JOURNAL OF VIROLOGY, Mar. 1986, p. 848–856
0022-538X/86/030848-09$02.00/0

Vol. 57, No. 3

Biosynthesis of Adenovirus Type 2 i-Leader Protein

JANEY S. SYMINGTON,[1] LYNNE A. LUCHER,[1] KARL H. BRACKMANN,[1] ANDERS VIRTANEN,[2] ULF PETTERSSON,[2] AND MAURICE GREEN[1]*

Institute for Molecular Virology, St. Louis University Medical Center, St. Louis, Missouri 63110,[1] and Department of Medical Genetics, The Biomedical Center, University of Uppsala, Uppsala, Sweden[2]

Received 19 August 1985/Accepted 4 November 1985

The i-leader is a 440-base-pair sequence located between 21.8 and 23.0 map units on the adenovirus type 2 genome and is spliced between the second and third segments of the major tripartite leader in certain viral mRNA molecules. The i-leader contains an open translational reading frame for a hypothetical protein of M_r about 16,600, and a 16,000-M_r polypeptide (16K protein) has been translated in vitro on mRNA selected with DNA containing the i-leader (A. Virtanen, P. Aleström, H. Persson, M. G. Katze, and U. Pettersson, Nucleic Acids Res. 10:2539–2548, 1982). To determine whether the i-leader protein is synthesized during productive infection and to provide an immunological reagent to study the properties and functions of the i-leader protein, we prepared antipeptide antibodies directed to a 16-amino acid synthetic peptide which is encoded near the N terminus of the hypothetical i-leader protein and contains a high acidic amino acid and proline content. Antipeptide antibodies immunoprecipitated from extracts of adenovirus type 2-infected cells a major 16K protein that comigrated with a 16K protein translated in vitro. Partial N-terminal amino acid sequence analysis by Edman degradation of radiolabeled 16K antigen showed that methionine is present at residue 1 and leucine is present at residues 8 and 10, as predicted from the DNA sequence, establishing that the 16K protein precipitated by this antibody is indeed the i-leader protein. Thus, the i-leader protein is a prominent species that is synthesized during productive infection. The i-leader protein is often seen as a doublet on polyacrylamide gels, suggesting that either two related forms of i-leader protein are synthesized in infected cells or that a posttranslational modification occurs. Time course studies using immunoprecipitation analysis with antipeptide antibodies revealed that the E1A 289R T antigen and the E1B-19K (175R) T antigen are synthesized beginning at 2 to 3 and 4 to 5 h postinfection, respectively, whereas the i-leader protein is synthesized starting at about 8 h postinfection and continues unabated until at least 25 h postinfection. The i-leader protein is very stable, as determined by pulse-chase labeling experiments, and accumulates continuously from 8 to 25 h postinfection, as shown by immunoblot analysis. The synthesis of i-leader protein does not depend upon viral DNA replication. Thus, the i-leader protein is a viral gene product of unknown function and high stability that is made in large quantities at intermediate times of productive infection. Immunofluorescence microscopy indicates that the i-leader protein is concentrated in the perinuclear region. Immunoblot analysis of subcellular fractions prepared in hypotonic buffer suggests that most of the i-leader protein accumulates in the cytoplasmic fraction.

Most late adenovirus type 2 (Ad2) mRNAs and some early intermediate mRNAs are initiated at the major late promoter at 16.5 map units (m.u.) (for a review, see reference 24). These mRNAs have a common 5′ terminus that is formed by splicing together three small segments totaling 203 nucleotides (nt) in length derived from sequences at 16.5, 19.6, and 26.6 m.u. Certain mRNAs contain an additional leader segment, termed the i-leader (3), which has been mapped by Virtanen et al. (27) between 21.75 and 22.96 m.u. on the Ad2 genome (9). The 440-nt-long i-leader contains an open translational reading frame starting with an ATG at nt 7968 that can encode a hypothetical protein of about M_r 16,600 (7, 9, 27). A 16,000-M_r polypeptide (16K protein) was identified by in vitro translation of mRNA selected by hybridization to DNA fragments containing i-leader sequences (27). The 16K protein probably corresponds to a 13,000-M_r polypeptide (13.6K protein) detected by in vitro translation of mRNA selected by hybridization to Ad2 DNA fragments which showed this species to map primarily at 21.5 to 22.0 m.u. (19). Partial sequence analysis of the 13.6K protein is consistent with it being encoded in the i-leader region (17).

To determine whether the i-leader protein that is translated in vitro is actually formed in virus-infected cells and to develop immunological reagents to further study the properties and function(s) of the i-leader protein, we prepared antibodies directed against a 16-amino acid synthetic peptide (peptide 65) encoded near the N terminus of the i-leader protein. In this report, we used immunoprecipitation and immunoblot analysis with antipeptide antibodies to show that the 16K i-leader is a new viral gene product that is synthesized in relatively large quantities at intermediate times during productive infection by Ad2 and does not require viral DNA replication for its synthesis. We describe the time course of synthesis and accumulation of the i-leader protein and the intracellular distribution of the protein as determined by subcellular fractionation and by immunofluorescence microscopy.

