1 subunit or the NS1 protein of influenza A virus allowed complete nuclear retention of the influenza A virus PA subunit (49). Interactions between the immediate-early regulatory proteins of herpes simplex virus type 1 both enhance and reduce the nuclear localization of each other as well as other herpes simplex virus type 1 proteins. For example, the nuclear localization of the transcriptional activator ICP0 was increased by ICP10 but reduced by ICP27 (76, 77). Similarly, ICP4 promotes the nuclear localization of ICP0 (45) as well as the DNA-binding protein, ICP8 (37).

The E4-34 kDa protein did not direct the nuclear localization of the E1B-55 kDa protein in any mouse and rat cell lines examined. Although these results were obtained by transfection of vaccinia virus-infected cells for most cell lines (Fig. 3 and Table 1), identical results were obtained in assays using REF-52 cells whether the proteins were transiently expressed from an intrinsically active promoter or stably expressed from integrated copies of the Ad genes (50a). Therefore, the failure of the E4-34 kDa protein to direct the nuclear localization of the E1B-55 kDa protein in the rodent cells appears to reflect an intrinsic difference between the primate and rodent cells. The nature of this difference that affects the interaction of the E1B-55 kDa and E4-34 kDa proteins is not apparent. However, the interactions of the E4-34 kDa and E1B-55 kDa proteins in the three hamster cell lines examined suggest a possible link.

Among the three hamster cell lines tested, the E4-34 kDa protein directed the nuclear localization of the E1B-55 kDa protein in BHK cells but not CHO-K1 or V79-4 cells. Group C Ad replicate poorly in all hamster cells; however, the BHK cell line has been reported to be moderately permissive for the replication of Ad2 (16, 59). Compared with viral yields from a permissive human cell line, Ad2 replicates with a 2-log-unit-reduced yield in BHK cells but replicates with a 3- to 5-log-unit reduction in CHO and V79 cells (18, 57, 78). Mouse and rat cells are even less permissive for the replication of group C Ad, typically yielding virus with a 3- to 5-log-unit reduction relative to human cells (3, 18, 65, 78). Finally, although Ad primarily establishes an abortive infection in monkey cells, Ad-infected monkey cells produced virus with only a 2-log-unit reduction compared with human cells (21, 36). These data suggest that the ability of the E4-34 kDa and E1B-55 kDa proteins to interact may be linked to the ability of the cell line to support Ad replication; those cell lines in which the E4-34 kDa protein can direct the nuclear localization of the E1B-55 kDa protein are predicted to support group C Ad replication with no more than a 2-log-unit reduction compared with human cells. Although clearly speculative, the principle defect for the replication of Ad in rodent cells might be due to the lack of an appropriate interaction between the E1B-55 kDa and E4-34 kDa proteins.

When REF-52 cells containing cytoplasmic E1B-55 kDa protein and nuclear E4-34 kDa protein were fused with HeLa cells, the E4-34 kDa and E1B-55 kDa proteins colocalized in both the rat and human cell nuclei (Fig. 6). Similarly, when REF-52 cells expressing only the E4-34 kDa protein were fused to HeLa cells, the E4-34 kDa protein was found in both rat and human cell nuclei in control experiments (data not shown). These results suggest that the nuclear E4-34 kDa protein might shuttle between the nucleus and the cytoplasm. Other nuclear proteins that show such behavior include the Rev protein of human immunodeficiency virus type 1 (33, 71), RNA-binding proteins (35, 56), nucleolar proteins (5, 43), and heat shock proteins (34, 72). Several investigators have suggested that such a shuttling protein could act as a chaperone to escort proteins into the nucleus (34, 50, 72) or escort RNA out of the nucleus (33, 35, 56, 71). Both activities would be consistent with the proposed functions of the E4-34 kDa protein as a mediator of RNA export and as an agent to direct the nuclear localization of the E1B-55 kDa protein.

The import of the E1B-55 kDa protein into the rat cell nuclei within the REF-52:HeLa heterokaryons suggests that the rat cell nucleus is competent for the entry and retention of the E1B-55 kDa protein. This was directly demonstrated with the closely related Ad2 E1B-55 kDa protein translated in vitro (52). In this study, rat liver nuclei were purified and tested for the ability to bind and internalize a variety of proteins. These investigators found that the E1B-55 kDa protein of Ad2 was internalized and retained by the rat liver nuclei as efficiently as other nuclear proteins. An interpretation consistent with these findings is that nuclear import of the E1B-55 kDa protein in the intact cell is prevented by a constituent of the cytoplasm. Our results suggest that the cytoplasmic restriction imposed on the E1B-55 kDa protein can be overcome by the E4-34 kDa protein in conjunction with a primate-specific factor.

