Biosynthesis and Properties of the Adenovirus 2 L1-Encoded 52,000- and 55,000- M_r Proteins

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The adenovirus type 2 L1 region, which is located at 30.7 to 39.2 map units on the viral genome, is transcribed from the major late promoter during both early and late stages of virus replication, and a 52,000- M_r (52K) protein-55K protein doublet has been translated *in vitro* on L1-specific RNA. To investigate the biosynthesis and properties of the L1 52K and 55K proteins, we prepared antibody against a synthetic peptide encoded near the predicted N terminus. As determined by immunoprecipitation and immunoblot analysis, the antipeptide antibody recognized major 52K and 55K proteins synthesized in adenovirus type 2-infected cells that appeared to be identical to the 52K-55K doublet translated *in vitro*. The immunoprecipitated 52K and 55K proteins were very closely related, as shown by a peptide map analysis. Both L1 proteins were phosphorylated, and they were phosphorylated at similar sites. No precursor-product relationship was detected between the 52K and 55K proteins by a pulse-chase analysis. Biosynthesis of the L1 52K and 55K proteins began about 6 to 7 h postinfection, after biosynthesis of the early region 1A and early region 1B 19K (175R) T antigens, and reached a maximum rate at about 15 h; the maximum rate was maintained until at least 25 h postinfection. At all times, the 55K protein appeared to be synthesized at a severalfold-higher level than the 52K protein. Both proteins were quite stable and accumulated until late times after infection. Viral DNA replication was not essential for formation of the L1 proteins. Thus, the L1 52K-55K gene appears to be regulated in a manner different from the classical early and late viral genes but similar to the protein encoded by the i-leader (Symington et al., *J. Virol.* 57:849-856, 1986). The L1 proteins were detected in the cell nucleus by immunofluorescence microscopy with antipeptide antibody and were found to be primarily associated with the nuclear membrane by an immunoblot analysis of subcellular fractions.

Two temporal stages of viral gene expression, early and late, have been described for productive infection of KB (HeLa) cells by adenovirus type 2 (Ad2); early viral genes are expressed prior to the onset of viral DNA replication at 6 to 7 h postinfection, and late viral genes are expressed subsequently (12). Six separate regions of the viral genome are transcribed during the early stage of Ad2 productive infection; these are early region 1A (E1A), E1B, E2, E3, E4, and late region 1 (L1) (for a review, see reference 33). The early viral genes encode proteins that appear to play primarily regulatory roles in virus replication (for a review, see reference 28). The proteins encoded by E1 and E2 have been well studied. There are six major E1A- and E1B-encoded proteins (the T antigens). These proteins perform functions that regulate gene expression and that can lead to cell transformation (for a review, see reference 10). The three E2-encoded proteins play important roles in the replication of viral DNA (for a review, see reference 16). The functions of the proteins encoded by E3 and E4 are poorly understood. E3 can code for up to eight proteins based on the occurrence of open translation reading frames (5). Only three E3-encoded proteins have been identified thus far, and two of these proteins are membrane proteins (28, 40). E3 appears to be dispensable for virus replication in cultured cells, but may play a role during natural infections in humans. From 8 to 16 putative E4 proteins have been identified by cell-free translation (20, 39). However, only two E4 proteins have been detected in productively infected cells, an 11,000- M_r (11K) or 14K protein that is found in the cell nucleus (6, 32) and a 25K protein that has been reported to be associated with the E1B 495R T antigen (31). Expression of the Ad12 E4 region

has been reported to be required for the establishment of soft-agar colony-forming rat cell lines transformed by Ad12 (35); these findings suggest that an E4 gene product may enhance virus-induced cell transformation. Recent studies have indicated that some of the E4-encoded proteins may have regulatory roles in the expression of other early genes (9).

