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Partition of E1A Proteins between Soluble and Structural Fractions of Adenovirus-Infected and -Transformed Cells

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The partition of E1A proteins between soluble and structural framework fractions of human cells infected or transformed by subgroup C adenoviruses was investigated by using gentle cell fractionation conditions. A polyclonal antibody raised against a *trpE*-E1A fusion protein (K. R. Spindler, D. S. E. Rosser, and A. J. Berk, J. Virol. 132–141, 1984) synthesized in *Escherichia coli* was used to measure the steady-state levels of E1A proteins recovered in the various fractions by immunoblotting. The relative concentration of E1A proteins recovered in the soluble fraction of adenovirus type 2-infected cells was at least fivefold greater than the relative concentration in the corresponding fraction of transformed 293 cells. The observed distribution of E1A proteins was not altered by the sulfhydryl-blocking reagent *N*-ethylmaleimide. E1A proteins were recovered in nuclear matrix, chromatin, and cytoskeleton fractions after further fractionation of the structural framework fraction. However, the E1A protein species that could be identified by one-dimensional gel electrophoresis were not uniformly distributed among the subcellular fractions examined. The results obtained when fractionation was performed in the presence of the oxidation catalysts Cu^{2+} or (*ortho*-phenanthroline)$_2$ Cu^{2+} indicate that E1A proteins can be efficiently cross-linked, via disulfide bonds, to the structural framework of both adenovirus-infected and adenovirus-transformed cells.

The adenovirus E1A transcription unit is the earliest to be expressed during productive infection (52, 60, 61). During the early phase of infection by subgroup C adenovirus type 2 (Ad2) or Ad5, two major mRNA species, usually termed 13S and 12S, are processed from E1A transcripts (3, 10, 40, 55, 63). These species encode proteins of 289 and 243 amino acids (R), respectively, that differ only in the presence of a unique, internal 46R sequence in the former (25, 55, 62, 71). It has been known for some time that several forms of both the 289R and the 243R proteins are produced in infected cells (14, 28, 30, 31, 33, 34, 45, 62), and recent high-resolution, two-dimensional separation has identified as many as 60 E1A polypeptide species (32). The E1A proteins can be phosphorylated (42, 66, 76), but whether additional modifications contribute to this myriad of E1A protein species is not known.

The E1A 243R protein is required for efficient viral DNA replication in human cells that are strictly growth arrested (65), whereas 289R products of the 13S mRNA are largely responsible for the E1A-mediated stimulation of transcription from other transcription units during the infectious cycle, an activity that is essential to efficient virus reproduction (2, 36, 49, 50, 57). The E1A proteins can also induce enhanced transcription of cellular genes or those of other viruses, introduced into cells synthesizing E1A proteins by transfection (1, 16, 24, 29, 68, 70) or as part of an adenovirus genome (24), and of certain endogenous cellular genes, including those encoding hsp70 (37, 51), β-tubulin (67), and an MHC H2K gene (58). More recently, both the 289R and 243R proteins have been reported to repress transcription dependent upon certain viral or cellular enhancer elements (5, 35, 72). In addition, both 289R and 243R E1A proteins play important roles in adenovirus transformation. Indeed, production of these proteins is sufficient to immortalize primary rodent cells and to induce a number of typical transformation phenotypes (see references 21 and 71 for reviews).

The molecular roles played by the E1A proteins to mediate efficient productive infection and transformation are less well understood. The available evidence suggests that the 289R E1A protein is not a sequence-specific transcription factor (see references 21a and 39 for reviews), and neither protein, at least when produced in *Escherichia coli*, exhibits DNA-binding activity (19). Various cell fractionation procedures have been used to determine the location of the E1A proteins and to identify their potential neighbors within the cell, but no consistent picture of their intracellular location(s) has emerged. Feldman and Nevins (18), using a gentle fractionation procedure designed to preserve cell structure, reported that the 289R proteins produced in H5*ts*125-infected 293 cells were predominately associated with the nuclear matrix, although these proteins were also found in the cytoplasm. The E1A proteins were also detected in both the nucleus and cytoplasm (in the latter as soluble and cytoskeletal-bound forms) of Ad5-infected cells by Rowe and colleagues (59). By contrast, Spindler and Berk (64) observed that E1A proteins extracted from Ad5-infected cells sedimented as monomers in glycerol gradients and therefore concluded that they do not form complexes with cellular components. The latter conclusion is difficult to reconcile with the recently reported immunoprecipitation of complexes containing both E1A proteins and specific cellular proteins (32a, 75). Moreover, few detailed studies of the distribution of the E1A proteins in transformed cells have been reported. Therefore, we have undertaken a more systematic study of the subcellular locations of the subgroup C adenovirus E1A proteins, as revealed by cell fractionation followed by immunoblotting, in particular comparing infected cells harvested during the early phase of infection and transformed human cells.

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MATERIALS AND METHODS

Cells and viruses. HeLa cells were maintained in suspension culture at a density of 3×10^5 to 5×10^5 cells per ml in S-MEM (GIBCO Laboratories) supplemented with 5% calf serum. The 293 cells were maintained in suspension culture at a density of 2×10^5 to 5×10^5 cells per ml in the same medium. HeLa and 293 cells were propagated in monolayer cultures in Dulbecco modified Eagle medium (GIBCO) supplemented with 10% calf serum.

