Nuclear Envelope Localization of an Adenovirus Tumor Antigen Maintains the Integrity of Cellular DNA

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The adenovirus early-region 1B 19,000-molecular-weight tumor antigen is required for oncogenic transformation of cells by adenovirus. We have demonstrated that this tumor antigen is located in the nuclear envelope of infected and transformed cells and that a fraction of the protein within the nuclear envelope is associated with the nuclear lamina. During cell division in the transformed cells, the nuclear envelope containing the tumor antigen dissociates at metaphase and then reforms around the separated daughter chromosomes at telophase. Adenovirus mutants carrying lesions in the gene encoding this tumor antigen cause degradation of host cell chromosomal DNA, and in these mutants, the intracellular localization of the 19,000-dalton protein is altered. These results demonstrate that components of the nuclear envelope function in the organization of chromatin in infected and transformed cells and that a virus-encoded protein plays a critical role in this process.

The genomes of DNA tumor viruses, such as the human adenoviruses, contain all of the information required for oncogenic transformation of cultured cells (reviewed in reference 44). The region of the adenovirus genome sufficient to cause transformation is early region 1 (E1), which consists of two distinct transcription units, E1A and E1B, that are expressed before DNA synthesis during lytic infection. The E1A gene products alone are capable of immortalizing primary cells (24), but expression of the complete transformation phenotype is not realized without other cooperating viral or cellular oncogene products such as E1B proteins, polyoma middle-T antigen, or the T24 Harvey *ras* protein (6, 23, 39, 41, 46, 47). The E1A gene products have been shown to function in regulating transcription of other early viral genes as well as some cellular genes (2, 13, 25, 27, 40). No specific functions have been assigned to any of the E1B gene products in either transformation or lytic growth of virus, but Babiss and Ginsberg (1) have assigned a role for the E1B 57,000-dalton protein (57K protein) in the shutoff of cellular protein synthesis. The two major mRNAs from the E1B transcription unit produce proteins with an M_r of 19,000 (175 amino acids) and an M_r of 57,000 (495 amino acids), with coding regions extending between nucleotides 1711 to 2236 and 2016 to 3500, respectively. These proteins are commonly called the adenovirus tumor antigens since animals with adenovirus-induced tumors have circulating antibodies directed against these antigens. We have sought to probe the function of the E1B tumor antigens in oncogenic transformation and lytic infection by analyzing adenovirus mutants which produce altered E1B gene products.

Initial studies of an adenovirus type 2 (Ad2) mutant, Ad2*ts*111 (7, 33), demonstrated that this mutant is temperature sensitive for viral DNA replication and causes degradation of host cell chromosomal DNA, a phenomenon not normally seen in cells infected with wild-type virus. These phenotypes are the result of two independent mutations; the temperature-sensitive DNA replication is the result of an altered DNA-binding protein encoded by the early region 2A (E2A) of the genome, and the DNA degradation phenotype

(*deg*), accompanied by an enhanced or abnormal cytopathic effect phenotype (*cyt*), is the result of a single point mutation in the gene encoding the E1B 19K tumor antigen (43, 48). The DNA replication defect has been genetically separated from the E1B 19K gene mutation by the construction of an Ad2*ts*111-adenovirus type 5 (Ad5) recombinant virus which contains E1 from Ad2*ts*111 and the E2A gene, and hence the DNA-binding protein, from wild-type Ad5. This recombinant, designated Ad2*cyt*106, is not temperature sensitive for DNA replication but does have the *deg* and *cyt* phenotypes (48).

A number of other adenovirus E1B 19K protein mutants were investigated and shown to possess the unusual *deg* and *cyt* phenotypes (48). The large-plaque mutants Ad2*lp*3 and Ad2*lp*5 (6), like Ad2*ts*111 and Ad2*cyt*106, have point mutations which result in amino acid substitutions in the 19K protein. Although both Ad2*lp*3 and Ad2*lp*5 have the *cyt* phenotype, Ad2*lp*3 is *deg*⁺ and does not degrade DNA. Therefore, the *cyt* phenotype can be independent of the *deg* phenotype even though both are caused by mutations in the same gene. It is unlikely that the DNA degradation seen in cells infected with E1B 19K mutants is a direct result of the altered cellular morphology, since Ad2*lp*3 induces the same abnormal morphology but DNA degradation does not occur. Ad2*ts*111, Ad2*cyt*106, Ad2*lp*3, and Ad2*lp*5 synthesize a gene product that can be detected by immunoprecipitation with antipeptide antibodies directed against the carboxy terminus of the protein (6, 48). Another E1B 19K mutant, Ad5*d*337 (S. Pilder, J. Logan, and T. Shenk, personal communication), which has an out-of-frame deletion in the gene encoding the 19K protein, also has the *cyt* and *deg* phenotypes although no detectable 19K gene product is made. Ad2*lp*3, Ad2*lp*5 (6), and Ad5*d*337 (Pilder et al., personal communication) are defective for transformation of rat cells, although Ad2*ts*111 is not defective for transformation of primary baby rat kidney cells (48). These mutants are viable and all produce virus in HeLa cells; therefore, under these conditions, a fully functional 19K protein is not required for lytic growth.

