Localization of the Adenovirus Early Region 1B 55-Kilodalton Protein during Lytic Infection: Association with Nuclear Viral Inclusions Requires the Early Region 4 34-Kilodalton Protein

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The distribution of the adenovirus early region 1B 55-kDa protein (E1B-55kDa) in lytically infected HeLa cells was determined. At the time of infection, when the E1B-55kDa protein facilitates the cytoplasmic accumulation of viral mRNA while simultaneously restricting the accumulation of most cellular mRNA, five distinct intracellular localizations of the protein were observed. Only one of these was disrupted when cells were infected with a mutant virus that fails to produce a second viral protein encoded by early region 4 (E4-34kDa). This protein normally forms a complex with the E1B-55kDa polypeptide, enabling it to influence RNA metabolism. This key localization of the E1B protein was within and about the periphery of nuclear viral inclusion bodies believed to be the site of viral DNA replication and transcription. In the absence of the E4-34kDa protein, the coincidence of E1B-55kDa-specific immunofluorescence and phase-dense viral inclusions was reduced compared with that in a wild-type infection. Similarly, by immunoelectron microscopy, the relative number of E1B-55kDa-specific immunogold particles associated with the clear fibrillar inclusion bodies was reduced. However, the E4-34kDa protein was not required for the close association of the early region 2A DNA binding protein with the viral inclusions. We propose that the viral 55-kDa-34-kDa protein complex interacts with a cellular factor required for cytoplasmic accumulation of mRNAs and directs it to the periphery of the transcriptionally active viral inclusion bodies. This model provides an explanation for the ability of these viral proteins to simultaneously enhance accumulation of viral mRNAs and inhibit accumulation of cellular mRNAs.

The adenovirus 55,000-Da protein encoded by early region 1B (E1B-55kDa) modulates the cytoplasmic accumulation of mRNA at late times after infection. In the course of a normal infection, only late viral mRNAs accumulate in the cytoplasm, even though transcription of the host cell genes continues largely unabated (1, 4, 28, 36) and the stability of cytoplasmic cellular mRNAs is unchanged (1, 28, 36, 39). In the absence of a functional E1B-55kDa protein, late viral mRNAs accumulate to reduced levels in the cytoplasm, while the accumulation of cellular mRNAs is not disrupted (2, 3, 39). A similar defect has been noted for viruses bearing mutations in the gene encoding the 34-kDa protein of early region 4 (22, 55). The functional moiety involved in the mRNA accumulation process is a physical complex of the E1B-55kDa and E4-34kDa proteins. Physical evidence for this complex was provided by Sarnow et al. (46), who showed that the E4-34kDa protein could be immunoprecipitated from infected cells or from appropriately reconstructed lysates *in vitro* by using an antibody for the E1B protein. Genetic evidence for this functional association can be inferred both from the similar phenotypes of E1B-55kDa and E4-34kDa mutants and from the properties of double mutants lacking the ability to synthesize both proteins. The double mutant defect was no more severe than that observed for viruses bearing either single mutation (9, 16).

The 55-kDa-34-kDa protein complex appears to exert its effect after an RNA molecule is spliced and polyadenylated. Viral late RNAs in the nucleus of cells infected with a virus unable to express the E1B product, *dI338*, are spliced and

polyadenylated (39). Further, a cDNA derivative of the viral fiber gene, which is expressed late after infection, produced mRNAs that did not undergo splicing and yet failed to accumulate to normal levels in the cytoplasm of cells infected with an E1B-55kDa mutant virus. Leppard and Shenk (30) were able to define more precisely the site of action of the E1B-55kDa protein on viral RNA metabolism. The movement of newly synthesized RNA sequences was monitored through a series of biochemically defined nuclear subfractions. In the absence of the E1B protein, viral late transcripts failed to accumulate in a DNase-labile fraction. Kinetically, RNAs reached this operationally defined fraction after their synthesis in association with the nuclear matrix and before their appearance in the nuclear membrane or cytoplasmic compartments. The work of Ketner et al. (9, 45, 55) suggested that the absence of either or both of the E1B-55kDa and E4-34kDa proteins resulted in a reduction of late viral mRNA in the nucleus as well as a defect in the transport of this nuclear mRNA. These observations are all consistent with the idea that the 55-kDa-34-kDa complex facilitates viral RNA metabolism at an intranuclear step, possibly by maintaining the stability of the pool of processed RNA molecules prior to translocation and perhaps even facilitating the translocational step itself.

Immunofluorescence studies showed the E1B-55kDa protein to be localized primarily within the nucleus and excluded from the nucleoli (5, 48). Similarly, the E4-34kDa protein was found to be in the nucleus late (24 h) in infection (16). Such a localization is consistent with the hypothesis that these proteins act on late viral RNA shortly after transcription but before translocation across the nuclear membrane (30, 45). The molecular mechanism by which the

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55-kDa–34-kDa complex simultaneously facilitates accumulation of late viral mRNAs while restricting accumulation of host mRNAs is unknown. In addition, not all cellular transcripts are blocked in the nucleus; the inducible major heat shock gene continues to be transcribed and transported in response to induction throughout adenovirus infection, as does β -tubulin mRNA (36). Furthermore, transcripts from the early viral promoters are not affected by the lack of either E1B or E4 product (22, 30, 39). Perhaps most significant is the observation that the discrimination is not based strictly on the identity of the RNA. When the cellular alpha-globin gene (24) or preproinsulin gene (19) was inserted into the viral chromosome, it was transcribed and its mRNA was moved to the cytoplasm late in the viral infection. In the case of the preproinsulin gene, this occurred at a time when the endogenous mRNA did not accumulate in the cytoplasm (19).

This study was designed to gain further insight into the function of the E1B-55kDa protein by detailed description of its intracellular localization. Indirect immunofluorescent and immunoelectron microscopy of cells infected with wild-type virus suggested several distinct distributions of the protein in the cytoplasm and within the nucleus. Perhaps the most striking localization of the protein was within and about the nuclear inclusions believed to represent viral replication and transcription centers. The significance of these distributions was examined by determining the localization of the E1B protein in cells infected with a virus lacking the E4-34kDa protein. In the absence of the E4 protein, the E1B-55kDa protein failed to associate efficiently with the centers of viral replication and transcription. The association of the E1B-55kDa protein with the replication centers suggests a model that could account for the simultaneous inhibition of host cell mRNA and facilitation of late viral mRNA accumulation.