HeLa cells grown in suspension culture were infected with Ad2, *dl*347, or *dl*348 at 50 PFU per cell and harvested 6 h after infection. HeLa cells in monolayer culture were infected at the same multiplicity, but were harvested 8 h after infection.

Cell fractionation. The fractionation of cells was done by the procedure of Cervera et al. (8) with minor modifications. Adenovirus-infected HeLa cells or 293 cells maintained in suspension culture were collected by centrifugation at 700 rpm for 3 min in a PRJ International centrifuge, and the medium was discarded. The cell pellet was suspended in ice-cold phosphate-buffered saline by gentle swirling of the tube, and the cells were again collected. After removal of the supernatant, cells were suspended in 20× the pellet volume of ice-cold extraction buffer (see below) by very gentle pipetting of the suspension with prechilled, wide-bore pipettes. The suspension was incubated at 0°C for 3 min before the nuclei and attached cytoskeletons were collected by centrifugation at 700 rpm (PRJ centrifuge) for 3 min at 0°C. The supernatant, termed the soluble phase, was separated, diluted fourfold with water, adjusted to 2% (wt/vol) sodium dodecyl sulfate (SDS) and 10% (vol/vol) glycerol and heated at 70°C for 30 min. The pellet, termed the structural framework fraction, was suspended in 10 mM Tris hydrochloride (pH 6.8) containing 20 mM NaCl and 5 mM $MgCl_2$ and treated with DNase I and RNase at 100 and 20 μg/ml, respectively, at room temperature for 15 min with occasional vortexing. This suspension was then adjusted to 2% (wt/vol) SDS and 10% (vol/vol) glycerol and heated at 70°C for 30 min. The structural fraction, when further fractionated, was first extracted with 10 mM Tris hydrochloride buffer containing Tween 40 and deoxycholate at concentrations of 1 and 0.5%, respectively (8), to produce the cytoskeleton fraction. The nuclei were then fractionated into a 2 M NaCl-extractable fraction after DNase and RNase treatment (20) and an insoluble nuclear pellet (matrix) fraction.

The extraction buffer was made by the procedure of Cervera et al. (8) by diluting to 1% a 10% stock of Triton X-100 (Fluka Chemical Corp.) into 10 mM piperazine-*N*, N′-bis(2-ethanesulfonic acid) (PIPES), pH 6.8, containing 100 mM KCl, 2.5 mM $MgCl_2$ and 0.3 M sucrose. Phenylmethylsulfonyl fluoride dissolved in ethanol was added to 1 mM just before the extraction buffer was added to the cell pellet. When required, the extraction buffer was made 0.2 to 0.5 mM in Cu^{2+}. (*ortho*-Phenanthroline)$_2$ (Sigma Chemical Co.) Cu II complex (CuP) was made by the procedure of Lothstein et al. (46). The extraction buffer was made 0.2 to 0.5 mM in Cu^{2+}, and two equivalents of *ortho*-phenanthroline dissolved in 50% ethanol in water were then added from a concentrated stock solution. After thorough mixing, the extraction buffer containing CuP was used directly for cell fractionation.

Electrophoresis and immunoblotting of proteins. Proteins of the various cell fractions were separated by electrophoresis in 12% polyacrylamide-SDS gels (41). Electrophoresis was for 20 h at 2.5 V/cm. Proteins were transferred from such gels electrophoretically to nitrocellulose paper by using a Hoefer Scientific Instruments transfer apparatus. For optimum transfer, a voltage gradient of 4 V/cm was applied for 2.5 h. Electrophoresis for longer periods resulted in passage of proteins of less than 20 kilodaltons through the filter, whereas shorter periods were not sufficient to transfer proteins of greater than 70 kilodaltons. After transfer, the nitrocellulose paper was soaked in a 5% nonfat dry milk solution in 10 mM Tris hydrochloride, pH 7.2, containing 0.9% NaCl (buffer A) for 90 min at 37°C with gentle shaking. At the end of this blocking step, nitrocellulose filters were enclosed in Seal-a-Meal bags (Dazey Corp.) and incubated with 0.15 ml of a rabbit E1A antiserum raised against a *trpE*-E1A fusion protein (66) in buffer A for 90 min. Filters were then washed twice at 37°C in a buffer A containing 0.05% nonfat dry milk, sealed in plastic bags containing 2 μCi of ^{125}I-labeled protein A (New England Nuclear Corp.) in the same solution and incubated at 37°C with shaking for 1 h. After two washes in buffer A containing 0.05% nonfat dry milk, filters were subjected to a more stringent wash in 10 mM Tris hydrochloride (pH 7.2) containing 0.5 M NaCl and 1% (vol/vol) Nonidet P-40 at 37°C with gentle shaking for 10 to 15 min. After a final wash in buffer A, filters were dried and wrapped in Saran Wrap before exposure to X-ray film (XAR or XRP; Eastman Kodak Co.) in the presence of intensifying screens.

RESULTS

Extraction of cells with nonionic detergents in buffers of near physiological ionic strength and pH solubilizes lipids to release soluble cellular proteins (approximately two-thirds of the total) but leaves intact the structural elements of the nucleus and the cytoplasm (4, 6, 44, 53). Such methods were designed to maximize preservation of structural elements of the cell (54). Therefore, we used them to investigate the associations of the E1A proteins with cellular components in both adenovirus-infected and -transformed cells.