As a first step toward understanding the function of the E1B 19K tumor antigen in lytic growth of virus and oncogen-

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ic transformation, we have investigated the intracellular localization of the 19K protein and have further characterized the E1B 19K mutants and the DNA degradation phenotype. The E1B 19K tumor antigen localizes in the nuclear envelope membranes and lamina, the structural component of the nucleus that has been implicated to be involved in the organization of interphase chromatin. This localization of the E1B 19K tumor antigen within the nuclear envelope is disrupted in mutant-infected cells in which the chromosomal DNA becomes degraded. We propose that one function of the nuclear envelope components is to control chromatin structure, and possibly function, in lytically infected and transformed cells.

MATERIALS AND METHODS

Cells and viruses. The growth of cell and viruses has been described in detail elsewhere (48).

Indirect immunofluorescence. HeLa cells, plated on glass cover slips, were infected with Ad2 at a multiplicity of 10 PFU per cell, when the cells were ca. 50% confluent. At various times postinfection, the cover slips were washed with phosphate-buffered saline (PBS; 150 mM NaCl, 20 mM sodium phosphate-potassium phosphate; pH 7.4) at 37°C and fixed in methanol at -10°C for 10 min. After rehydration in PBS, the cover slips were incubated with the anti-peptide antibodies directed against the carboxy terminus of the E1B 19K tumor antigen (19) or human autoantibodies specific for lamins A and C (35). The cover slips were then washed for 25 min in five changes of PBS and incubated with fluorescein-conjugated goat anti-rabbit immunoglobulin G (IgG) (anti-19K stained) or rhodamine-conjugated rabbit anti-human IgG (anti-lamin stained) at 37°C. All antibodies were diluted in PBS with 1% bovine serum albumin (BSA), and antibody incubations were at 37°C for 1 h. The cover slips were washed with five changes of PBS, rinsed once in water, and mounted in Aquamount with 0.1% *p*-phenylenediamine. Slides were examined with a Zeiss Photomicroscope III equipped with epifluorescence optics, and photographs were taken with Tri-X film. Other fixation methods, such as 3.7% formaldehyde, produced inferior results with both the anti-19K protein and anti-lamin antibodies; therefore all indirect immunofluorescence was performed on methanol fixed cells.

Electron microscopy. HeLa cells at 50% confluency were either processed directly (mock infected) or infected with Ad2 at a multiplicity of 50 PFU per cell. At 41 h postinfection, the cells in the dishes were washed with PBS at 37°C and made permeable when the dish was flooded with ice-cold PBS containing 0.1% Nonidet P-40-15 mM β -mercaptoethanol-1 mM phenylmethylsulfonyl fluoride for 6 min. The cells in the dishes were washed several times with PBS and fixed with 2% formaldehyde-0.01% glutaraldehyde in PBS at room temperature for 20 min. After being washed with four changes of PBS, the aldehyde groups were reduced by an incubation with 50 mM NH_4Cl in PBS for 10 min at room temperature. The cells were washed with PBS and incubated with either anti-19K protein or anti-lamin antibodies diluted in PBS-1% BSA at room temperature for 1 h with gentle agitation. Control dishes that were stained with the second antibodies alone were incubated with PBS-1% BSA during this time. Washing with five changes of PBS containing 0.01% saponin, BSA, and sheep gamma globulin preceded a 1.5-h incubation with the horseradish peroxidase-conjugated second antibodies. Dilution of goat anti-rabbit IgG to 100 $\mu\text{g}/\text{ml}$ in PBS-1% BSA followed staining with the

anti-19K protein antibody, and addition of rabbit anti-human IgG followed staining with the anti-lamin autoantibody. The cells were then washed extensively, first with four changes of PBS containing saponin, BSA, and gamma globulin with gentle agitation overnight. After three changes of PBS, the cells were then fixed for 10 min with 2% glutaraldehyde in 75 mM sodium cacodylate-4.5% sucrose. After being washed with three changes of PBS, the cells were incubated with 300 mM glycine (pH 10) in PBS for 10 min and then rinsed with three changes of 100 mM Tris-hydrochloride (pH 7.6) at room temperature. The diaminobenzidine (DAB) reaction was conducted for 40 min by incubating the cells with freshly made and filtered solution of 1 mg of DAB per ml in 50 mM Tris-hydrochloride (pH 7.6)-0.01% H_2O_2 at room temperature in the dark. When the reaction product had developed, the dishes were rinsed with Tris-hydrochloride (pH 7.6) and then with PBS before a 20-min postfixation with 1% OsO_4 in 50 mM cacodylate. The cells were washed with distilled water, block stained with saturated aqueous uranyl acetate, dehydrated in ethanol, and embedded in Epon (5). Thin sections were examined in a Philips 201 electron microscope.