Most late viral genes are organized into five 3'-coterminal families, L1 through L5, that are abundantly transcribed from the common major late promoter at 16.8 map units during late times after infection. The late genes encode mainly viral structural proteins, as well as several nonstructural proteins having poorly defined functions (28). The major late promoter is also active early after infection and selectively expresses mRNA encoding a 52K-55K doublet, as demonstrated by *in vitro* translation (1, 3, 17, 19, 21, 24, 34). To determine whether the 52K and 55K proteins are synthesized during productive infection and to further study the properties and functions of these proteins, we prepared a strong antibody to a synthetic peptide encoded near the N terminus of the 52K-55K gene, as predicted from the DNA sequence (1, 8, 30). We determined the time course of synthesis of the 52K and 55K proteins and found that both proteins are phosphorylated and that these proteins are closely related, relatively stable, and do not appear to have a precursor-product relationship. They are primarily localized in the nucleus or are associated with the nuclear membrane or both.

MATERIALS AND METHODS

Preparation of antipeptide antibody targeted to the L1 52K and 55K proteins. Peptide 406 (Tyr-Met-Arg-Pro-Pro-Gln-Gln-Arg-Gln-Glu), which encodes a 10-amino acid

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sequence that includes amino acids at positions 8 through 17 from the N terminus of the open translational reading frame of the putative Ad2 52K-55K gene at about 30 map units (1, 8, 30), was synthesized by the solid-phase procedure (11). Tyrosine was placed at the N terminus to facilitate coupling by diazotization to bovine serum albumin (2). We raised antibodies to peptide 406 coupled to bovine serum albumin in rabbits by using previously described methods (11). Peptide 34 (Gly-Glu-Glu-Phe-Val-Leu-Asp-Tyr-Val-Glu-His-Pro-Gly-His-Gly-[Cys]), which is directed to the unique region of the Ad2 E1A 289R T antigen, was also synthesized by the solid-phase procedure, and antibodies were prepared in rabbits (Symington and Green, unpublished data). The preparation of anti-peptide 12 antibody targeted to the E1B 19K (175R) protein has been described previously (11).

Preparation of [^{35}S]methionine-labeled, $^{32}\text{P}_i$ -labeled, and unlabeled extracts of Ad2-infected cells for immunoprecipitation and for immunoblot analysis. KB cells were grown in suspension cultures in Joklik modified Eagle minimal essential medium containing 5% horse serum and were infected with 50 to 200 PFU of Ad2 strain 38-2 per cell (13). For labeling with [^{35}S]methionine, 10 ml of cells (4×10^6 to 5×10^6 cells) was centrifuged at $300 \times g$ for 5 min at various times postinfection, washed twice with methionine-free Eagle minimal essential medium containing 2% dialyzed horse serum, and labeled with 400 μCi of [^{35}S]methionine (1,100 Ci/mmol) for 1 h. For labeling with $^{32}\text{P}_i$, at 15 h postinfection 6-cm dishes containing cells were washed twice with P_i -free medium containing 2% dialyzed calf serum and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid) (pH 7.1) and labeled with 5 mCi of $^{32}\text{P}_i$ for 3 h. The cells were centrifuged, washed with phosphate-buffered saline containing 0.1% Trasylol, solubilized by sonication in 3 ml of cell sonication buffer (20 mM Tris hydrochloride, pH 7.4, 10% glycerol, 50 mM NaCl, 5 mM EDTA, 1 mM 2-mercaptoethanol, 1% deoxycholate, 0.5% Nonidet P-40, 0.5 M urea, 0.1% Trasylol, 1 mM phenylmethylsulfonyl fluoride), and clarified by centrifugation at $100,000 \times g$ for 60 min.

For pulse-chase labeling, monolayers of KB cells were grown on 6-cm petri dishes and infected with 200 PFU of Ad2 per cell. After 1 h for virus absorption, medium containing 2% agamma calf serum was added. At 13 h postinfection, the cells were washed with methionine-free Eagle minimal essential medium and labeled with 200 μCi of [^{35}S]methionine per plate for 30 min; after this, the plates were harvested immediately for no chase or washed and allowed to incubate in unlabeled medium for various times before harvesting. The cells were washed, scraped, centrifuged, and solubilized as described above.