Distribution of E1A proteins in infected and transformed cells. HeLa cells in suspension culture were infected with Ad2 at a multiplicity of infection of 50 PFU per cell and harvested 6 h after infection. Such infected HeLa cells and Ad5-transformed 293 cells (27), also grown in suspension culture, were fractionated in parallel by the procedure of Cervera et al. (8) to generate two fractions, one containing detergent-soluble proteins and the second containing the proteins associated with the structural framework of the cell. After electrophoresis in 12% polyacrylamide-SDS gels, proteins were electrophoretically transferred to nitrocellulose paper (7, 69) under the optimized conditions described in Materials and Methods. The filters were then reacted with rabbit immune serum raised against a *trpE*-E1A fusion protein synthesized in *Escherichia coli* from plasmid pKRS103 (66), kindly provided by K. R. Spindler and A. J. Berk, and ^{125}I-protein A. The filters were washed under the conditions described in Materials and Methods, minimizing their exposure to the nonionic detergent Nonidet P-40; although prolonged washes in the presence of the detergent reduced background labeling of proteins, some protein was lost in the process, as judged by examination of prestained proteins.

Typical results obtained under these conditions are shown in Fig. 1, which illustrates the distribution of E1A proteins between the soluble and structural framework fractions recovered from Ad2-infected HeLa and 293 cells. In this and all subsequent experiments, half of the soluble fraction

proteins were compared with the total structural framework fraction proteins, so that the total amount of protein applied to each lane was similar. In addition to several E1A proteins, apparent molecular sizes 36 to 46 kilodaltons, both cell lines contained the four proteins designated C_1 to C_4, of which C_3 and C_4 were largely restricted to the structural framework fraction (Fig. 1). These four proteins represent cross-reacting cellular proteins for their labeling (but not that of the E1A proteins), could be almost completely eliminated by extensive washing of the filters (data not shown), and were detected in uninfected HeLa cells (Fig. 1, lane 5). It is clear from the data shown in Fig. 1 that 293 cells contained significantly smaller quantities of the E1A proteins than did Ad2-infected HeLa cells harvested during the early phase of infection. However, a number of other, potentially more interesting differences in the E1A proteins detected in the infected and transformed human cells were observed. Thus, the spectrum of E1A proteins observed in 293 cells appeared to be simpler than that of infected HeLa cells, and some of the E1A proteins recovered from the two cell types appeared to display different mobilities (compare lanes 1 and 2 with lanes 3 and 4, Fig. 1). These results suggest that the E1A proteins may be modified differently in the two cell types examined. This observation complicates quantitative comparisons of the E1A proteins detected in adenovirus-infected HeLa cells and adenovirus-transformed 293 cells. Nevertheless, it is clear that the partition of the E1A proteins between the soluble and structural framework fractions was strikingly different in infected compared with transformed human cells (compare lanes 1 and 2 with lanes 3 and 4, Fig. 1). The ratio of the sum of the intensities of the E1A protein bands detected in the soluble fraction to the sum of those present in the structural fraction was at least 10:1 for adenovirus-infected HeLa cells, compared with a ratio of less than 1:1 for 293 cells.

The transformed 293 cells contained a smaller total quantity of E1A proteins than did the productively infected HeLa cells examined in the experiment shown in Fig. 1, no doubt because the former contained fewer copies of the E1A transcription unit than the latter, which were infected at 50 PFU per cell. The larger fraction of soluble E1A proteins observed in productively infected cells (Fig. 1) may, therefore, represent polypeptides for which no binding sites exist in the structural framework fraction. In other words, the larger quantity of E1A polypeptides might saturate available binding sites present in the structural fraction. However, when HeLa cells infected with 5 or 50 PFU per cell were fractionated in parallel, the distribution of E1A proteins observed was identical to that shown in lanes 1 and 2 of Fig. 1, even though the cells infected at the lower multiplicity contained two- to threefold less total E1A proteins (data not shown), that is, a level similar to that present in 293 cells (Fig. 1).

In some previous reports, E1A proteins, at least those encoded by the 13S mRNA, have been reported to be preferentially associated with the nuclear matrix, the insoluble material remaining when nuclei were extracted with 2 M NaCl after DNase I digestion (18). To examine in more detail the subcellular distribution of the E1A proteins associated with structural elements of the cell, the structural framework fractions recovered from infected or transformed cells were further fractionated as described in Materials and Methods to yield cytoskeletal, chromatin, and nuclear-insoluble fractions. These were examined for the presence of E1A proteins by immunoblotting as described previously. Typical results obtained in this way are shown in Fig. 2. As expected