Nuclear lamina preparations. (i) **Method I.** Suspension cultures (1 liter) of HeLa cells grown to 5×10^5 cells per ml were infected with Ad2 at a multiplicity at 20 PFU per cell, and at 25 h postinfection, mock- and virus-infected cells were harvested and washed in PBS. Nuclei were isolated by subjecting cells to hypotonic Dounce homogenization and purified by sequential centrifugation through 1.6 and 2.2 M sucrose solutions by the method of Blobel and Potter (4). The nuclei were examined microscopically and were free from all visible cytoplasmic contamination. The nuclear pore complex-lamina fraction was isolated from purified nuclei by successive DNase I digestions, Triton X-100 extraction, and 2 M NaCl treatment of the nuclei (8).

(ii) **Method II.** Nuclear lamina were also isolated by similar treatments (see text) from monolayer cells (35) that were mock infected or Ad2 infected for 40 h. Dilute protein samples from supernatant fractions were concentrated by precipitation with trichloroacetic acid, and all samples were suspended in sample buffer and boiled before electrophoresis (30).

For Western blotting, 15% polyacrylamide gels of nuclear lamina preparations were electrophoretically transferred to nitrocellulose (45) and incubated with anti-19K protein or anti-lamin antibodies; the immune complexes were detected with ^{125}I -labeled protein A. The amount of the 19K tumor antigen in various cellular fractions was quantitated by excising the radioactive bands after Western blotting and counting them with a gamma counter. All samples fell within the linear range of transfer.

Analysis of degraded DNA. HeLa cells were infected with Ad2 or Ad2cyt106 at a multiplicity of 50 PFU per cell, and total and Hirt supernatant DNAs were isolated from each at 48 h postinfection. Total genomic DNA was isolated by lysing cells in a solution of 150 mM NaCl, 10 mM EDTA, 10 mM Tris-hydrochloride (pH 7.5), and 0.4% sodium dodecyl sulfate and incubating the lysate with 200 μg of proteinase K per ml overnight at 37°C. The DNA was extracted with phenol followed by chloroform-isoamyl alcohol (24:1), spooled after the addition of 2 volumes of ethanol, and redissolved in Tris-EDTA. Low-molecular-weight DNAs were isolated by the method of Hirt (22) with minor modifications (48). Total DNA (10 μg) and variable amounts of Hirt DNAs from mock-, Ad2-, and Ad2cyt106-infected cells were digested with *EcoRI* and electrophoresed through 1% agar-

FIG. 1. Localization of the E1B 19K tumor antigen during lytic infection. HeLa cells infected with wild-type Ad2 were fixed at various times postinfection and stained by indirect immunofluorescence with antibodies either to the E1B 19K tumor antigen (A, C, and E) or to the nuclear lamins (G). Fixation was at (A) 12 h, (C) 17 h, and (E) and (G) 40 h postinfection. (B, D, F, and H) represent the corresponding phase-contrast images of the same fields as in (A, C, E, and G). Magnification, $\times 1,113$.

ose gels. The DNAs were transferred to nitrocellulose filters by using standard procedures, and the blots were hybridized at 42°C with nick-translated plasmid probes in a solution of 50% formamide, 50 mM HEPES (*N*-2-hydroxyethylpiper-

zine-*N'*-2-ethanesulfonic acid; pH 7.4), 1× Denhardt solution, 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and 150 µg of alkali-hydrolyzed salmon sperm DNA per ml. Blots were washed at 50°C in 0.1× SSC–0.1%

sodium dodecyl sulfate and exposed to X-ray film for 24 h (p β Pst), 4 days (p α HCG), and 2 h (pblur2). The probes used were: p β Pst, a 4.4-kilobase-pair *Pst*I genomic DNA fragment encoding a human β globin gene plus 3'- and 5'-flanking sequences cloned into pBR322 with a deletion between the *Ava*I site at 1429 and the *Pst*I site at 2490; p α HCG, a partial cDNA copy of mRNA encoding the α subunit of human chorionic gonadotrophin cloned into pBR322; and pblur2, a 300-base-pair human repetitive element cloned into the *Bam*HI site of pBR322.

RESULTS

Localization of the E1B 19K tumor antigen. Since intracellular localization of a protein can give clues to possible functions, HeLa cells lytically infected with wild-type adenoviruses were examined by indirect immunofluorescence with antipeptide antisera directed against the predicted carboxy-terminal amino acid sequence of the 19K protein. At early times (12 h) postinfection, weak fluorescent staining was observed at the nuclear periphery, as well as some faint staining of the cytoplasm (Fig. 1A). At 17 h postinfection, the cytoplasmic staining was more pronounced and reticular in appearance, in addition to an intense staining of the nuclear surface (Fig. 1C). This pattern of nuclear staining is characteristic of antibodies that stain the nuclear envelope, which can appear as a nuclear ring and depends upon the plane of focus. The cytoplasmic distribution of the 19K tumor antigen was indistinguishable from the staining pat-

tern seen when infected cells were stained with antibodies specific for the endoplasmic reticulum (ER) (data not shown). By 40 h postinfection, the 19K tumor antigen localized exclusively on the nuclear surface with no discernible staining of the cytoplasm (Fig. 1E). Identical immunofluorescence results were obtained with antipeptide antibodies directed against the predicted amino terminus of the E1B 19K antigen (data not shown), and both anti-19K protein antibodies stained only adenovirus-infected cells, giving background staining of mock-infected control cells (data not shown) or the occasional uninfected cells within the population of infected cells (Fig. 1C and E). The perinuclear distribution of the 19K tumor antigen in infected cells is very different from the distribution of staining seen with antibodies to the E1B 57K tumor antigen or the 72K DNA-binding protein, which stain structures in the nuclear interior (data not shown). These results are consistent with the localization of the 19K protein at the nuclear periphery, possibly the nuclear envelope.