When cells were to be treated with arabinofuranosylcytosine (araC) to prevent DNA synthesis, they were grown in monolayers and infected with 200 PFU of Ad2 per cell. At 1 h postinfection, medium containing 20 μg of araC per ml was added, and this medium was renewed after 12 h. The cells were washed in methionine-free medium, labeled with [^{35}S]methionine from 25 to 26 h postinfection, and harvested as described above.

For immunoblot analysis, unlabeled Ad2-infected cells were centrifuged and washed as described above at various times after infection. A total of 5×10^6 cells were lysed by adding 400 μl of boiling sodium dodecyl sulfate (SDS) sample buffer (0.125 M Tris hydrochloride, pH 6.8, 5% 2-mercaptoethanol, 3% SDS, 12.5% glycerol, 0.001% bromophenol blue) and heating the preparation for 10 min at 95°C prior to electrophoresis.

In vitro translation. Cytoplasmic RNA isolated from KB cells infected with Ad2 at 21 h postinfection was hybridized to Ad2 *Hind*III fragment B DNA (17.0 to 31.5 map units) that was immobilized on a nitrocellulose filter. The RNA was eluted and translated for 60 min at 30°C with 20 μCi of [^{35}S]methionine per tube in a rabbit reticulocyte system by using a translation kit (New England Nuclear Corp.).

Immunoprecipitation analysis. Portions of extracts from [^{35}S]methionine-labeled or $^{32}\text{P}_i$ -labeled infected cells containing 5×10^5 trichloroacetic acid-insoluble cpm or portions from in vitro translation mixtures containing 2×10^5 trichloroacetic acid-insoluble cpm were immunoprecipitated with 5 to 20 μl of antipeptide antiserum in a 1-ml reaction volume (14). For competition studies, the peptide used to generate the antipeptide antibody was included in the reaction mixture at a concentration of 10 $\mu\text{g}/\text{ml}$. Immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis (14).

Cell fractionation. KB cells infected with Ad2 or mock-infected cells were harvested at 22 h postinfection by washing the preparations twice in phosphate-buffered saline containing 0.1% Trasylol. The cells were ruptured by Dounce homogenization, and the components were separated by using a dextran-polyethylene glycol two-phase system and a 1% Triton X-100 nuclear wash as described previously (15, 38).

Immunoblot analysis. Samples were electrophoresed on SDS-polyacrylamide gels containing 12.5% acrylamide and 0.12% bisacrylamide. After gel electrophoresis, the gels were equilibrated with transfer buffer for 5 min and used for protein transfer analysis as described previously (37, 38).

Indirect immunofluorescence. KB cells were grown on cover slips for 1 day prior to infection with 200 PFU of Ad2 per cell. At various times postinfection, the cells were rinsed with cold phosphate-buffered saline and cold methanol and fixed with methanol for 10 min at -20°C . The cells were stained with antipeptide antiserum and photographed as described elsewhere (38).

RESULTS

Preparation of antibody directed to a peptide near the N termini of the hypothetical Ad2 L1 52K and 55K proteins. To prepare an immunological reagent suitable for investigating the properties and synthesis of the L1 52K and 55K proteins, we prepared antibodies targeted to a 10-amino acid sequence that is located between amino acid residues 8 and 17 of the 52K and 55K proteins, as deduced from the Ad2 DNA sequence (nucleotides 11061 to 11090), and contains three proline, one glutamic acid, and two arginine residues (8, 30). Tyrosine was added at the N terminus of peptide 406 to facilitate coupling of the peptide to bovine serum albumin with diazotized benzidine (2), and antibodies were generated in rabbits as described in Materials and Methods. Enzyme-linked immunosorbent assay titers of 1:640 were obtained after 4 months, and titers of 1:800,000 were obtained after 6 months. The 52K and 55K proteins could be detected by radioimmunoprecipitation of infected cell extracts with serum obtained after 4 months (see below).

To determine whether anti-peptide 406 antibody recognized the authentic 52K and 55K proteins, we prepared an in vitro translation mixture programmed with L1-specific RNA that was hybridization selected from 21-h Ad2-infected cytoplasmic RNA. This translation mixture should have included the L1 proteins, based on previous reports (1, 19, 21). Immunoprecipitation of the translation mixture with anti-peptide 406 antibody yielded a prominently labeled doublet

at M_r s of about 52,000 and 55,000, which presumably represented the L1 proteins (Fig. 1A, lane 2).