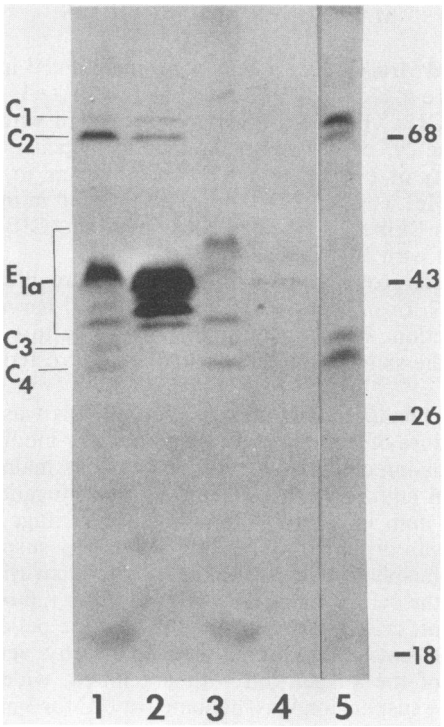

FIG. 1. Distribution of E1A proteins in adenovirus-infected and -transformed cells. Equal quantities of Ad2-infected HeLa cells (lanes 1 and 2) or 293 cells grown in suspension (lanes 3 and 4) were fractionated, as outlined in the text, into soluble (lanes 2 and 4) and structural framework (lanes 1 and 3) fractions. To keep the total quantity of protein in each lane approximately equal, half of the soluble-phase proteins was applied to lanes 2 and 4. After electrophoresis, immunoblotting was performed as described in Materials and Methods. Lane 5 shows a mock-infected, whole-cell extract probed with the E1A antiserum. Numbers at right indicate molecular size (in kilodaltons).

on the basis of the results of experiments like those shown in Fig. 1, the soluble fraction recovered from Ad2-infected HeLa cells contained a substantially greater proportion of the E1A proteins than the corresponding fraction prepared from 293 cells. In both infected and transformed cells, however, more than half of the E1A proteins not released into the soluble fraction were recovered with the cytoskeleton (lanes 3 and 7, Fig. 2). Thus, only small quantities of the E1A proteins were found in either the fraction generated by DNase digestion of purified nuclei (chromatin) or associated with the remaining insoluble nuclear material (nuclear matrix) (lanes 1 and 2 and 5 and 6, Fig. 2).

Various experiments have been performed to establish that the most striking difference observed in the distribution of the E1A proteins in infected compared with transformed human cells, the much greater concentration of these proteins in the soluble fraction in the former, was not the result of an artifact introduced by the fractionation procedures employed. To rule out the possibility that the composition of the cell fractions depended upon the volume of detergent-containing buffers used for cell lysis and to check for the linearity of signals on immunoblots, two samples each of adenovirus-infected HeLa cells or 293 cells, one set containing twice as many cells as the other, were extracted in parallel with an identical volume of buffer. The distribution of E1A proteins between the soluble and structural fractions

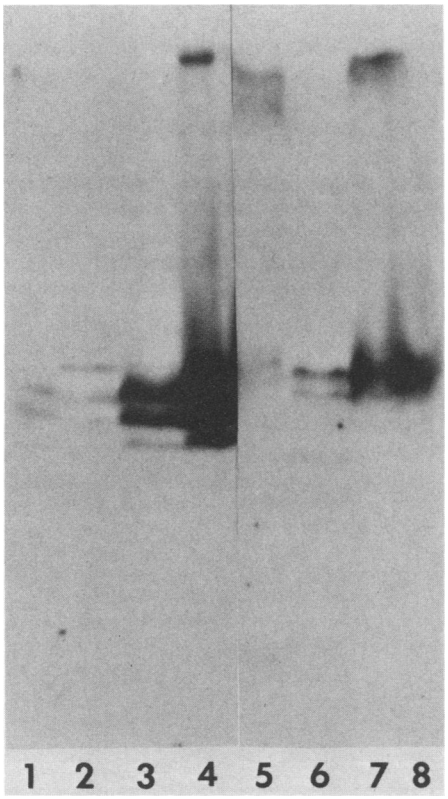

FIG. 2. Distribution of the E1A proteins of the structural framework fraction among cytoskeletal, nuclear-soluble, and nuclear-insoluble fractions. Ad2-infected HeLa cells or 293 cells grown in suspension were first fractionated into a soluble fraction (lanes 4 and 8), and the remaining structural framework fraction was separated into the cytoskeletal fraction (lanes 3 and 7), by extraction with low-ionic-strength, double-detergent-containing buffer, and the nuclear fraction. The nuclear fraction was further separated into a fraction that was high salt extractable after DNase and RNase treatment (lanes 2 and 6) and the insoluble nuclear pellet (matrix) (lanes 1 and 5). Half the soluble fraction protein was applied to lanes 4 and 8.

was analyzed as described previously. It is clear from the results of this experiment (Fig. 3A) that the quantities of E1A proteins analyzed in these experiments were within the linear range of detection. Moreover, because approximately twice the quantity of E1A protein could be detected when twice the number of cells was extracted (compare any pair of lanes in Figure 3A), it is unlikely that the volume of buffer in which cells were extracted contributed to the distribution pattern of the E1A proteins described above. HeLa cells are epithelial in origin, whereas 293 cells were derived by Ad5 DNA-mediated transformation of human embryonic kidney fibroblasts (27). Nevertheless, examination of cellular proteins by staining (Fig. 3B) indicates that the major cellular proteins of the two cell types partitioned between the soluble and structural fractions in identical fashion. Thus, the observed difference in the distribution of the E1A proteins in 293 compared with infected HeLa cells cannot be readily attributed to differences in the effects of the fractionation protocol upon the two cell types.

A number of variations in the protocol and the source of the cells were tested to rule out trivial explanations of the results. For example, 293 cells grown in monolayers were fractionated and compared with Ad2-infected HeLa cells, also grown in monolayers. In another experiment, 293 cell monolayers were trypsinized, the protease was quenched with excess serum, and the cell suspension was fractionated identically and in parallel to Ad2-infected HeLa cells in suspension culture. In some experiments, the degree of confluence of 293 cells grown as monolayers was varied. Within the bounds of experimental error, the same result was always obtained: the ratio of E1A proteins recovered in the soluble fraction of the cell compared with the structural fraction was higher for Ad2-infected cells by a factor of 5 to 10 than the corresponding ratio for adenovirus-transformed 293 cells (data not shown).