The nuclear envelope of eucaryotic cells is the structural organelle which serves to separate and regulate the interaction between the nuclear components and the contents of the cytoplasm (for reviews, see references 11 and 21). The basic structure of the nuclear envelope consists of a double membrane system, where the outer nuclear membrane is continuous with the rough ER membranes and with the inner nuclear membrane at the site of the pore complexes. Subjacent to the inner nuclear membrane, and extending a short distance into the nuclear interior, is the lamina. The lamina is

FIG. 2. Distribution of the 19K tumor antigen during the cell cycle of transformed cells. 293 cells, plated on cover slips, were labeled with Hoechst dye #33258 (10 μ g/ml) for 4 h at 37°C when they were 50% confluent and then fixed and stained for indirect immunofluorescence with anti-19K protein or anti-lamin antibodies as described in the legend to Fig. 1. (B), (D), (F), and (H) represent DNA staining by the Hoechst dye and correspond to the 19K protein (19K) or lamin (LS1) immunofluorescence micrographs (A), (C), (E), and (G). (A and B) Interphase; (C and D) single cell in the center of each field represents metaphase; (E and F) anaphase; and (G and H) telophase stages of mitosis. Photographs were taken under epiillumination with a Leitz microscope. Magnification, $\times 752$.

a structural component of the nuclear envelope, being in close physical association with the inner surface of the inner nuclear membrane, and it also serves as an anchoring point for the nuclear pore complexes (for a review, see reference 15). Together with the nuclear membranes, the lamina and associated pore complexes cover the cortical area of the nucleus, and likely serve as an attachment point for DNA.

Very few nuclear envelope components have been characterized. An exception is a group of antigenically related phosphoproteins, termed lamins A, B, and C, which range between 60,000 and 70,000 daltons and are the major components of the nuclear envelope lamina (8, 16). Using human autoantibodies specific for lamins A and C (35), we have

compared the distribution of these known nuclear envelope proteins to that of the E1B 19K tumor antigen in lytically infected HeLa cells. The indirect immunofluorescence localization of the lamina polypeptides is identical to the localization of the E1B 19K tumor antigen at the level of the light microscope (Fig. 1G). Collectively, these results indicate that there is a change in the distribution of the E1B 19K tumor antigen during the course of adenovirus lytic infection, with its eventual accumulation at, or near, the nuclear envelope.

Distribution of the E1B 19K tumor antigen during the cell cycle. One prediction that could be made about the localization of the 19K protein, if it is a nuclear envelope protein, is

FIG. 3. Immunoperoxidase electron microscopic localization of the E1B 19K tumor antigen in lytically infected HeLa cells at 41 h postinfection. Electron micrographs of thin sections through nuclei of Ad2 (A to D and G) or mock-infected HeLa cells (E, F, and H) stained with anti-19K protein (A and G) or anti-lamin (C, E, and H) antibodies. For comparison, the control panels represent reactions with goat anti-rabbit (B) or rabbit anti-human (D and F) horseradish peroxidase-conjugated second antibodies alone. The nucleus (Nu), pore complex (P), virion (V), inner (in) and outer (out) nuclear membranes, and lamina (L), are indicated. The arrows in (G) and (H) indicate the reaction product at the nuclear surface and the asterisk in (G) marks an uninfected cell nucleus adjacent to infected cell nuclei in the same section. Magnifications: (A to F), $\times 86,432$; (G and H), $\times 7,147$.

that this localization should be disrupted with the disintegration of the nuclear envelope at mitotic prophase (9, 14, 16, 28). This cannot be tested in lytically infected HeLa cells which are no longer progressing through the cell cycle. But 293 cells, which are adenovirus-transformed human embryo kidney cells (18) and which constitutively express E1A and E1B gene products, are suitable cells for such analysis.