To determine whether the L1 52K and 55K proteins that are observed in *in vitro* translation mixtures (1, 19, 21) were indeed present in infected cells and whether they could be detected with anti-peptide 406 antibody, we immunoprecipitated extracts of [35 S]methionine-labeled Ad2-infected KB cells (20 h postinfection) with anti-peptide antibody in the presence and absence of peptide 406. A major band at 52K to 55K was immunoprecipitated by anti-peptide 406 antibody (Fig. 1B, lane 1); a shorter exposure of the X-ray film showed that the band was a doublet (data not shown). Incubation with peptide 406 completely blocked immunoprecipitation of the 52K and 55K proteins (Fig. 1B, lane 2), whereas an unrelated peptide had no effect (Fig. 1B, lane 3), thus confirming the specificity of the immunoprecipitation reaction and providing evidence that the immunoprecipitated species were the L1 proteins.

Close relationship between L1 52K and 55K proteins as established by two-dimensional tryptic maps of [35 S]methionine-labeled proteins. An extract of [35 S]methionine-labeled Ad2-infected KB cells was immunoprecipitated with anti-peptide 406 antibody, and the 52K and 55K species were resolved by SDS-polyacrylamide gel electrophoresis (Fig. 2, lane 1). Immunoprecipitation of the 52K and 55K bands, but not of the contaminating bands, was blocked by inclusion of peptide 406 in the immunoprecipitation reaction mixture (Fig. 2, lane 2). Each of the polypeptide bands was digested with trypsin and peptide mapped by two-dimensional elec-

FIG. 1. Immunoprecipitation with anti-peptide 406 antibody of Ad2 52K and 55K proteins from *in vitro* translation mixtures (A) and from extracts of Ad2-infected KB cells (B). (A) Cytoplasmic RNA from Ad2-infected KB cells at 21 h postinfection was hybridized selected with Ad2 *Hind*III fragment B (17.0 to 31.5 map units) and translated *in vitro* by using the rabbit reticulocyte system and [35 S]methionine as the labeled amino acid, as described in Materials and Methods. The translation mixture was immunoprecipitated with anti-peptide 406 antibody, and the precipitate was electrophoresed on a 12.5% polyacrylamide gel and visualized by autoradiography. Lane 1, 14 C-labeled protein markers; lane 2, proteins translated *in vitro* and immunoprecipitated with anti-peptide antibody. (B) Immunoprecipitation with anti-peptide 406 antibody of extracts of KB cells labeled from 20 to 21 h postinfection. Lane 1, anti-peptide 406 antibody; lane 2, competition with peptide 406; lane 3, competition with an unrelated peptide.

FIG. 2. Immunoprecipitation of [35 S]methionine- and 32 P-labeled Ad2 L1 52K and 55K proteins from extracts of Ad2-infected cells with anti-peptide 406 antibody and competition with peptide 406. An Ad2-infected suspension culture of KB cells was labeled with [35 S]methionine from 15 to 16 h postinfection. A 60-mm monolayer of Ad2-infected cells was labeled with 32 P_i from 15 to 18 h postinfection. Cell extracts were prepared, immunoprecipitated with anti-peptide 406 antibody in the presence or absence of peptide 406, and electrophoresed on an SDS-polyacrylamide gel as described in Materials and Methods. Lanes 1 and 3, Immunoprecipitates prepared in the absence of peptide 406; lanes 2 and 4, immunoprecipitates prepared in the presence of peptide 406.

trophoresis and chromatography. The theoretical number of methionine-containing tryptic peptides encoded by the L1 52K-55K gene predicted from the DNA sequence is nine (8, 30). Very similar patterns of about eight or nine methionine-containing tryptic peptides were observed for the 55K and 52K proteins (Fig. 3A and B, respectively). Thus, the 52K and 55K proteins are very closely related species, differing from each other in an unknown manner.