The distribution of E1A proteins was also examined in several lines of Ad2-transformed rat embryo cells, including F17, REM, and T2C4 (22, 23). In all cases, a greater proportion of the E1A proteins was recovered in the structural framework fraction than when Ad2-infected HeLa cells were similarly fractionated (data not shown). Thus, a greater association of E1A proteins with the cytoskeleton and nucleus appears to be a general property of adenovirus-transformed cells compared with adenovirus-infected cells.

Identification of 289R and 243R E1A proteins. During the early phase of adenovirus infection, two major E1A mRNA species, 13S and 12S mRNA, encoding proteins of 289 and 243R, respectively, are produced (see Introduction). Therefore, we wished to determine whether the products of either E1A mRNA species were preferentially associated with the structural elements of the cell, and we examined the distribution of E1A proteins in fractions recovered from HeLa cells infected by the Ad5 cDNA-virus *dl*348 and *dl*347 (74). These viruses express only the 289R or 243R proteins, respectively. As illustrated in Fig. 4, two of the most abundant E1A proteins detected in Ad2-infected HeLa cells, designated V and X in Fig. 4, were produced in cells infected by H5*dl*348 but not by H5*dl*347. Thus, these proteins must be encoded by the 13S E1A mRNA species. The E1A species designated Z, also abundant in wild-type virus-infected cells, was by contrast not produced in cells infected by H5*dl*348 (Fig. 4, lanes 3 and 4) but could be detected after infection by *dl*347 (lanes 5 and 6, Fig. 4) and must therefore be encoded by the 12S E1A mRNA species. The substantially lower concentration of E1A proteins observed after H5*dl*347 infection, compared with H5*dl*348 or Ad2 infection, is not unexpected: the former virus cannot produce the 289R E1A proteins that stimulate E1A transcription (2, 36, 50, 57) and grows less efficiently than H5*dl*348 (74). Proteins corresponding to the species designated Y in Ad2-infected cells were apparently present in cells infected by either mutant, suggesting that Y contained at least two E1A proteins. The species designated W could be detected in cells infected by neither mutant (compare lanes 2, 4, and 6, Fig. 4), suggesting that it is probably a product of the E1A 12S mRNA present in H5*dl*347-infected cells at levels too low to detect.

Several of the E1A proteins that could be assigned to one or other of the E1A mRNA species were concentrated in the soluble or structural fractions. Species largely recovered in the former fraction included X (13S mRNA) and Z (12S mRNA), whereas the 13S mRNA-encoded protein designated V was preferentially associated with the structural elements of the cell (Fig. 4).

Fractionation of E1A proteins after blocking or oxidation of sulfhydryl groups. The internal 46R segment unique to the 289R products of the E1A 13S mRNA contains five cysteine residues (55) and is, therefore, extremely cysteine rich. Cysteine sulfhydryl groups can be oxidized by dissolved oxygen present in buffers and can participate in exchange