MATERIALS AND METHODS

Cell and virus culture. HeLa cells were maintained as monolayers in Dulbecco modified Eagle's minimal essential medium supplemented with 10% calf serum (GIBCO Laboratories, Grand Island, N.Y.), penicillin, and streptomycin. 293 cells, which constitutively express the E1A and E1B gene products (20), were maintained in the same medium. The phenotypically wild-type adenovirus type 5 used in these studies, *dl309* (27), served as the parent to the variant containing a deletion in the carboxyl terminus of the E1B-55kDa protein, *dl338* (39). *dl355* contains a deletion in the gene encoding the E4-34kDa protein. Its construction was described by Halbert et al. (22), and it was further characterized by Cutt et al. (16).

Cells were infected by seeding HeLa cells at a 1:3 dilution 24 h prior to infection. Cultures were infected with 20 PFU per cell in 1/10 to 1/5 the normal volume of phosphate-buffered saline (PBS; 137 mM NaCl, 3 mM KCl, 16 mM Na_2HPO_4 , 2 mM KH_2PO_4) supplemented with 2% calf serum, 1 mM CaCl_2 , and 1.5 mM MgCl_2 with intermittent agitation for 1 h at 37°C. The infection medium was removed and replaced with normal growth medium. Infected cultures were then analyzed microscopically at various times after infection. To produce viral stocks, infections were allowed to proceed until the cells were killed; at that point, the cells were disrupted by multiple freeze-thaw cycles, and virions were purified by sequential centrifugation in discontinuous and equilibrium CsCl_2 gradients (26). Viral titers were determined by plaque assay on 293 cells.

Cell fractionation. For immunogold labeling and some immunofluorescence studies, cells were extracted by methods previously described (17, 18). In brief, after an initial room temperature wash in PBS containing 1.5 mM MgCl_2 (PBS + Mg), the cells were washed twice with PBS + Mg at 4°C and then extracted for 3 min on ice with CSK buffer, which consisted of 100 mM KCl, 300 mM sucrose, 3 mM MgCl_2 , 1 mM EGTA [ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid], 10 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid); pH 6.8], 1.5 mM phenylmethylsulfonyl fluoride, and 0.5% Triton X-100. In some studies, the cytoskeletal preparations were further extracted by 0.25 M ammonium sulfate (replacing the KCl in the CSK buffer) for 5 min on ice.

Indirect immunofluorescence. A 40% solution of formaldehyde in water was prepared from electron microscope-grade paraformaldehyde (Polysciences Inc., Warrington, Pa.) immediately before use. At the indicated times, the cells were washed twice with PBS + Mg at room temperature and either extracted or fixed as intact cells. Intact cells were immediately fixed in 4% formaldehyde in PBS + Mg at room temperature for 20 min, washed three times for 5 min each time with PBS + Mg, and then permeabilized with 1% Triton X-100 in PBS + Mg for 5 min. Identical results were obtained by fixation in ice-cold 80% acetone or 1:1 acetone-methanol; however, the degree of morphological preservation was highest in cells fixed with freshly prepared formaldehyde. Extracted cells were rinsed briefly with CSK buffer and fixed for 20 min in 4% formaldehyde in CSK buffer at 4°C prior to immunolabeling.

After fixation, all steps were performed at room temperature (22 to 24°C). The cells were washed two times for 5 min each with Tris-buffered saline (20 mM Tris chloride [pH 7.6], 137 mM NaCl, 3 mM KCl, 1.5 mM MgCl_2) containing 5 mg of bovine serum albumin per ml, 1 mg of glycine per ml, and 0.05% sodium azide (TBS-BG) with 0.05% Tween 20. The inclusion of bovine serum albumin and Tween 20 in TBS-BG was found to significantly reduce nonspecific background staining arising from amine-reactive elements, particularly when low concentrations of glutaraldehyde were used for fixation. Samples were incubated in the primary antibody (undiluted hybridoma cell medium supplemented with 0.05% sodium azide, 25 mM Tris chloride [pH 8.0], and 0.05% Tween 20) for 1 to 3 h at room temperature with constant agitation. The E1B-55kDa-specific monoclonal antibody (clone 2A6) was described by Sarnow and associates (48); the E2A-72kDa-specific monoclonal antibody (clone B6-8) was described by Reich et al. (43). Both antibodies were kindly provided by A. J. Levine (Princeton University). After binding took place, the samples were washed five times in TBS-BG and then incubated with 8 to 15 μg of fluorescein-conjugated goat antibodies specific for mouse immunoglobulin G (Jackson ImmunoResearch, West Grove, Pa.) per ml in TBS-BG with 10% normal goat serum for 60 min. After being washed five times in TBS-BG, the samples were mounted in PBS-glycerol with 10% polyvinyl alcohol and 1 mg of *p*-phenylenediamine per ml to inhibit bleaching of the fluorochrome (40). Samples were examined and photographed with a Zeiss Photomicroscope III by using epifluorescence illumination. Exposures were standardized for each experiment and were typically between 15 and 60 s with Kodak T-Max 400 film (Eastman Kodak, Rochester, N.Y.) developed to an effective exposure index of 1,600 by using T-Max liquid developer.

Indirect immunogold labeling prior to embedment. Cells

were grown on flexible Mylar coverslips that were treated first with Du Pont Teflon dry lubricant (Polysciences) and then with 0.1% poly-L-lysine (Sigma Chemical Co., St. Louis, Mo.) and sterilized. The cells were infected, extracted with CSK buffer, fixed, and incubated with the primary antibody as described for indirect immunofluorescence. Nonspecific binding of the 10-nm colloidal gold-goat anti-mouse immunoglobulin G complex (Janssen, Piscataway, N.J.) was blocked by treating the samples with 5% (wt/vol) nonfat dry milk and 10% normal goat serum in TBS-BG for 1 to 3 h at room temperature. The gold-conjugated antibodies were diluted 1:1 or 1:3 in TBS-BG with 10% normal goat serum and 0.05% Tween 20 and then applied to the coverslips for 3 to 6 h at room temperature.