Phosphorylation of the L1 52K and 55K proteins. The L1 52K and 55K proteins could represent different posttranslational forms of the same primary gene product. Since biologically important proteins are often posttranslationally modified by phosphorylation, we performed experiments to determine whether the L1 proteins were phosphorylated. Ad2-infected KB cells were metabolically labeled with 32 P_i from 15 to 18 h postinfection, and extracts were prepared and immunoprecipitated by using anti-peptide 406 antibody. Two phosphorylated species which comigrated with [35 S]methionine-labeled 52K and 55K proteins were resolved by SDS-polyacrylamide gel electrophoresis (Fig. 2, lane 3). Immunoprecipitation of both phosphorylated species was blocked by peptide 406 (Fig. 2, lane 4), providing evidence that the phosphorylated species were indeed the 52K and 55K proteins. We found that there was quantitatively a severalfold-higher level of [35 S]methionine-labeled and 32 P_i-labeled 55K protein than of 52K protein; similar results were obtained with increasing levels of anti-peptide antibody, indicating that the anti-peptide antibody was present in excess. The higher rate of synthesis of the 55K protein was observed also in pulse-chase experiments and at different times after infection (see below).

To determine whether phosphorylation occurs at the same sites in the 52K and 55K proteins, we performed a phospho-

FIG. 3. Two-dimensional tryptic peptide maps of [^{35}S]methionine- and $^{32}\text{P}_i$ -labeled Ad2 L1 52K and 55K proteins isolated by immunoprecipitation with anti-peptide 406 antibody. Immunoprecipitates were prepared from extracts of Ad2-infected KB cells as described in the legend to Fig. 2. The labeled 52K and 55K bands were excised from the gel, digested with trypsin, and peptide mapped as described in Materials and Methods. (A) [^{35}S]methionine-labeled 55K protein. (B) [^{35}S]methionine-labeled 52K protein. (C) $^{32}\text{P}_i$ -labeled 55K protein. (D) $^{32}\text{P}_i$ -labeled 52K protein.

peptide mapping analysis of the individual immunoprecipitated bands of the L1 proteins. The patterns of tryptic phosphopeptides on the two-dimensional autoradiograms were virtually the same for the 55K and 52K proteins (Fig. 3C and D, respectively). Thus, within the limits of phosphopeptide mapping, differences in phosphorylation do not appear to account for the two forms having different molecular weights observed for the L1 proteins.

Pulse-chase analysis of L1 52K and 55K protein synthesis. To obtain evidence as to whether a precursor-product relationship exists between the 52K and 55K proteins, Ad2-infected KB cells were pulse-labeled at 13 h postinfection for 30 min and chased with unlabeled methionine for 60, 120, and 180 min. Extracts were prepared and analyzed by immunoprecipitation with anti-peptide 406 antibody. For comparison, synthesis of the Ad2 E1A 289R T antigens was analyzed with the same extracts by using anti-peptide 34, which is targeted to the unique region of the 289R protein encoded by the E1A 13S mRNA (unpublished data). The multiple E1A T antigen species with apparent M_r s of 47,000 to 52,000 were prominent after a 30-min pulse and no chase (Fig. 4A, 0 min). The level of radioactive T antigen decreased dramatically with increasing chase times due to the higher turnover rate of the E1A T antigens (Fig. 4A) (36; Lucher, Blackman, Symington, and Green, *J. Virol.*, in press). In contrast, nearly the same levels of labeled L1 52K and 55K proteins were found after chase times of 60, 120, and 180 min (Fig. 4B). Furthermore, the same relative amount of each protein was observed at each time point (Fig.

4B). Thus, these experiments provided no evidence for the existence of a precursor-product relationship between the two L1 proteins.