DNA of 293 cells was stained *in vivo* with Hoechst dye to identify the condensed chromosomes of cells at different stages of mitosis, and the cells were fixed and stained by indirect immunofluorescence with anti-E1B 19K protein or anti-lamin antibodies. Interphase 293 cells stained for the E1B 19K tumor antigen revealed ER-like cytoplasmic staining and a prominent nuclear ring (Fig. 2), similar to the pattern in lytically infected HeLa cells (Fig. 1C). During metaphase, by which time the nuclear envelope had broken down as determined by phase-contrast microscopy, nuclear envelope fluorescence was absent from cells stained with the anti-19K protein- or anti-lamin-specific antisera (Fig. 2, C panels). Anaphase mitotic cells were similar, except that some of the 19K protein had returned to the spindle pole sides of the chromosomes (Fig. 2, E panels). At telophase, there was a return of nuclear envelope fluorescence with both anti-19K protein and anti-lamin antibodies, commensurate with reassembly of the nuclear envelope (Fig. 2 G panels). From this experiment, it is clear that the distribution of the 19K tumor antigen is not the same as that of DNA, and in fact, the chromosomes exclude the stain, making its distribution clearly different from the histones and other chromatin-binding proteins. Most importantly, there is a direct correlation between the assembly and disassembly of the nuclear lamins and the distribution of the E1B 19K tumor antigen during the cell cycle of adenovirus-transformed cells, although with the 19K antigen, the diminution in staining as cells progress into mitosis is less obvious than with the lamina antigens due to the presence of additional 19K protein localized in the cytoplasm.

Ultrastructural localization of the E1B 19K tumor antigen. Localization of the E1B 19K tumor antigen by indirect immunofluorescence suggested that there was little 19K protein present in the nuclear interior and that it was found primarily in the envelope or around the perimeter of the nucleus. To establish the precise localization of the 19K tumor antigen, infected cells were stained by indirect immunoperoxidase labeling, and thin sections were viewed in the electron microscope.

Wild-type Ad2 or mock-infected HeLa cells were stained with anti-19K protein or anti-lamin antisera, followed by peroxidase-labeled second antibodies, and the reaction was developed with diaminobenzidine. In the electron microscope, the peroxidase reaction product appeared as an electron-dense precipitate at the site of the immune complexes. In infected and uninfected HeLa cells the anti-lamin antibodies stained the lamina (Fig. 3C, E, and H), a thick layer on the inner surface of the inner nuclear membrane, as previously demonstrated (16, 29). No major differences were seen in the distribution of lamins in adenovirus-infected cells versus the mock-infected control, although there were structural changes in the nucleus which were brought on by the viral infection, most notably an increase in the size and irregular shape of the nucleus. In contrast to the distribution of the lamins, the 19K tumor antigen localized exclusively to the inner and outer nuclear membranes of infected cells (Fig. 3A). There was no appreciable staining of the nuclear interior or pore complexes of infected cells stained with anti-19K protein antibodies, and no reaction with uninfected cells

that are in the population (Fig. 3G, asterisk) or in control experiments (data not shown). The lack of staining of the interior of the nucleus cannot be due to a lack of antibody penetration, since the anti-lamina antibodies were able to stain the more interior lamina. When the reactions were done with the second antibodies alone, no staining was observed in any of the sections (Fig. 3B, D, and F).

Distribution of the E1B 19K tumor antigen within the nuclear envelope substructure. To determine the relationship between the E1B 19K protein and the other components of the nuclear envelope, two different cell fractionation experiments were performed. With method I, the nuclear lamina and associated pore complexes were isolated from mock-infected or Ad2 infected HeLa cells by the method of Dwyer and Blobel (8). By immunofluorescence, virtually all the 19K protein was located in the nuclear envelope. Briefly, this involves treating purified nuclei in suspension with DNase, extracting with nonionic detergents to remove membranes, and finally, treating the nuclei with high salt to remove peripheral and chromosomal proteins (mainly histones). The remnants of these treatments are the structural components of the nuclear envelope containing the lamina, nuclear pore complexes, and associated proteins, with little DNA. The lamins were the major proteins in such preparations of both uninfected and infected cells (Fig. 4A, lanes c and d). There were however, differences between the protein composition of the lamina preparations from adenovirus-infected and mock-infected HeLa cells, and among these additional proteins found in the lamina of infected cells, a protein of 19,000 was detectable.

Similar results were obtained with the more rapid method II, which involves isolating the nuclear structural proteins by extraction of cells attached to tissue culture dishes (35). With method II, whole cells were extracted on a plastic surface with a nonionic detergent, followed by nuclease treatment and then high salt. There is enrichment for lamins as well as the cytoskeletal elements actin and vimentin which remain insoluble under these conditions. As with method I, additional proteins were found in the lamina in method II preparations of adenovirus- but not mock-infected cells (Fig. 4A, lane f).

The identity of the 19K protein found in the lamina of infected cells was confirmed as the E1B 19K tumor antigen by Western blotting with the anti-19K carboxy-terminal peptide antisera. The 19K tumor antigen was present in adenovirus-infected total cell lysates and in method I and II nuclear lamina preparations, but not in similar preparations from uninfected cells (Fig. 4B). In addition, the distribution of the E1B 19K protein was monitored by indirect immunofluorescence during these cell fractionation experiments. A fraction of the 19K protein remained associated with the nuclear envelope and lamina throughout method I (data not shown) and method II fractionations, and its distribution closely resembled that of lamina (Fig. 4D).