Electron microscopy. After being labeled, the samples were washed extensively and postfixed in 2.0% glutaraldehyde and then in 0.1% osmium tetroxide in PBS + Mg, and then they were dehydrated in a graded series of alcohol. Resin-embedded specimens were infiltrated with epon-anhydride-propylene oxide and then with three changes of epon-anhydride and embedded by inverting filled Beem capsules on the coverslips and curing at 60°C. Diethylene glycol distearate (DGD) was used as a removable embedding medium as described previously (12). Alcohol-dehydrated samples were transferred to *n*-butanol and then infiltrated with DGD, first through a series of DGD-*n*-butanol mixtures and then through pure DGD (60°C). The samples were embedded in DGD by quickly inverting filled Beem capsules on the coverslips before allowing the DGD to harden at room temperature. Gold resin sections (50 to 100 nm thick) were cut with a diamond knife; gold-purple to purple-green DGD sections (150 to 250 nm thick) were cut with glass knives on an LKB Ultratome II. Sections were collected on poly-L-lysine- and carbon-coated, Formvar-covered nickel grids. Resin-containing sections were stained with aqueous uranyl acetate and lead citrate before being examined at 80 kV with a JEOL 100C or Philips 400 electron microscope. DGD was removed by immersing the grids in *n*-butanol overnight with three changes of *n*-butanol. The embedment-free samples were then transferred to 100% ethanol through butanol-ethanol mixtures, dried through the CO₂ critical point, coated with evaporated carbon, and viewed without further staining or contrast enhancement at 100 kV in an electron microscope.

Morphometric analysis. Three separate immunogold labeling experiments were performed. In each experiment, two to three independently labeled preparations (coverslips) were generated for each antibody-virus combination. Sections from several (three to six) blocks were collected from each independent preparation. For infected cultures, the sections were surveyed for the presence of infected cells; clearly infected cells were systematically photographed at $\times 16,000$ magnification and then examined after printing at $\times 44,000$ magnification. The exact position of each gold particle was recorded by means of a Summagraphics digitizing tablet and the MacMeasure program (kindly provided by W. Rasban, National Institutes of Health). A grid (25 by 32) was superimposed on the micrograph, and each grid square (representing $0.025 \mu\text{m}^2$) was scored for the presence of the feature of interest (clear fibrillar inclusion [CFI] body), other distinct morphological features (nucleolus, condensed chromatin, and nuclear lamina) and location (nucleus or cytoplasm). This method, although overestimating the area represented by the selected morphological feature, included a margin approximately 120 nm wide about the selected feature. Thus, particles closely associated with, through not contained

within, the feature were included. These data, stored and analyzed with a Macintosh II microcomputer, were used to derive the absolute density of gold particles in regions of the cells containing the distinct morphological features. To compare results obtained from independent labeling preparations, the results were scaled such that the density of gold particles in the nucleoplasm exclusive of the nucleoli and highly condensed cellular chromatin was normalized to 1 per unit area. Regions of high electron density were not included in the analysis because of the difficulty in unambiguously identifying the gold particles. In each preparation, between 6 and 20 cells were analyzed and the positions of approximately 1,200 (minimum of 259 and maximum of 8,765) gold particles were recorded.

RESULTS

Indirect immunofluorescence staining of the E1B-55kDa protein in cells infected with wild-type adenovirus type 5. Five distinct patterns of localization were seen for the E1B-55kDa protein 15 h after infection of HeLa cells with phenotypically wild-type *d1309* (Fig. 1a and b). First, all cells that stained positively for the viral protein exhibited a diffuse nuclear stain that was excluded from the nucleoli. The diffuse nuclear staining pattern was not homogeneous and under close examination appeared as a fine granular distribution. This is in contrast with the homogeneous diffuse nuclear distribution of the E2A-72kDa DNA binding protein (compare Fig. 1b with Fig. 2b and 3b). The significance of such a difference is not clear but suggests that the E1B protein is not freely distributed in the nucleoplasm.

A second characteristic staining pattern observed included brightly staining, filamentous structures of discrete lengths and limited curvature. These filamentous structures or spicules were observed in the nuclei of approximately one-fourth of the wild-type virus-infected cells. By focusing through the nucleus, it was possible to determine that these spicules were distributed throughout the nuclear interior at apparently random orientations. Because of their random orientation with respect to the focal plane, many of these bodies appear as bright, diffuse spots. In cells displaying a very intense diffuse nuclear stain, the spicules were often obscured. Unlike the reticular network of E1A proteins observed in some infected HeLa cells (56), the nuclear spicules of E1B-55kDa protein did not interconnect and had an apparent length of no more than 3 μm . The fine arrows in Fig. 1c indicate examples of this structure.

A third localization of the E1B-55kDa protein occurred as round bodies or clusters about less intensely stained bodies in the nucleus. These structures are indicated by unlabeled arrowheads in Fig. 1 and are most evident in the cell seen in Fig. 1b. At its earliest appearance (12 h), this distribution was primarily represented as discrete round bodies, often near the periphery of the nucleus. At later times, a larger number of brightly stained globular bodies, as well as larger, vesicular structures, appeared. When stained for the E1B-55kDa protein, these bodies were not uniform in appearance but generally had irregular borders or appeared to be composed of clusters of brightly stained smaller structures about a more uniformly stained vesicular body. These globular and vesicular structures, identified by immunofluorescence, often had a visible phase-dense counterpart (Fig. 2). Similar vesicular structures have been identified in adenovirus-infected cells (43, 52, 54) as well as cells infected by other DNA viruses (42). These structures have been shown to be the site of viral DNA synthesis and accumulation (10, 33, 37,

FIG. 1. Indirect immunofluorescent staining of adenovirus- and mock-infected HeLa cells with an E1B-55kDa-specific monoclonal antibody. HeLa cells grown on glass coverslips were infected at a multiplicity of 20 PFU of wild-type virus (a and b) or the E4-34kDa-defective virus, *dl355* (c and d) per cell, or mock infected (e and f). At 15 h postinfection, the cells were fixed, permeabilized, and analyzed by indirect immunofluorescent staining using the E1B-55kDa-specific monoclonal antibody 2A6. Representative fields are shown for all cases, except panel d, which represents approximately 1 of 500 positively staining cells. The micrographs in panels a through e were photographed and printed under identical conditions. The micrograph in panel f represents an overexposure (approximately eightfold) of the field in panel e. Representative structures indicated by arrows and arrowheads are discussed in the text. Bar, 10 μ m.