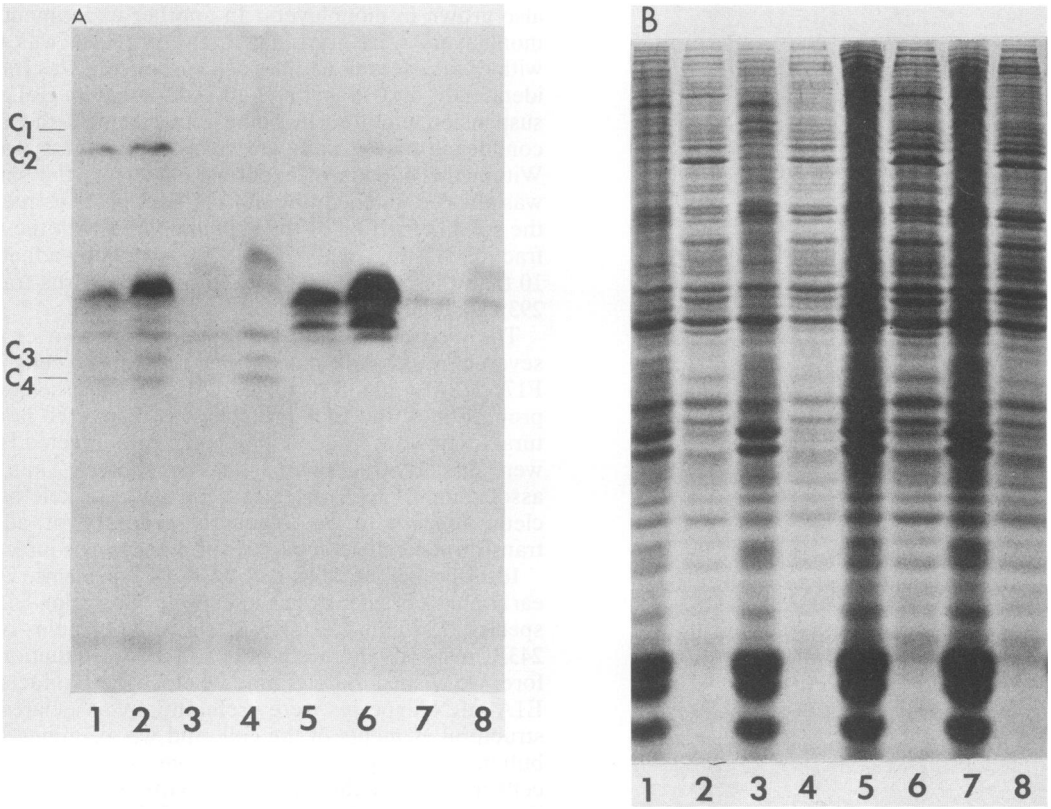

FIG. 3. Immunoblotting of E1A proteins extracted under different conditions. (A) HeLa cells in suspension culture infected with Ad2 (lanes 1, 2, 5, and 6) or 293 cells in suspension culture (lanes 3, 4, 7, and 8) were fractionated into soluble (lanes 5 to 8) and structural framework (lanes 1 to 4) fractions. Lanes 5 to 8 were loaded with half the total protein of the soluble fraction. The even-numbered lanes show proteins recovered from twice the number of cells used in the odd-numbered lanes. (B) Coomassie blue stain of the cellular proteins from the same samples immunoblotted in panel A. Samples applied to lanes 5 to 8 show the proteins of the fractions corresponding to those shown in lanes 1 to 4, but obtained by extracting twice the number of cells in the same volume of buffer. Lanes 2, 4, 6, and 8 show proteins of soluble fractions, whereas lanes 1, 3, 5, and 7 show structural framework fraction proteins from infected HeLa (lanes 1, 2, 5, and 6) or 293 (lanes 3, 4, 7, and 8) cells.

reactions with disulfide bonds present in proteins, properties that might have complicated studies of nuclear matrices and scaffolds in the past (38, 43, 47). Therefore, we examined the effect of the presence of the sulfhydryl-blocking reagent *N*-ethylmaleimide in the extraction buffer upon the distribution of E1A proteins, to determine whether the structure-bound E1A proteins observed in previous experiments were the result of such artifacts. The results obtained when the extraction buffer contained 5 mM *N*-ethylmaleimide, conditions that specifically and quantitatively modify free sulfhydryl groups (17), are shown in Fig. 5. Comparison of lanes 3 and 4 of Fig. 5 indicates that addition of *N*-ethylmaleimide did not alter the ratio of soluble to structure-bound E1A proteins. Thus, we can conclude that the usual extraction conditions, without *N*-ethylmaleimide, used in these experiments were not sufficiently oxidizing to make a significant difference in the quantity of E1A proteins recovered in the structural fraction.

Stabilization of the filamentous networks of the cell has been achieved in previous studies by using oxidation catalysts such as micromolar concentrations of Cu^{2+} or CuP (43, 46, 47, 56). Therefore, we wished to learn whether similar oxidation catalysts added with the extraction buffer would cross-link the cysteine-rich E1A proteins to their nearest neighbors through the formation of disulfide bonds. Addition of 0.5 mM Cu^{2+} to the extraction buffer induced a dramatic change in the partition of the E1A proteins (Fig. 6A, lanes 1 and 2): virtually all were recovered in the structural framework fraction in Ad2-infected HeLa cells. Identical results were also obtained with 293 cells (data not shown). A comparison of the E1A proteins recovered in the structural fraction from identical numbers of Ad2-infected suspension HeLa cells exposed for 5 min to our usual extraction buffer or to extraction buffer containing 0.2 mM Cu^{2+} or 0.2 mM CuP yielded similar results (Fig. 6A, lanes 3, 5, and 4, respectively). The dramatic change in the partition of E1A proteins between the soluble and structural framework fractions of infected cells induced by Cu^{2+}-catalyzed oxidation (Fig. 6A) was not the result of a general redistribution of cellular proteins under these conditions: the majority of cellular proteins recovered from cells fractionated with untreated extraction buffer and those exposed to Cu^{2+}-catalyzed oxidation were present in the structural fraction at similar concentrations, as judged by Coomassie blue staining (Fig. 6B).

In the experiments whose results are shown in Fig. 6, protein samples were extensively reduced with excess β-mercaptoethanol before electrophoresis. When samples were denatured, but not reduced, the E1A proteins were recovered in complexes too large to enter 12% polyacryl-

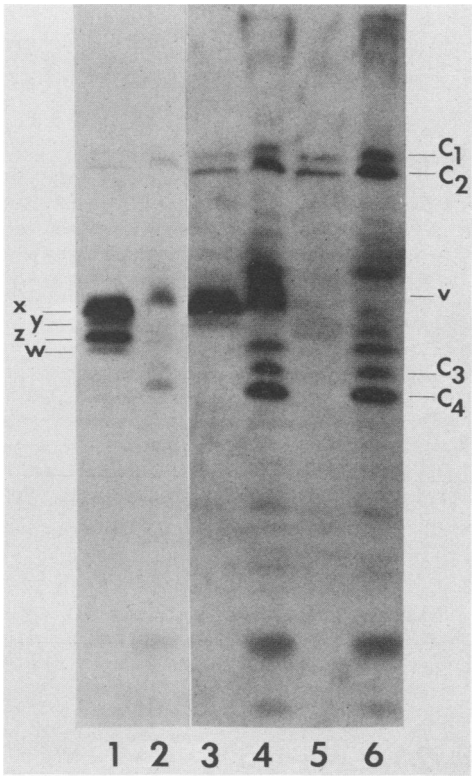

FIG. 4. Comparison of the partition of the E1A proteins of Ad2-, H5*dl*347-, and H5*dl*348-infected HeLa cells between the soluble and structural framework fractions. Equal numbers of HeLa cells in suspension culture were infected with 50 PFU of Ad2 (lanes 1 and 2), H5*dl*348 (lanes 3 and 4), or H5*dl*347 (lanes 5 and 6) per cell, and the cells were harvested 6 h after infection. Infected cells were fractionated as described previously into structural framework (lanes 2, 4, and 6) and soluble (lanes 1, 3, and 5) fractions. The soluble fractions shown in lanes 1, 3, and 5 contained half the total proteins of the fraction derived from the same number of cells, whose structural fractions are shown in lanes 2, 4, and 6.