These experiments indicated that a portion of the nuclear envelope-associated 19K tumor antigen fractionated with the nuclear lamina subfraction of the envelope, but it was necessary to determine what percentage of the total cellular 19K protein this represented. The distribution of the 19K protein was monitored by using the rapid method II fractionation procedure from cells that had been infected for 40 h. The amount of 19K protein released by detergent, nuclease, and high-salt extraction, and the amount that remained insoluble in infected HeLa cells, was measured in quantitative Western blots (Fig. 4C). The initial detergent extraction solubilized 2% of the total 19K protein (Fig. 4C, lane h),

FIG. 4. Biochemical fractionation of the E1B 19K tumor antigen. (A) 15% polyacrylamide gel, stained with Coomassie blue, of mock- (lanes a, c, and e) or Ad2-infected HeLa cells (b, d, and f to m) fractionated as follows: lanes a and b, total protein; lanes c and d, method I nuclear pore complex-lamina fraction; lanes e and f, method II nuclear lamina plus cytoskeleton. Indicated in the margin are the lamins (a, b, and c), and the arrow marks the migration position of the E1B 19K tumor antigen in this gel system. The numbers to the left represent molecular weight standards ($\times 10^3$). (B) Western blot of a duplicate gel as in (A) probed with anti-19K protein antipeptide antibodies. (C) Western blot of Ad2 infected HeLa cells (40 h postinfection) with anti-19K protein antibodies of fractions from a method II nuclear lamina preparation; lane g, total cell protein; lane h, proteins released by a 5 min 0.1% Nonidet P-40 lysis; lane i, proteins released by micrococcal nuclease digestion; lane j, proteins released by a 5-min 2 M NaCl-extraction; lane k, proteins remaining insoluble throughout these treatments; lane l, as in lane k, except that the final 2 M NaCl extraction was for 20 min; lane m, 19K remaining insoluble as in lane k, except that the initial extraction was with 1% Nonidet P-40. (D) Indirect immunofluorescence of insoluble nuclear material, as in (C) (lane k), of Ad2-infected cells with anti-19K protein (a) or anti-lamin (c) antibodies and the corresponding phase-contrast images (b and d).

another 2% was rendered soluble by digestion with micrococcal nuclease (Fig. 4C, lane i), with the remaining bulk of the 19K protein divided equally between the high-salt-soluble fraction and the insoluble nuclear lamina (Fig. 4C, lanes j and k). When treated in parallel with the 19K protein, the lamins were not extractable by these treatments (data not shown), indicating a difference in the fractionation behavior between the 19K protein and lamins. The portion of the 19K protein retained in the lamina was very tightly associated, remaining insoluble after extraction with a 10-fold-higher detergent concentration or after prolonged extraction with 2 M NaCl (Fig. 4C, lanes l and m). In addition, when method II was used with 293 cells, more 19K protein was released by the initial detergent extraction consistent with the presence of some 19K protein in the cytoplasm of these transformed cells, although an appreciable amount remained within the insoluble lamina (data not shown).

In summary, early in lytic infection and in transformed cells, the E1B 19K protein localized in the nuclear envelope and in the cytoplasm, possibly with the membranes of the ER. Later in infection, the E1B 19K tumor antigen was found in both the inner and outer nuclear membranes of the nuclear envelope. Of the 19K protein, ca. one-half was

tightly bound to the nuclear lamina and presumably corresponds to the 19K protein which localized to the inner nuclear membrane.

Alterations in the E1B 19K tumor antigen cause chromosomal DNA degradation. Infection of HeLa cells with Ad2cyt106 mutant virus results in DNA degradation which is apparent in Hirt supernatants as a smear of low-molecular-weight DNA on an agarose gel (Fig. 5A, lane 9 and reference 48). Degraded DNA is not found in mock- or wild-type Ad2-infected cells (Fig. 5A, lanes 7 and 8). Previous experiments have demonstrated that the bulk of the degraded DNA is of cellular origin (7, 43, 48). We have investigated the molecular composition of this degraded DNA in Southern blots, using probes for two specific cellular genes that encode β globin and α human chorionic gonadotrophin (α HCG), as well as a probe for the human *Alu* repetitive element.

The β globin and α HCG probes hybridized to *Eco*RI restriction fragments of the expected sizes in total genomic DNA from mock-, Ad2-, and Ad2cyt106-infected HeLa cells (Fig. 5B and C, lanes 1 to 3). Under these conditions, the β globin probe also hybridized with a 2.2-kilobase δ globin fragment (12). The human *Alu* sequence probe detected a smear of DNA in the whole genomic digests as expected.