54), and evidence suggesting that these are also the sites of late viral RNA synthesis has been presented (32, 37, 57).

A fourth staining pattern was observed in the cytoplasm, where the E1B protein exhibited a fine reticular distribution. Staining was not evident about the periphery of the cell, nor

did the staining coincide with any of the major filament systems of the cytoskeleton (data not shown). As shown in Fig. 1a, typical of intermediate and late times in infection, the intensity of cytoplasmic staining largely reflected the overall staining intensity of the particular cell. At early times

in infection (prior to 9 h), staining for the E1B-55kDa protein was fainter and appeared to be localized primarily in the cytoplasm (data not shown).

Finally, in many cells, a small number (typically one to five) of discrete cytoplasmic bodies were strongly stained with E1B-55kDa-specific antibodies (Fig. 1a to d). When more than one cytoplasmic body was evident, they were often closely associated, in all cases appearing in a perinuclear position approximately 2 to 5 μ m from the nuclear border. These structures are indicated by the double arrowheads in Fig. 1. Similar discrete cytoplasmic bodies containing the E1B protein have been observed in virally transformed cells and have been shown to contain the cellular tumor antigen p53 (6, 59). Although the cytoplasmic bodies observed in the transformed cells were similar in appearance by immunofluorescent- and immunoelectron-microscopic analysis (see Fig. 10), the significance of these structures during lytic infection is not understood.

Distribution of the E1B-55kDa protein in the absence of a functional E4-34kDa protein. Physical (46) and genetic (9, 39) evidence suggests that the E1B protein carries out its role in RNA metabolism as a complex with the E4-34kDa protein. In the absence of detailed information about the localization of the E4 protein, we sought to determine if the complex distribution of the E1B protein described above was dependent on the presence of a functional E4-34kDa protein. The virus used for this study, *dl355*, was constructed to lack a portion of the gene encoding the E4-34kDa protein (22). Identical results (not shown) were obtained using *dl1003*, a virus in which the entire E4-34kDa gene was detected (8).

Several differences in the localization of the E1B protein were evident at 15 h after infection with *dl355* when compared with the wild-type distribution (Fig. 1c and d). The extent and intensity of diffuse nuclear staining observed in the *dl355*-infected cells were somewhat less than those observed in wild-type virus-infected cells; this is evidenced by the lack of clearly excluded nucleoli. However, examples of cells demonstrating a more extensive diffuse nuclear distribution were infrequently observed, as seen in Fig. 1d. (The cell in Fig. 1d was one of approximately 500 examined). Also, *dl355*-infected cells contained a greater number of brightly stained nuclear spicules, and the fraction of cells containing globular or vesicular bodies stained for the E1B protein was significantly lower than that in a comparable population of cells infected with wild-type virus. The fraction of cells containing the globular or vesicular structures stained for the E1B protein varied with the multiplicity of infection and increased through the course of the infection (data not shown). Nevertheless, the globular or vesicular staining pattern was typically observed 10-fold less frequently in *dl355*-infected cells (5 of 380) than in wild-type virus-infected cells (49 of 345). Within the cytoplasm, the diffuse reticular staining found in the mutant virus-infected cells was comparable to or slightly less extensive than that of the wild-type virus-infected cells. Finally, the number and staining intensity of the cytoplasmic perinuclear bodies appeared similar to or perhaps slightly greater than those observed in the wild-type virus-infected cells.

Identical results (not shown) were obtained with other E1B-55kDa-specific antibodies, including the rat monoclonal antibodies 9C10 (59) and RA1 (5) and another mouse monoclonal antibody, IC6 (gift of A. Zantema, University of Leiden, Leiden, The Netherlands). None of the antibodies specific for the E1B protein stained uninfected cells, nor did any of the antibodies stain cells infected with the E1B-55kDa-deficient virus *dl338* (data not shown and Fig. 7e).

The specificity of the E1B-55kDa antibody used in these studies can be seen in Fig. 1e and f. In the experiment whose results are illustrated in Fig. 1e, mock-infected cells were stained for the E1B-55kDa protein, photographed, and printed under conditions identical to those of the virus-infected samples; no background staining was evident. The same field of control cells is seen in Fig. 1f, except that the combined exposure obtained through the microscope and enlarger reflects an approximately eightfold overexposure. The weak background staining observed is primarily reticular in nature throughout both the cytoplasm and the nucleoplasm. The 2A6 monoclonal antibody used for this study also exhibits a faint perinuclear staining in the overexposure similar to that observed with antibodies that recognize epitopes in the Golgi stack (11). This pattern was not seen with the other E1B-55kDa-specific antibodies.

Colocalization of the E1B-55kDa protein with phase-dense viral inclusion bodies requires the E4-34kDa protein. As noted above, the globular and vesicular structures which were stained with E1B-55kDa-specific antibody corresponded to phase-dense inclusion bodies specific to virus-infected cells. Others have previously reported that the E2A-72kDa DNA binding protein is closely associated with these structures (41, 54). We also found this to be the case. All wild-type virus-infected cells containing distinct inclusion bodies, identified by phase illumination microscopy, showed a coincidence of these structures with stain for the E2A DNA binding protein (in 15 of 15 cells). The nature of the association, illustrated in Fig. 2a and b, suggests that the DNA binding protein is very tightly associated with the phase-dense structures. By focusing through the depth of the cell, we could observe that the rim-staining pattern (Fig. 2b) arises from the spherical, shell-like organization of the larger vesicular bodies.

A close association of the E1B-55kDa protein with the viral inclusion bodies in wild-type virus-infected cells was also observed. Cells with prominent viral inclusion bodies generally displayed a coincidence of staining for the E1B-55kDa protein (Fig. 2c to h). Although some examples of rim staining could be seen (arrowheads in Fig. 2d and f), the E1B-55kDa protein was not as closely associated with the viral inclusion bodies as the E2A-72kDa DNA binding protein was, nor was the E1B protein as uniformly distributed about the inclusion bodies. Rather, the E1B protein appeared to be organized in nodules or crenulations about the periphery of the inclusion body (25 of 35 cells) or, as noted in several cells (7 of 35), loosely associated with the phase-dense inclusion bodies (Fig. 2g and h). Finally, approximately 10% (3 of 35) of the cells containing vesicular inclusion bodies did not exhibit associated E1B-specific staining. Within the nuclei of all these cells, the E1B-55kDa protein was also diffusely distributed. It should be noted that the extent of association between the E1B protein and the inclusion bodies varied slightly between experiments and more substantially through the course of the infection. At earlier times in the infection (12 to 14 h), the association between the E1B protein and inclusion bodies appeared greater, although it was more difficult to positively identify the less developed viral inclusion bodies by phase microscopy. At later times (18 to 32 h), the viral inclusion bodies were more prominent but the colocalization of E1B protein and viral inclusion bodies was less extensive (data not shown).