Rate of synthesis of the Ad2 L1 52K and 55K proteins during productive infection. Extracts of Ad2-infected KB cells labeled with [^{35}S]methionine for 1 h at various times after infection were immunoprecipitated with anti-peptide 406 antibody. To relate the time course of synthesis of the L1 proteins to the time course of synthesis of typical early viral proteins, the same extracts were immunoprecipitated with anti-peptide 34 antibody directed to the unique region of Ad2 E1A 289R T antigen (unpublished data) and with anti-peptide 12 antibody directed to the N terminus of the Ad2 E1B 19K (175R) T antigen (11). The synthesis of E1A T antigens was first detected at 2 to 3 h postinfection (faint band) and reached a maximum rate at about 4 to 5 h; this maximum rate was maintained until at least 10 h postinfection. Synthesis decreased thereafter, becoming negligible by 15 h postinfection (Fig. 5A). The synthesis of the 19K 175R T antigen was first detected at 4 to 5 h postinfection, was maintained at high levels up to at least 15 h, and decreased somewhat at 20 and 25 h postinfection (Fig. 5B). The synthesis of the L1 52K and 55K proteins followed a different pattern. Both proteins were barely discernable at 6 to 7 h (Fig. 5C), but were detected readily at 8 to 9 h postinfection. The maximum rate of synthesis of the L1 proteins occurred at 15 h postinfection and was essentially unchanged at 20 and 25 h postinfection (Fig. 5C).

Accumulation of the L1 52K and 55K proteins during productive infection. The relative levels of accumulation of the L1 proteins were estimated from 2 to 25 h postinfection

FIG. 4. Pulse-chase analysis of the Ad2 L1 52K and 55K proteins. KB cells were labeled with [^{35}S]methionine for 30 min at 13 h postinfection, and portions were chased with an excess of unlabeled methionine for 60, 120, and 180 min. Extracts of cells were immunoprecipitated with anti-peptide 406 antibody directed to the 52K and 55K proteins or with anti-peptide 34 antibody directed to the unique region of the E1A 289R T antigen.

by an immunoblot analysis in which anti-peptide 406 antibody was used. For comparison and as a control, the accumulation of the E1B 175R T antigen was determined with the same extracts by using anti-peptide 12 antibody. The E1B T antigen was detected at 6 h postinfection and accumulated up to 20 to 25 h postinfection (Fig. 6A). Sufficient levels of L1 proteins accumulated to be readily observed at 15 h postinfection by immunoblot analysis (low levels were often seen at 10 h postinfection) (Fig. 6B). Approximately constant levels of the L1 proteins were found between 15 and 25 h postinfection.

Effect of an inhibitor of viral DNA replication, araC, on the synthesis of the L1 proteins. To determine whether viral DNA replication was required for the synthesis of the 52K and 55K proteins, Ad2-infected KB cells were maintained in the presence of araC from 1 to 26 h postinfection and were labeled with [35 S]methionine from 25 to 26 h postinfection. A cell extract was prepared and immunoprecipitated with (i) anti-peptide 406 antibody to measure the synthesis of the L1 proteins and (ii) anti-peptide 12 antibody to measure the synthesis of E1B 19K 175R T antigen which served as a control for possible toxicity by the drug. The same levels of the E1B 19K 175R T antigen were synthesized in the absence and presence of the drug (Fig. 7). Low but significant levels of the 52K and 55K proteins were synthesized in the presence of araC (Fig. 7). In other experiments and in an

FIG. 5. Time of synthesis of the Ad2 L1 52K and 55K proteins, E1A 289R T antigens, and E1B 175R T antigen during productive infection of KB cells with Ad2. Extracts of Ad2-infected KB cells were labeled for 1-h periods at 2, 4, 6, 8, 15, 20, and 25 h postinfection and immunoprecipitated with antipeptide antibodies specific for the 52K and 55K proteins (C), the Ad2 E1B 175R T antigen (B), and the Ad2 289R T antigen (A), as described in Materials and Methods.

FIG. 6. Accumulation of Ad2 L1 52K and 55K proteins and the E1B 175R T antigen during productive infection of KB cells with Ad2, as determined by an immunoblot analysis with anti-peptide 406 antibody. (A) Extracts of KB cells prepared at different times after infection with Ad2 were electrophoresed on a 12.5% SDS-polyacrylamide gel, transferred to nitrocellulose, and treated with anti-peptide 406 serum and then with 125 I-labeled protein A, as described in Materials and Methods. After autoradiography, the immunoblot was probed with anti-peptide 12 antibody directed to the Ad2 E1B 175R T antigen. (B) Densitometer scan of the relative concentrations of the 52K and 55K proteins in the autoradiogram shown in panel A. Vertical axis represents percent of total 52K and 55K proteins found at various times postinfection; 25-h value normalized to 100%.

immunoblot analysis, higher levels of 52K and 55K proteins were formed in the presence of araC.