amide-SDS gels (data not shown). The susceptibility of such E1A protein-containing complexes to reduction indicates that Cu^{2+} or CuP indeed induced cross-linking of these viral proteins to cellular components via the formation of disulfide bonds.

DISCUSSION

Knowledge of the locations and interactions with cellular components of the adenovirus E1A proteins in infected and transformed cells could be expected to provide important clues about the molecular functions of these important proteins. As discussed in the Introduction, no clear picture of these properties of the E1A protein had emerged from application of cell fractionation procedures when the studies reported here were undertaken. Most, if not all, cell fractionation methods provide a less than ideal representation of the interactions of cellular components in vivo (see reference 12 for discussion). The procedure used in these experiments relies upon a cell lysis buffer that is close to physiological in ionic strength and contains a mild, nonionic detergent. Extraction in this medium for 3 min at 0°C was neither sufficiently oxidizing nor sufficiently reducing to induce artifactual partitioning of the E1A proteins, for addition of saturating quantities of the sulfhydryl blocking reagent *N*-ethylmaleimide produced no significant alteration in the distribution of these cysteine-rich proteins (Fig. 5). A similar conclusion has been reached previously (9). Therefore, we believe that the partition of the E1A proteins among subcellular fractions shown in Fig. 1 and 2 provides as reasonable a representation as is possible by cell fractionation procedures of the locations of the subgroup C adenovirus E1A proteins in infected and transformed cells.

Although most of the E1A proteins present in Ad2-infected HeLa cells during the early phase of infection were recovered in the soluble phase, a significant fraction, 10 to 20%, was associated with the nucleus and the structural framework of the cytoplasm (Fig. 1 and 2). It seems likely that this subset of the E1A proteins was overlooked in the experiments of Spindler et al. (66), in which extraction was performed in buffers containing low concentrations of salt, conditions known to disrupt a subset of the structural filaments of the cell, the microfilaments (8). Moreover, the extraction buffer employed by Spindler and colleagues contained 10 mM dithiothreitol, a potent disulfide bond cleaving agent (11), suggesting that natural interactions among E1A proteins and cellular components mediated by disulfide bonds might be disrupted. In this context, it may well be significant that we have demonstrated that E1A proteins can be cross-linked to cellular components by Cu^{2+}-catalyzed air oxidation (Fig. 6). The susceptibility of the E1A proteins to such oxidation may also account, at least in part, for the discrepancy between the data reported here and a previous report of the association of a large fraction of the 289R E1A

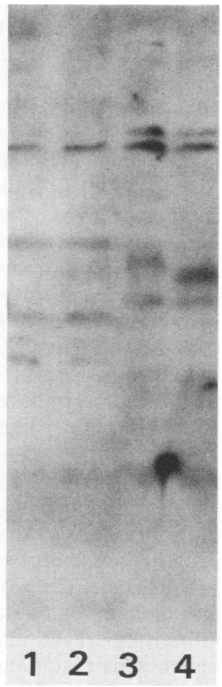

FIG. 5. Fractionation of the E1A proteins in the presence of *N*-ethylmaleimide. Ad2-infected HeLa cells in suspension culture were fractionated into soluble (lanes 3 and 4) and structural framework (lanes 1 and 2) fractions by using extraction buffer containing 5 mM *N*-ethylmaleimide (lanes 1 and 3) or no blocking agent for free sulfhydryl groups (lanes 2 and 4). Lanes 3 and 4, showing the soluble fractions, contained half the total quantity of protein present in the corresponding structural framework fractions.

proteins with the nuclear matrix by Feldman and Nevins (18), who used extraction conditions more likely to permit air oxidation than those used here. In addition, the use of H5*ts*125-infected 293 cells (Fig. 1 and 2) and an antibody raised against a portion of the unique segment of the 289R protein could have biased against detection of all forms of the 289R proteins normally present in adenovirus-infected cells. Despite the large quantitative differences in the distribution of the E1A proteins seen here and reported previously (18), we also found that one E1A 289R protein species, probably that designated V in Fig. 5, is preferentially, if not exclusively, associated with the nuclear matrix fraction recovered from adenovirus-infected cells (Fig. 2).