The association between the phase-visible viral inclusion bodies and viral antigens was also examined with cells infected with virus lacking the E4-34kDa protein (Fig. 3). By

FIG. 2. Phase-dense vesicular structures coincide with indirect immunofluorescent staining for both E2A-72kDa and E1B-55kDa proteins in wild-type virus-infected cells. HeLa cells were infected at a multiplicity of 20 PFU per cell with wild-type virus and processed 15 h later for indirect immunofluorescence with antibodies specific for the E2A-72kDa protein (a and b) or the E1B-55kDa protein (c through h). The phase-contrast micrograph in the left panel corresponds to the fluorescent micrograph on the right. Selected phase-dense vesicular structures are indicated by arrowheads, and the corresponding locations are indicated similarly in the fluorescent image. Shown are representative samples selected by phase-contrast microscopy for the appearance of prominent phase-dense viral structures. Bar, 10 μ m.

FIG. 3. Phase-dense vesicular structures in virus-infected cells lacking the E4-34kDa protein coincide with indirect immunofluorescent staining for the E2A-72kDa but not the E1B-55kDa protein. HeLa cells were infected at a multiplicity of 20 PFU per cell with the E4-34kDa defective virus, *dl355*, and processed 15 h later for indirect immunofluorescence with antibodies specific for the E2A-72kDa protein (a and b) or the E1B-55kDa protein (c through h). The fields were selected and the micrographs are marked as described in the legend for Fig. 2. The fine arrows in panels g and h mark a phase-dense vesicle in a region devoid of staining for the E1B protein. Bar, 10 μ m.

FIG. 4. Localization of the E1B-55kDa protein in sequentially extracted virus-infected HeLa cells 15 h postinfection. Cells were infected with wild-type virus (a and c) or the E4 mutant virus, *dl355* (b and d). At 15 h postinfection the cells were sequentially extracted with Triton X-100 (a and b) or Triton X-100 followed by 0.25 M ammonium sulfate (c and d). The resulting structures were then fixed and processed for indirect immunofluorescence with an antibody for the E1B-55kDa protein. Bar, 10 μ m.

phase illumination microscopy, cells infected with the E4 mutant virus appeared identical to the wild-type virus-infected cells (compare the phase panels of Fig. 2 with Fig. 3). Vesicular or globular viral inclusion bodies were apparent in approximately the same fraction of cells. When these cells were stained for the E2A DNA binding protein, all (10 of 10) demonstrated a tight association between the inclusion body and DNA binding protein (Fig. 3a and b).

However, a close association between the viral inclusion bodies and the E1B-55kDa protein was not observed in *dl355*-infected cells. Approximately 60% of the cells displaying phase-visible inclusion bodies failed to show a significant colocalization of staining for the E1B-55kDa protein and the inclusion bodies (Fig. 3c to h). Of 47 cells, 25 showed no coincidence and 5 showed no significant nuclear staining. The fine arrows in Fig. 3g and h mark an inclusion body in a region devoid of staining for the E1B-55kDa protein. The arrowheads in Fig. 3c to h mark vesicular inclusion bodies that occur in regions of the nucleus containing diffuse stain for the E1B protein. The diffuse staining observed in the regions containing the inclusion bodies is not the result of variations in the plane of focus, as this could easily be ascertained during photography. Moreover, the arrowheads in Fig. 3d, f, and h point to an apparent shadow of the vesicular inclusion body in the diffuse fluorescence. The appearance of such a shadow indicates that the plane of focus is the same for both phase and fluorescent micrographs. In addition, such images suggest that staining for the E1B-55kDa protein not only fails to coincide with but is also excluded from the phase-dense aspect of the vesicular inclusion.

The requirement for the E4-34kDa protein for association of the E1B-55kDa protein with viral inclusion bodies was further examined with infected cells that were extracted with either Triton X-100 alone (Fig. 4a and b) or Triton X-100 followed by 250 mM ammonium sulfate (Fig. 4c and d). These treatments are elements of an extraction protocol (13, 17, 18) developed to remove freely soluble components while preserving the morphology and organization of underlying subcellular structure. A distinct reduction in the reticular cytoplasmic and diffuse nuclear stain occurred in extracted samples, enhancing the contrast of the remaining patterns. The lack of E1B-55kDa staining in association with viral inclusion bodies in extracted *dl355*-infected (Fig. 4b and d) compared with wild-type virus-infected cells (Fig. 4a and c) is striking.

Immunoelectron-microscopic localization of the E2A-72kDa DNA binding protein and the E1B-55kDa protein. The discrete structures identified by indirect immunofluorescent staining for the E1B-55kDa protein were preserved after extraction with Triton X-100 (Fig. 4). Consequently, indirect immunogold staining was performed on extracted cells before they were embedded and sectioned. Initial experiments indicated that this preembedding labeling protocol afforded a greater degree of recognition by the antibodies employed, particularly the E1B-55kDa-specific monoclonal antibody. In addition, the level of ultrastructural preservation was greater when a preembedding labeling approach was used.