Localization of the L1 52K and 55K proteins within infected cells by immunofluorescence microscopy of fixed cells and by immunoblot analysis of cell fractions using anti-peptide 406 antibody. To localize the L1 proteins within Ad2-infected cells by immunofluorescence microscopy, cover slip preparations of KB cells at 15 h postinfection were incubated with anti-peptide 406 antibody. The nuclear region of the infected cells was stained predominantly by antipeptide antibody (Fig. 8A). Preincubation of the antibody with peptide 406 largely eliminated the staining of the nucleus (Fig. 8B), providing evidence that the nuclear localization was specific for the L1 proteins. As a control for immunofluorescence which might have been due to cross-reactions with other proteins (e.g., the host protein, which was visible on an immunoblot (Fig. 9), cells which had been infected for just 2 h (before synthesis of 52K and 55K began) were treated. These cells showed only a faint general glow with anti-peptide 406 antibody (data not shown). It is significant that

FIG. 7. Synthesis of the Ad2 L1 52K and 55K proteins and the E1B 175R T antigen in the presence and absence of araC. Ad2-infected KB cells were maintained in the presence or absence of 20 μ g of araC per ml from 1 h postinfection and were labeled with [35 S]methionine from 25 to 26 h postinfection. Extracts containing 5×10^6 cpm and representing approximately the same number of cells were immunoprecipitated with anti-peptide 406 antibody directed to the 52K and 55K proteins or with anti-peptide 12 antibody directed to the E1B 19K 175R T antigen.

anti-peptide 406 antibody did not cross-react with the E2 72K DNA-binding protein (data not shown), as has been reported for antibody to another L1 52K-55K peptide (18). This improves the usefulness of anti-peptide 406 antibody for immunofluorescence studies.

To further study the intracellular distribution of the 52K and 55K proteins, KB cells at 22 h after infection with Ad2 were fractionated into cytosol, membranes (plasma membranes and endoplasmic reticulum), nuclear wash (outer nuclear membranes), and nuclei (15, 25). Each fraction was subjected to an immunoblot analysis in which anti-peptide 406 antibody was used. The major portion of the 52K and 55K proteins was found in the nuclear membrane fraction (Fig. 9, nuclear wash) and was therefore either an integral component of the nuclear membrane or tightly bound. A lesser amount was found in the cytosol, and a very small amount was found in the nuclear fraction.

DISCUSSION

We found that antibody generated against a synthetic peptide, peptide 406, which was directed to a 10-amino acid sequence encoded close to the putative N terminus of the L1 52K and 55K proteins, recognized two protein species, the 52K and 55K proteins. The same two proteins were immunoprecipitated by anti-peptide 406 antibody from extracts of Ad2-infected cells and from *in vitro* translation mixtures programmed by viral RNA selected by hybridization to a DNA fragment containing L1 sequences. Our findings indicate that the L1 52K and 55K proteins, which were previously identified by *in vitro* translation (1, 19, 21), represent major viral protein species that are synthesized during productive infections.

The relationship between the L1 52K and 55K proteins has not been fully elucidated. Both proteins appear to contain the same N-terminal sequence, because each is immunoprecipitated by anti-peptide 406 antibody. A tryptic peptide map analysis of [35 S]methionine-labeled proteins

showed that the 52K and 55K species are very closely related in amino acid sequence. The 52K and 55K species are both phosphoproteins, and a phosphopeptide map analysis did not detect differences in phosphorylation sites. Thus, differential phosphorylation does not appear to account for the different electrophoretic mobilities of the 52K and 55K proteins. There does not appear to be a precursor-product relationship between the two species, at least as revealed by a pulse-chase analysis. Perhaps some unknown posttranslational modifications account for the two L1 species (52K and 55K). It is also conceivable that the 52K species is translated from a minor L1 mRNA containing an internal spliced-out sequence. Further studies should help clarify this issue.