FIG. 5. Degradation of cellular DNA induced by the adenovirus E1B mutant Ad2cyr106. Agarose gel electrophoresis of total chromosomal and Hirt supernatant DNAs isolated from mock- (lanes 1, 4, and 7), Ad2- (lanes 2, 5, and 8), and Ad2cyr106- (lanes 3, 6, and 9) infected HeLa cells. Total chromosomal DNA was cut with *Eco*RI (lanes 1 to 3), and Hirt DNAs were either digested with *Eco*RI (lanes 4 to 6) or run undigested (lanes 7 to 9). (A) Ethidium bromide-stained gel; (B to D) Southern blots of identical gels hybridized with probes: (B) β globin (p β Pst); (C) α HCG cDNA (p α HCG); (D) *Alu* repeat (pblur2). p β Pst hybridizes to form 5.2-, 3.6-, and 2.2-kilobase-pair fragments of *Eco*RI-digested human DNA (12), and p α HCG hybridizes to a 5-kilobase-pair *Eco*RI fragment (10). The pblur2 300-base-pair repetitive element hybridizes to *Alu* repeat sequences throughout the genome (38). The asterisks indicate cross-hybridization of pBR322 sequences with mitochondrial DNA that is greatly enriched in the Hirt supernatant DNAs, and in lane 7 of (B) and (C), the supercoiled (lower band) and relaxed circle (upper band) forms can be seen. This cross-hybridization can be eliminated by using more stringent washing conditions (55°C).

The same pattern of hybridization is seen in *Eco*RI digests of Ad2cyr106 Hirt supernatant DNAs, indicating that these sequences are represented in the degraded DNA (Fig. 5B, C, and D, lane 6). No lower-molecular-weight or unexpected bands were generated by the Ad2cyr106-induced DNA deg-

radation which would be indicative of nuclease-hypersensitive sites. Specific bands corresponding to either β globin or α HCG sequences could not be detected in Hirt supernatants that had not been digested with *Eco*RI, presumably because the sequences were present in high-molecular-weight frag-

FIG. 6. Localization of the E1B 19K tumor antigen in E1B mutant-infected cells. HeLa cells infected with the E1B 19K protein mutants at 50 PFU per cell were fixed and stained by indirect immunofluorescence with the anti-19K protein antipeptide antibodies at 40 h postinfection as described in the legend to Fig. 1. (A) Ad2ts111, (B) Ad2cyr106, (C) Ad2lp3, and (D) Ad2lp5. Not shown is deletion mutant Ad5dl337 which gave only background fluorescence because of the lack of detectable 19K gene product. Magnification, $\times 671$.

ments that could not be resolved on these gels. This suggests that the *in vivo* cutting sites were sufficiently removed from these genes so that abnormal bands could not be generated. Hybridization of β globin sequences in a region of the gel corresponding to the high-molecular-weight DNA was detected (Fig. 5B, lane 9) and might represent such fragments. Another possibility is that the nuclease cleavage in the degraded DNA is totally random and that fragments of a discrete length containing these genes are not produced. *Alu* sequences could be detected in Hirt supernatant DNAs that had not been digested *in vitro* (Fig. 5D, lane 9), but because of the repetitive nature of this DNA, no specific bands could be detected. Moreover, no significant hybridization with any of the probes, including the *Alu* probe, could be detected in mock- or Ad2-infected cell Hirt supernatant DNAs (Fig. 5B, C, and D, lanes 4, 5, 7, and 8), because the DNA degradation only occurs in the mutant-infected cells. In all lanes of Hirt supernatant DNAs in Fig. 5B and C, hybridization to the mitochondrial DNA was observed and was due to hybridization with plasmid vector sequences (asterisk in Fig. 5B and C). The mitochondrial DNA was not present in sufficient quantities to detect this hybridization in total genomic digests (Fig. 5B, and C, lanes 1 to 3).

Distribution of the E1B tumor antigen in adenovirus mutant-infected cells. The intracellular localization of the E1B 19K tumor antigen was examined in HeLa cells infected with the E1B 19K mutants, of which all but Ad2/p3 result in the degradation of chromosomal DNA. The distribution was followed by indirect immunofluorescence with the 19K antipeptide antibodies at 17 and 40 h postinfection. At 17 h postinfection, the distribution of the E1B 19K protein, the E1B 57K protein, the lamins A and C, and the E2A DNA-binding protein was not significantly different from the distribution of these proteins found in wild-type-infected cells (data not shown). By 40 h postinfection, however, the distribution of 19K protein in all but Ad2/p3 was drastically altered (Fig. 6). In cells infected with Ad2ts111, Ad2cyr106, and Ad2/p5, the 19K protein appeared in fragmented structures, separated from the nuclear envelope. This altered distribution of the E1B 19K protein in mutant-infected cells correlated with the degradation of cellular DNA and also occurred at the same time postinfection at which DNA degradation was observed. The distribution of lamins A and C was normal in the mutant-infected cells, as was the distribution of the E1B 57K protein and the 72K DNA-binding protein (data not shown). Fluorescence was not seen with Ad5dl337 (data not shown), since it does not make a detectable 19K gene product.

DISCUSSION

At intermediate times postinfection with wild-type adenovirus, the 19K tumor antigen was localized in both the nuclear envelope and the cytoplasm, and at later times, the localization was exclusively in the nuclear envelope. By indirect immunofluorescence, the cytoplasmic distribution was identical to that of the ER in infected cells. Consistent with these results, Persson et al. (37) have reported that at intermediate times in infection, the E1B 19K protein localized to cytoplasmic membranes and to the nucleus. The 19K protein is not a typical membrane protein in that no signal sequence is evident from the predicted amino acid sequence, derived from the DNA sequence (17). The 19K protein is not labeled with tritiated sugar residues and is therefore not likely to be glycosylated (37). There are, however, internal hydrophobic domains which may be sites of membrane interaction. Since the rough ER is continuous with the outer

and inner nuclear membranes, the 19K protein probably remains attached to the cytoplasmic membranes as it is transported from the ER to the nuclear envelope and the lamina. This could occur by incorporating ER-19K protein complexes directly into the nuclear envelope.