It is now clear that a number of reagents can influence the behavior of proteins during subcellular fractionation. Certain cellular proteins can, for example, be precipitated onto the nuclear matrix by the high salt concentrations normally used during matrix preparation (47). The human c-*myc* p62 protein, originally thought to be a matrix-associated protein (13), has been shown recently to become irreversibly associated with an insoluble nuclear complex after exposure of intact nuclei to temperatures of greater than 35°C (15). Such insolubilization of the c-*myc* protein appears to be independent of the conditions under which heating is performed and is probably the result of the precipitation or polymerization of a subset of nuclear proteins (15). The oxidation-induced association of adenovirus E1A proteins with the structural framework fraction of the cell reported here (Fig. 6) appears to be the result of covalent attachment of the viral proteins to cellular components. The formation of air-oxidation-induced disulfide bonds among protein constituents of the intermediate filaments and between the glucocorticoid receptor and components of the nuclear matrix has been described previously (56; S. H. Kaufman, S. Oket, A.-C. Wikstrom, J.-A. Gustafsson, and J. H. Shaper, J. Biol. Chem., in press), and it seems likely that this process also contributes to the stabilization of nuclear scaffolds (43, 47).

The readiness with which all forms of the E1A proteins can undergo oxidation to become cross-linked to elements of the cytoskeletal framework (Fig. 6) indicates that not only the cysteine-rich, unique region of the 289R protein but regions common to the two proteins contain reduced cysteine residues. The latter might include the segment comprising the C-terminal 50 residues, a region that contains 5 cysteine residues (25, 55). The presence of cysteine residues that can be readily oxidized also suggests that at least some of the 60 or so E1A protein species identified by high-resolution, two-dimensional electrophoresis might represent different conformers of the proteins, fixed by internal disulfide bonds. Indeed, *N*-ethylmaleimide noticeably alters the migration in conventional SDS-polyacrylamide gels of the most abundant form of the 289R protein detected (Fig. 5).

It has been reported previously that adenovirus E1A mRNA species and proteins are more stable in transformed than in adenovirus-infected cells (64, 73). Nevertheless, an approximately threefold higher steady-state concentration of E1A proteins was routinely observed in infected HeLa cells (see, for example, Fig. 1). This result suggests that the E1A transcription unit must be more efficiently expressed in infected cells. More striking than this quantitative difference in production of E1A proteins were the differences in the distribution of E1A proteins in infected cells compared with transformed cells: in transformed 293 cells, about half of the total E1A protein was recovered in the soluble phase and about half in the structural framework fractions, whereas 80 to 90% of the E1A proteins produced during the early phase of infection was present in the soluble fraction (Fig. 1 and 2). As discussed in Results, this difference cannot be attributed to artifacts of the cell fractionation conditions, differences in the growth states of infected HeLa and 293 cells, or differences in the total quantity of E1A proteins synthesized in transformed and infected cells, but rather appears to be a general property of adenovirus-transformed cells. It is not yet known whether as many forms of the E1A proteins are present in transformed as in infected cells (32), nor has the nature of the differences responsible for the large number of E1A proteins identified by Harlow and colleagues (32) been established. Therefore, we do not yet know whether the different subcellular distributions of the E1A proteins in transformed and infected cells correlate with the synthesis of a particular subset of the E1A proteins made in the latter. Nevertheless, it is clear from these observations that properties displayed by adenovirus E1A proteins in infected cells are not necessarily typical of transformed-cell E1A proteins. Therefore, it seems quite possible that at least some E1A protein species do not perform strictly analogous molecular functions in infected and transformed cells.

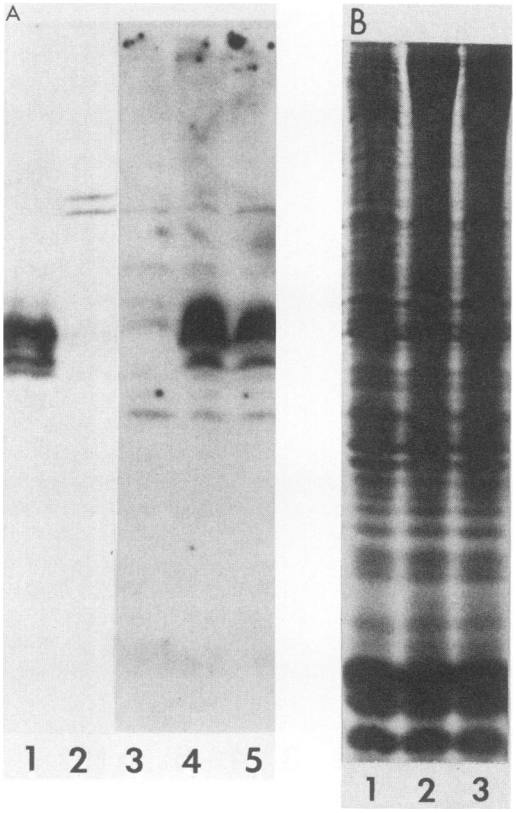

FIG. 6. Fractionation of the E1A proteins of Ad2-infected HeLa cells in the presence of Cu^{2+} or CuP. (A) Lanes 1 and 2 show an immunoblot analysis of the structural framework and soluble fractions, respectively, recovered from the same number of Ad2-infected HeLa cells extracted for 3 min at 0°C with extraction buffer containing 0.5 mM Cu^{2+}. Lanes 3, 4, and 5 show the structural fractions from an identical number of Ad2-infected cells that had been extracted for 5 min at 0°C with extraction buffer alone, extraction buffer containing 0.2 mM CuP, or extraction buffer containing 0.2 mM Cu^{2+}, respectively. All samples were extensively reduced with β-mercaptoethanol before electrophoresis. Lanes 1, 2, and 3 of panel B show a Coomassie blue stain of the structural fraction proteins immunoblotted in lanes 3, 4, and 5, respectively, of panel A.

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