The survey micrograph of Fig. 5a illustrates the characteristic features of a HeLa cell infected for 15 h with adenovirus. In this example, the cell was infected with the E4 mutant virus, *dl355*, extracted with Triton X-100, and

FIG. 5. Survey electron micrograph of a virus-infected HeLa cell extracted and immunogold stained for the E1B-55kDa protein. A monolayer HeLa cell culture was infected with the E4 mutant virus, *dl355*, at a multiplicity of 20 PFU per cell. At 15 h postinfection, the cells were extracted and immunogold stained with an antibody for the E1B-55kDa protein prior to embedment. (a) In the low-magnification survey, the cytoplasm (Cy), CFIs and DFI are indicated. Some nucleoli of the infected cells fail to remain intact through the immunogold staining procedure, and the presumptive residual nucleolus is indicated (rNu). The scattered dense grains throughout the nucleus are clusters of 10-nm gold beads. Bar, 2 μ m. (b) Higher-magnification view of the detail indicated in panel a. Single 10-nm gold beads are evident throughout the CFI, and numerous clusters of beads occur in the free nucleoplasm. Bar, 0.25 μ m.

immunostained for the E1B-55kDa protein before being embedded and sectioned. Detailed analysis of the cytoskeletal framework obtained from both uninfected and adenovirus-infected HeLa cells has been published (29, 61). As previously discussed, the filamentous networks of the cytoplasm are poorly visualized by standard embedding and staining procedures—hence the sparse cytoplasm seen in Fig. 5a. The empty region in the center of the nucleus appears to represent a remnant of the nucleolus. Apparently the paraformaldehyde fixation used before immunostaining was not adequate to preserve the nucleoli of all infected cells, and such empty regions similar in size and number to nucleoli were observed in some infected cells. Other nucleic acid-containing structures of the extracted cell appeared well preserved and retained their staining properties. The structures in Fig. 5 labeled CFI and DFI appear identical to the clear and dense fibrillar inclusions initially identified by Moyne and associates (37) and further characterized by Puvion-Dutilleul et al. (41). Similar structures were previously described in adenovirus-infected cells by Martinez-Palomo and associates (32–34) and Phillips and Raskas (38). Further evidence for the identity of the structures labeled CFI with those identified by previous investigators derives from their association with the E2A-72kDa DNA binding protein (41; Fig. 6).

The results presented in Fig. 5 also demonstrate the penetration of the nuclei and CFIs by the 10-nm colloidal gold particles. The region of the large CFI marked in Fig. 5a is enlarged in Fig. 5b. Numerous clusters of 10-nm particles are evident surrounding the inclusion body, and many individual gold particles are visible within the body itself.

Similar distributions of gold particles were observed for each of the CFIs seen in the survey micrograph of Fig. 5a. The presence of gold particles within the boundary of each CFI indicates that the antibody-gold complex is able to penetrate the CFI. If the association of gold particles with the CFI were the result of surface or shell staining, the staining pattern seen would require the unlikely event that each CFI present was sectioned tangentially. Moreover, in serial sections of similarly treated specimens, gold particles were observed throughout the depth of the CFI (data not shown).

The localization of the E2A DNA binding protein visualized by immunogold staining in extracted cells (Fig. 6) was consistent with that determined by immunofluorescence microscopy (Fig. 2b and 3b). Immunofluorescence studies of the distribution of the E2A-72kDa protein in extracted cells demonstrated that the diffusely distributed DNA binding protein is removed after the cell is extracted with nonionic detergents (54). Consistent with this observation, no staining was observed in the nucleoplasm outside of the CFI. Essentially all gold particles were restricted to a limited margin about the periphery of the CFI that was no wider than the 10-nm gold particle itself. When staining was observed within the fibrillar inclusion, the small size and irregular border of the CFI suggested that the staining was the result of a tangential section to the inclusion body. Identical distributions of the E2A DNA binding protein were observed in cells infected with wild-type virus (Fig. 6a), virus lacking the E4-34kDa protein (Fig. 6b), and virus lacking the E1B-55kDa protein (Fig. 6c). The last virus, *dl338*, contains a large deletion in the 55-kDa protein-coding region and was

used in the experiments described below as a control for nonspecific staining by the E1B-55kDa-specific antibody.

Next, the association of the E1B-55kDa protein and the CFIs was studied by immunogold electron microscopy. Representative micrographs of infected cells containing both high and low labeling densities are shown in Fig. 7. In cells infected with wild-type virus, labeling for the E1B protein was enriched around the CFIs relative to that observed in other regions of the nucleus. Unlike the distribution of the E2A-72kDa DNA binding protein, the E1B protein was not restricted to the CFIs in the extracted cells, as suggested by the residual diffuse nuclear stain observed by immunofluorescence microscopy after extraction (Fig. 4a). Immunogold labeling patterns that appeared to represent the nuclear spicules and perinuclear cytoplasmic body were also observed and are discussed below. The E1B protein associated with the CFIs was not limited to the margins of the CFIs (arrowheads in Fig. 7a and b), as was observed for the DNA binding protein. Gold particles were frequently observed within inclusion bodies as well as more loosely distributed about the margins of the CFIs. Gold particles appearing away from the CFIs were not associated with any readily identified structure, although they did associate with features exhibiting an intermediate electron density. In samples containing a larger number of gold particles (Fig. 7a), the increased staining was reflected in a greater number of clustered gold particles away from the CFIs as well as an increase in single particles associated with the CFIs. The E1B-55kDa protein was not associated with condensed chromatin, such as that along the nuclear lamina or in the nucleolus (Fig. 7a). The DFI bodies were also devoid of staining for the E1B protein.

The nonspecific staining associated with immunogold labeling for the E1B-55kDa protein was extremely low. To control background labeling, cells were infected with a virus that was unable to express a complete E1B-55kDa protein. Cells infected with this mutant virus (*dl338*) proceed through the early and intermediate stages of the infection in a wild-type fashion and exhibit a wild-type distribution of the E2A-72kDa DNA binding protein (Fig. 6c). However, very few (mean = 4.0; $n = 12$) gold particles were observed in sections of nuclei from *dl338*-infected cells stained for the E1B-55kDa protein (Fig. 7e).

In the absence of the E4-34kDa protein, immunogold staining for the E1B-55kDa protein was not enriched about the CFIs. Figure 7c represents the typical labeling pattern for the E1B protein observed in cells infected with the E4 mutant virus, *dl355*. Numerous clusters of gold particles were seen in the nucleoplasm away from the CFIs. The distribution of the E1B protein in the *dl355*-infected cells failed to suggest a preferential association with the CFIs (Fig. 7c and d).