By late times in infection, there has been a complete translocation of the 19K protein to the nuclear envelope, where it is found in both the inner and outer nuclear membranes. Biochemical fractionation demonstrated a close association of half of the 19K tumor antigen with the lamina in infected cells. This probably represents the fraction of the 19K protein located in the inner nuclear membrane, since it is the inner nuclear membrane that is intimately associated with the lamina. Lamin B for example, is an integral inner nuclear membrane protein (31) as well as a lamina protein and is thought to be a link between these two structures.

In transformed cells the 19K is localized in both the cytoplasmic membranes and the nuclear envelope (this report; unpublished experiments; 37). Like the nuclear lamins, the 19K tumor antigen is reversibly disassembled and assembled during mitosis in transformed cells, coincident with nuclear envelope breakdown and reformation. Hyperphosphorylation of the lamins occurs at the time of lamina disassembly; this process may regulate the state of polymerization of the lamins (14). Lamin B remains associated with membranes when the envelope is disassembled, whereas lamins A and C are soluble (14); however, the fate of the 19K protein during mitotically induced nuclear envelope breakdown is not known.

Although the adenovirus E1B 19K tumor antigen is in an interesting location in the nuclear envelope, the function of this tumor antigen cannot be determined by this localization alone. We have therefore examined viral mutants which carry lesions in the gene which encodes this protein. Examination of such mutants has established that they cause the degradation of chromosomal DNA and enhanced cytopathic effect in infected cells (48a). Mutants which produce an altered E1B 19K tumor antigen gene product, owing to amino acid substitutions in the protein (Ad2ts111, Ad2cyr106, and Ad2/p5), or a mutant that does not synthesize the 19K gene product at all because of an out-of-frame deletion (Ad5dl337) have the two phenotypes *cyt* and *deg*. In these mutants, intracellular localization of the 19K tumor antigen is either grossly perturbed or the tumor antigen is totally absent. Another mutant that carries a point mutation in the E1B 19K gene is Ad2/p3. This mutant has the *cyt* phenotype but does not cause the degradation of chromosomal DNA (*deg*⁺) and the localization of the E1B 19K protein in Ad2/p3 is indistinguishable from that of the wild-type virus. Thus, there is a direct correlation between an aberrant localization of the 19K tumor antigen or its absence from the nuclear envelope in cells infected by these adenovirus mutants and the degradation of chromosomal DNA. The 19K protein, however, is not directly responsible for this DNA degradation because Ad5dl337, which does not produce a 19K gene product, has the DNA degradation phenotype. We have postulated that another viral or cellular protein which normally interacts with the E1B 19K protein is directly responsible for degrading chromosomal DNA in the absence of the 19K protein, and we are currently searching for the protein(s) responsible. These results also suggest that the presence of the 19K protein in the nuclear envelope is important, directly or indirectly, for maintaining the structural integrity of the interphase DNA during lytic infection.

It has been proposed that nuclear structural components function in the topological organization of interphase DNA

in uninfected cells for important cellular events such as DNA replication and transcription (for review, see references 20 and 26). Evidence supporting the role of nuclear structure in the organization of interphase chromatin is mainly indirect. First, structural proteins at the nuclear periphery, most probably the lamins, may determine the structure of interphase chromatin because they interact with DNA (15, 20, 32). Second, the dissociation of the lamins correlates with chromosome condensation at the onset of mitosis and meiosis, and the lamins reassociate before reconstruction of the nuclear envelope and the reestablishment of the interphase organizational state of the chromatin (9, 16, 28, 42). Third, specific loci of *Drosophila* polytene chromosomes have been found to be associated with the nuclear envelope in vivo at discrete points (34). Finally, a cellular protein called perichromin, which is associated with the condensed chromosomes during mitosis, is also associated with the nuclear envelope at interphase and has been implicated in the organization of chromatin (36). These results suggest that interphase chromatin of uninfected cells is spatially organized by the nuclear envelope and its associated structures. This organization may determine the metabolic fate of DNA sequences at interphase. Our finding that a nuclear envelope-associated virus protein is required to maintain the structural integrity of cellular DNA in infected cells is consistent with the proposed role of the nuclear envelope in organizing chromatin. Whether the nuclear envelope-associated tumor antigen influences cell DNA transcription or replication will be of particular interest.

The function of the E1B 19K tumor antigen is still not known. Since the protein is required for tumorigenicity in vivo (3), our results suggest that components of the ER or nuclear envelope play an important role in the biochemical pathway leading to stable transformation of cells. It will be of interest to determine the function of the protein in these structures and whether any cellular oncogenes have a similar localization and function.

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