The synthesis of the 52K and 55K proteins was found to begin at about 6 to 7 h postinfection, after the synthesis of the E1A T antigens and the synthesis of the E1B 19K 175R T antigen, which began at 2 to 3 and 4 to 5 h postinfection, respectively. By 15 h postinfection, the synthesis of the 52K and 55K proteins reached a maximum rate, which was maintained until at least 25 h postinfection. Very recently, Lewis et al. (18) detected the L1 52 and 55K proteins in 24-h extracts of Ad2-infected cells by using a different antipeptide serum. The earlier detection shown here may have been due to the greater strength of our antipeptide antiserum.

The L1 52K-55K gene appears to be regulated in a manner different from the typical early and late classes of viral genes. Early genes (e.g., E1 through E4) are transcribed from 1 to 2 h postinfection (23) and are expressed as mRNA and protein prior to the onset of viral DNA replication at 6 to 7 h postinfection (3, 7, 12, 27). Late genes are expressed as mRNA from the major late promoter at 16.8 map units after the onset of viral DNA replication, and the synthesis of late proteins, mainly virion structural proteins, is detected beginning at about 10 h postinfection (29). The L1 52K-55K gene has been reported to be expressed as cytoplasmic mRNA at early times, at 1 and 5 h postinfection (1, 24, 34). However, as shown here, the synthesis of the 52K and 55K proteins does not begin early but rather begins at intermediate times (6 to 7 h postinfection); we believe that if low levels of the 52K and 55K proteins had been synthesized at earlier times, they would have been detected by the strong anti-peptide 406 antibody. Furthermore, the synthesis of most early proteins is curtailed at late times postinfection, whereas the synthesis of the 52K and 55K proteins continues at high levels until at least 25 h postinfection. The regulation of the L1 52K-55K gene appears to be similar to the regulation of the intermediate class of viral genes, which includes those that encode proteins IX, a minor virion structural protein, and protein IVa2, a possible maturation protein. The intermediate genes are expressed beginning at about 6 to 12 h postinfection, and the expression of these genes can occur independently of viral DNA replication (4, 26). The gene encoding the i-leader protein, which is transcribed from the major late promoter at early times, as the L1 52K-55K gene is, appears to be regulated in a manner similar to the L1 52K-55K gene, as described elsewhere (38). It is possible that early viral gene products are necessary to activate the expression of the intermediate class of genes. This is consistent with studies suggesting that transcription of the L1 52K-55K gene is dependent upon the expression of an E1A gene product (22).

The functions of the 52K and 55K proteins are unknown. The results of our studies on the time course of synthesis, accumulation, and phosphorylation of these L1 proteins should prove valuable in addressing this question. The

FIG. 8. Localization of the Ad2 L1 52K and 55K proteins in cell nuclei by immunofluorescence microscopy, using anti-peptide 406 antibody. Cells at 15 h postinfection were incubated with anti-peptide 406 antibody (A) or with anti-peptide 406 antibody that had been preincubated with peptide 406 (B).

FIG. 9. Intracellular distribution of Ad2 L1 52K and 55K proteins by subcellular fractionation and immunoblot analysis. KB cells infected with Ad2 for 22 h (lanes I) or mock-infected cells (lanes M) were fractionated into cytosol, membranes (plasma membranes and endoplasmic reticulum), nuclear wash (outer nuclear membranes), and nuclei as described in Materials and Methods. The equivalent of 5×10^5 cells of each fraction was electrophoresed on a 12.5% SDS-polyacrylamide gel and probed with anti-peptide 406 antibody and then with ^{125}I -labeled protein A.

availability of a high-titer antibody to these proteins, which is devoid of cross-reaction with E2A 72K DNA-binding proteins, should also be useful. The results of fractionation and immunofluorescence studies with this antibody are relevant as well; they suggest that the 52K and 55K proteins are primarily nuclear proteins or nuclear membrane proteins or both or that they are tightly bound to the nucleus. Studies with mutants having mutations in the L1 52K-55K gene will be critical in determining the functions of these proteins.

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