Differences in the apparent immunogold labeling efficiency between samples were noted (compare Fig. 7c and d). This variation, reflected in the absolute number of gold particles

FIG. 6. Immunogold localization of the E2A-72kDa protein about the margins of CFIs. Cells were infected at a multiplicity of 20 PFU per cell with wild-type virus (a), the E4-34kDa-deficient virus, *dl355* (b), or the E1B-55kDa-deficient virus, *dl338* (c). The cells were extracted 15 h after infection and immunogold stained with an antibody for the E2A-72kDa protein prior to embedment. E2A-72kDa-specific immunogold staining was found exclusively along the margins of the CFI bodies in both solid and vesicular forms. Bar, 0.25 μ m.

seen in otherwise similar cells, prevented direct comparisons based on the number of gold particles associated with a particular structure in different samples. To enable such comparisons to be made, a morphometric index measuring the relative density of gold particles associated with the CFIs was developed. The absolute area density of immunogold particles over the CFIs and over regions of the nucleus that excluded the CFIs was determined. The particle densities from each preparation were scaled such that the density of immunogold particles over the nucleoplasm (exclusive of the CFIs) was normalized to 1 per unit area. From three experiments, nine separately labeled preparations of wild-type virus-infected cells and eight preparations of E4 mutant, *d/355*-infected cells were analyzed (Fig. 8). The results indicate a moderate degree of variability. Nonetheless, within each experiment, the relative density of E1B-55kDa-specific labeling associated with the CFIs in cells infected with wild-type virus was clearly greater than that measured in mutant virus-infected cells. The normalized particle density about the CFIs in all *d/355*-infected cells was nearly equal to that measured in the nucleoplasm in general, further suggesting that the E4-34kDa product was responsible for the increased concentration of the E1B protein about the CFIs.

Infected cells that were immunogold stained for the E1B-55kDa protein displayed two characteristic staining patterns in addition to that associated with the CFIs and the diffuse distribution in the nucleoplasm. Within the nucleus, linear arrays of gold particles were observed. The density of E1B-specific gold labeling along these arrays, together with the frequency and apparent size of these arrays, suggested that these structures represent the nuclear spicules observed by immunofluorescence microscopy. Also in agreement with the immunofluorescent images, a heavily decorated body was observed in the cytoplasm of infected cells. Both structures appear to be filamentous in nature and were poorly differentiated by the uranyl-lead stains used for the conventional resin-containing sections. Consequently, embedment-free electron microscopy was also employed to visualize these structures (Fig. 9 and 10). This procedure employs the contrast obtained in the absence of any embedding material which arises from the electron density of the object relative to the vacuum of the specimen chamber. Heavy metal stains are not required for contrast enhancement, and poorly staining structures such as protein filaments of the cytoplasm and nuclear matrix are better visualized by embedment-free electron microscopy (12, 58).

The intranuclear, linear array is seen in Fig. 9. This pattern was common in cells infected with the E4 mutant virus, *d/355* (two to eight arrays per nuclear section), but was also observed (in approximately 25% of the sections) in cells infected with wild-type virus. These structures occurred throughout the nuclear volume and did not appear to associate with any readily identified intranuclear component. When viewed in conventional, resin-containing sections (Fig. 9a), the gold particles decorate a faintly staining filamentous structure. Similar structures viewed by embed-

FIG. 8. Normalized E1B-55kDa-specific immunogold particle density associated with CFI in wild-type virus- and E4-34kDa mutant virus-infected cells. In three separate experiments, generating 17 independently labeled preparations, the density of E1B-55kDa-specific immunogold particles was measured in regions of the nucleus containing and excluding CFIs. The density of gold particles in the nuclear regions containing the CFIs was normalized by dividing by the particle density determined for regions of the nucleus that excluded the CFIs. Each value plotted in the histogram represents an independent labeling preparation determined by analyzing between 259 and 8,765 gold particles in 6 to 20 cells.

ment-free microscopy also suggest the filamentous nature of the structure (Fig. 9b). The gold particles appear to decorate filaments that are 12 to 15 nm in diameter and often intertwine. These filaments further differ from the highly branched core filaments of the nuclear matrix in that they form limited junctions and are fairly straight (23).

The cytoplasmic, perinuclear body identified by immunofluorescence microscopy appears to be represented by the heavily decorated structures shown in Fig. 10. The concentration of E1B-55kDa-specific label associated with this structure was often high, even in cells containing few gold particles in the nucleus. In a conventional resin-containing section, the perinuclear body was typically seen in the vicinity of intermediate-sized filaments (Fig. 10a). By embedment-free microscopy, the intermediate-sized filaments were seen to enmesh the perinuclear body, which itself appeared to be composed of a closely knit tangle of intermediate-sized (12- to 15-nm) filaments (Fig. 10b). A perinuclear body composed of 8-nm filaments was previously determined to contain the E1B-55kDa protein in adenovirus-

FIG. 7. Immunogold localization of the E1B-55kDa protein in virus-infected cells. Cells were infected at a multiplicity of 20 PFU per cell with wild-type virus (a and b), the E4-34kDa-deficient virus, *d/355* (c and d), or the E1B-55kDa-deficient virus, *d/338* (e). The cells were extracted 15 h postinfection and immunogold stained with an antibody for the E1B-55kDa protein prior to embedment. Representative micrographs illustrating the immunogold staining seen in cells displaying solid-appearing clear fibrillar bodies are shown. The CFI bodies are indicated by unlabeled arrowheads; the presumptive residual nucleolus (rNu) and a DFI are also indicated. (e) The specificity of the stain and background level of staining can be seen. Two gold beads are indicated by the arrows. Bar, 0.2 μ m.

FIG. 9. Linear array of E1B-55kDa-specific immunogold staining present in *d*/355-infected cells. Cells were infected at a multiplicity of 20 PFU per cell with the E4-34kDa-defective virus and processed for immunogold staining of the E1B-55kDa protein 15 h later. The linear array of gold beads visualized in the resin-embedded section (a) appears coincident with a weakly staining filamentous structure. The similar pattern of immunogold staining visualized in the embedment-free section (b) also appears coincident with intertwined filamentous structures with diameters of 12 to 15 nm that have a limited electron density. Similar structures were observed in wild-type virus-infected cells at an approximately 50-fold-lower frequency. Bar, 0.1 μ m.

transformed cells (59). The relationship between the perinuclear body observed here in the lytically infected cells and that observed in the transformed cells is unknown.