Because the E1B-55 kDa protein is restricted to the cytoplasm, it seems likely that a key interaction between the E4-34 kDa and E1B-55 kDa proteins occurs in the cytoplasm. Such an interaction has been described for proteins that gain entry to the nucleus by having a nuclear localization signal unmasked by the action of a second factor. For example, the family of c-Rel/NF-κB transcriptional activators require the release of

FIG. 6. PEG-mediated fusion of HeLa cells with REF-52 cells expressing the E1B-55 kDa and E4-34 kDa proteins leads to the nuclear localization of the E1B-55 kDa protein. Expression of the E1B-55 kDa and E4-34 kDa proteins in REF-52 cells was established with the vaccinia virus/T7 system. Donor cells were obtained after prelabeling of the nuclei of either REF-52 or HeLa cells with bisbenzimide. The donor cells were harvested by trypsinization and allowed to adhere to the substrate containing recipient cells. Donor and recipient cells were then fused with PEG 6.5 h after transfection, and the heterokaryons were maintained in the presence of cycloheximide for an addition 4 to 5 h before processing for double-label immunofluorescence. (A) Montage of phase-contrast micrographs of uninfected REF-52 cells fused with infected REF-52 cells; (B) montage of phase-contrast micrographs of HeLa cells that were plated and exposed to PEG; (C to H) REF-52 cells expressing the E1B-55 kDa and E4-34 kDa proteins fused to REF-52 cells; (I to N) REF-52 cells expressing the E1B-55 kDa and E4-34 kDa proteins fused to HeLa cells. (C, F, I, and I) Phase-contrast image; (D, G, J, and M) staining for the E4-34 kDa protein; (E, H, K, and N) staining for the E1B-55 kDa protein. Donor cell nuclei (identified by morphology and bisbenzimide staining) are marked with arrows; recipient cell nuclei are marked with arrowheads. Bar = 25 μm.

an inhibitory factor that blocks the intrinsically active nuclear localization signal present on the transcription factor (23). Unlike the c-Rel-related proteins, the E1B-55 kDa protein does not have a obvious nuclear localization signal. Thus, it seems improbable that interaction with the E4-34 kDa protein unmasks cryptic nuclear localization signals. The Ad DNA polymerase was reported to enter the nucleus by "piggyback" with the preterminal protein (75). A similar mechanism could account for the E4-directed nuclear localization of the E1B-55 kDa protein. Consistent with this possibility, the E4-34 kDa and E1B-55 kDa proteins occur as a stable complex in transfected as well as infected cells. Furthermore, because the E4-34 kDa protein can shuttle between nucleus and cytoplasm, the E4-34 kDa protein could continue to escort any E1B-55 kDa protein that was not retained in the nucleus or initially transported into the nucleus.

New protein synthesis was not required for the E4-mediated nuclear import of the E1B-55 kDa protein in the rat-human heterokaryons. This finding suggests that an existing activity or diffusible factor present in the human cell is required for the E4-mediated localization of the E1B-55 kDa protein. Since coexpression of the E1B-55 kDa and E4-34 kDa proteins in *Saccharomyces cerevisiae* did not lead to the nuclear localization of the E1B-55 kDa protein (41), we would predict that yeast cells lack such a factor. The nature of this unknown activity or factor warrants further investigation. For example, we previously hypothesized the existence of a diffusible factor required for the efficient export of newly synthesized late viral mRNA (51). This factor would be directed to the sites of late viral RNA synthesis through an interaction with the E1B-55 kDa–E4-34 kDa complex. Therefore, a human cell-specific factor that mediates the interaction of the E4-34 kDa and E1B-55 kDa proteins might be important in the control of mRNA transport. Such a factor has been suggested by studies of the Rev protein of human immunodeficiency virus type 1. The Rev protein was reported to require a factor found in human cells that is absent in mouse cells to mediate export of mRNA containing the Rev-responsive element (67, 70). It would be of considerable interest to determine if viruses as divergent as Ad and human immunodeficiency virus type 1 usurp the control of mRNA transport through related or even identical cellular factors.

ACKNOWLEDGMENTS

This work was supported in part by Public Health Service grant AI 35589 from the National Institute of Allergy and Infectious Disease to D.A.O. and grant CA 41086 from the National Cancer Institute to T.S. Oligonucleotide synthesis was performed in the DNA Synthesis Core Laboratory of the Comprehensive Cancer Center of Wake Forest University, supported in part by NIH grant CA-12197. Tissue culture reagents and services were provided by the Tissue Culture Core Laboratory of the Comprehensive Cancer Center of Wake Forest University, supported in part by NIH grant CA-12197.

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