DISCUSSION

The E1B-55kDa polypeptide exhibits a variety of localizations in HeLa cells infected with wild-type adenovirus. Within the cytoplasm, it was found in a diffusely distributed, reticular pattern (Fig. 1a) and in an intensely stained fibrous body adjacent to the nucleus (Fig. 1 and 10). Within the nucleus, the polypeptide was present in a granular, diffuse distribution that was excluded from the nucleoli (Fig. 1 and 4), within discrete spicules that were randomly distributed throughout the nuclear volume (Fig. 1 and 9), and in local concentrations within and about the periphery of viral inclusion bodies (Fig. 2 and 7). This complex distribution is in agreement with the presence of the protein in multiple cellular compartments generated by biochemical fractionation (44, 50). The variety of localizations may result, at least in part, from specific interactions of the E1B-55kDa polypeptide with other proteins such as p53 (6, 47, 59, 60) and by oligomerization (21). Possibly the different subcellular localizations of the E1B protein reflect different functional forms of the protein such as have been suggested for the simian virus 40 large T antigen (e.g., in reference 49).

As previously noted, mutant viruses that cannot express the E1B-55kDa protein exhibit reduced cytoplasmic accumulation of viral mRNAs and fail to prevent accumulation of cellular mRNAs late after infection. Since the viral protein

appears to exert its effect on RNA metabolism at an intranuclear step (9, 30), it seems unlikely that the E1B protein in the cytoplasm is involved. Further, the diffuse cytoplasmic distribution of the E1B protein is predominant very early in infection (data not shown) before it exerts an observable effect on host cell mRNA accumulation (2, 39). The perinuclear fibrous body containing the E1B protein also seems a poor candidate, not only because of its cytoplasmic location but also because a similar structure has been observed in transformed cells (6, 59) where cellular mRNA metabolism is not known to be influenced by the E1B product.

The population of E1B protein in the nucleus that appears most likely to play a key role in mRNA metabolism is that which is concentrated within and about the periphery of viral inclusion bodies. This follows from the simultaneous disruption of this localization (Fig. 3 and 7c and d) and loss of effects on RNA metabolism (22, 45) in the absence of the E4-34kDa polypeptide, which is known to form a complex with the E1B-55kDa polypeptide (46). Apparently, a key sequence or structural element required to achieve this localization is present either in the E4-34kDa polypeptide or in the 55-kDa-34-kDa protein complex that is not present in the E1B protein alone. The lack of the 34-kDa protein does not cause a general disruption of protein associations with the viral inclusion bodies, since the tight association of the E2A-72kDa DNA binding protein with these structures is independent of its presence (Fig. 3 and 6). It seems clear that neither of the remaining two nuclear localizations of the E1B-55kDa polypeptide is sufficient to mediate effects on

FIG. 10. High concentration of E1B-55kDa-specific immunogold labeling in a cytoplasmic body. Cells were infected with wild-type virus at a multiplicity of 20 PFU per cell and processed for E1B-55kDa immunogold staining 15 h later. Nearly every cell specifically stained for the E1B-55kDa protein contained several of the cytoplasmic bodies illustrated here. In the resin-containing section (a), the cytoplasmic body appears more densely stained than the intermediate-sized filaments. The embedment-free section (b) shows the body to be enmeshed in a network of intermediate-sized (12- to 18-nm) filaments and appears itself to be composed of a closely knit tangle of intermediate-sized filaments. The nuclear lamina (NL) is indicated from within the nucleus. Bars: (a) 0.25 μm , (b) 0.1 μm .

RNA metabolism. The amount of diffusely distributed nuclear E1B protein is nearly unchanged, while the amount present as discrete spicules is increased in the absence of the E4 protein when the E1B protein does not appear to properly influence RNA metabolism. However, it remains possible that the E1B protein in diffuse granules and spicules can affect RNA metabolism, but only when the E4-34kDa protein is also present to localize the 55-kDa-34-kDa complex at viral inclusion bodies.

Given its role in viral RNA metabolism within the nucleus, it seems reasonable that the E1B-55kDa polypeptide is localized in the vicinity of the viral inclusion bodies. Autoradiographic studies of adenovirus-infected cells have established that the CFIs are sites of both DNA replication (32, 33, 37) and transcription (37). The E2A-72kDa DNA binding protein is very tightly associated with the periphery of these inclusion bodies (Fig. 2 and 3; 7, 41, 54) and functions directly in viral DNA replication (25, 53) and viral transcription (15). The E1B-55kDa polypeptide exhibits a looser and more diffuse association with the inclusions. It functions after transcription, polyadenylation, and splicing (39) but before the RNA leaves the nucleus (9, 30). Thus, the E1B-55kDa protein associated with the viral inclusion bodies is spatially situated in a manner consistent with its action

after transcription but before translocation across the nuclear membrane.

We have previously suggested that viral chromosomes may dominate microenvironments within the nucleus that are favorable for transport of processed RNAs to the cytoplasm (30, 36, 39). Such environments might represent sites normally occupied by active genes that are gated via nuclear channels to pores, as proposed by Blobel (7). Such a model would account for facilitated expression of cellular genes when they are included on the viral chromosome (19, 24) and would explain the requirement for viral DNA replication to block cytoplasmic accumulation of host cell mRNAs, since it would be necessary to colonize and dominate almost all of these transport-gated microenvironments.

Localization of the E1B-55kDa polypeptide at the limited number of fairly large replication-transcription centers is consistent with its ability to enhance cytoplasmic accumulation of late viral mRNAs. But how does this localization prevent cytoplasmic accumulation of host cell mRNAs? We propose that the complex of the E1B-55kDa and E4-34kDa proteins interacts with and draws to the periphery of viral replication-transcription centers a nuclear factor required for movement of RNAs from their site of processing to the nuclear pore for transport to the cytoplasm. Factors which

could play such a role have been identified in studies employing in vitro transport systems (35, 51). Further, the Sm antigen (31) and a nuclear matrix-associated antigen, P11 (14), become associated with inclusion bodies during adenovirus infection (13a).

Relocation of a cellular factor required for intranuclear movement of newly synthesized and processed RNAs could explain the ability of the 55-kDa–34-kDa protein complex to simultaneously inhibit accumulation of RNAs transcribed from cellular locations and stimulate accumulation of transcripts derived from the viral chromosome.

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