MATERIALS AND METHODS

Peptide synthesis, coupling of peptide to carrier protein, and preparation of antipeptide antibody. Peptide 65 (Asp-Arg-Glu-Glu-Leu-Asp-Leu-Pro-Pro-Pro-Val-Gly-Gly-Val-Ala-Val-[Cys]) which contains a 16-amino acid sequence (see Fig. 1) encoded near the N terminus of the Ad2 i-leader open translational reading frame around 22 m.u. (27) and peptide 34 (Gly-Glu-Glu-Phe-Val-Leu-Asp-Tyr-Val-Glu-His-Pro-

* Corresponding author.

Gly-His-Gly-[Cys]), unique to the Ad2 E1A 289-amino acid residue (289R) protein, were synthesized by the solid-phase procedure (1) by methods recently described (11). Cysteine was placed at the C terminus to facilitate coupling through the sulfhydryl group if desired (11). Peptides were prepared based on the amino acid sequence deduced from the appropriate DNA sequence: for peptide 65, nt 7974 to 8021; for peptide 34, nt 973 to 1020 (7, 23, 27). The amino acid composition of the synthesized peptides agreed with the predicted values. The synthesis of peptide 7, targeted to the C terminus of the E1B-19K (175R) protein, and the production of antibodies to this peptide have been reported previously (11).

Peptides were coupled to keyhole limpet hemocyanin by glutaraldehyde, and antibodies were raised in rabbits by methods described previously (11). The enzyme-linked immunosorbent assay titer of anti-peptide 65 was 2,560 by 3 months and reached 800,000 by 6 months. The 16K i-leader protein could be detected by radioimmunoassay by 3 months. The enzyme-linked immunosorbent assay titer of anti-peptide 34 antibody was also 2,560 by 3 months and reached 800,000 by 7 months. The E1A proteins could be detected by radioimmunoassay as well by 3 months.

Preparation of [^{35}S]methionine-labeled, [^{3}H]leucine-labeled, and unlabeled extracts of Ad2-infected cells for immunoprecipitation analysis and for immunoblot analysis. KB cells were grown in suspension culture in Eagle minimal essential medium (EMEM) containing 5% horse serum and infected with 50 to 200 PFU of Ad2 (strain 38-2) per cell (13). For immunoprecipitation analysis, cells were labeled with [^{35}S]methionine or [^{3}H]leucine at various times after infection. Samples (10 ml) of infected cells (about 4.3×10^6 cells total) were centrifuged at $300 \times g$ for 5 min, washed twice with 1 ml of methionine-free or leucine-free EMEM containing 2% dialyzed horse serum and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.1), and incubated with 400 μCi of [^{35}S]methionine (1,100 mCi/mmol) or 500 μCi of [^{3}H]leucine (120 Ci/mmol) in the same medium for 1 h at 37°C with shaking. Cells were harvested by centrifugation and washed with phosphate-buffered saline (PBS) containing 0.1% Trasylol. The pellet was solubilized by sonication in 3.0 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 centrifuged at $100,000 \times g$ for 60 min. The supernatant was stored at −20°C for use in immunoprecipitation experiments as described below.

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, cells were washed with methionine-free EMEM and labeled with 200 μCi of [^{35}S]methionine per plate for 30 min; after this, plates were harvested immediately for no chase or washed and allowed to incubate in unlabeled complete EMEM for desired times before harvesting. 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 was renewed at 12 h by the method of Gaynor et al. (8). Cells were washed in methionine-free medium and labeled with [^{35}S]methionine from 25 to 26 h postinfection and harvested as described above.

For immunoblot analysis, at various times after infection unlabeled cells were harvested by centrifugation and washed as described above. The cell pellet was stored at −70°C. Cells were lysed by heating in 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) for 10 min at 95°C before electrophoresis.

In vitro translation. Total cytoplasmic RNA was prepared from KB cells infected with Ad2 at 21 h postinfection and hybridized to the Ad2 *Hin*dIII B fragment (17.0 to 31.5 m.u.) immobilized on a nitrocellulose filter. Eluted RNA was translated in a rabbit reticulocyte system with a translation kit (New England Nuclear Corp., Boston, Mass.) with 20 μCi of [^{35}S]methionine per tube. The reaction was run for 60 min at 30°C.

Immunoprecipitation analysis of in vivo- and in vitro-labeled extracts. Samples of extracts from [^{35}S]methionine-labeled or [^{3}H]leucine-labeled infected cells containing 5×10^6 to 1×10^7 trichloroacetic acid-insoluble cpm or samples from in vitro translates containing 2×10^5 trichloroacetic acid-insoluble cpm were used for immunoprecipitation 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 (homologous peptide) was included in the reaction mixture at a concentration of 10 μg/ml. An unrelated peptide (heterologous peptide) was sometimes included in the immunoprecipitation mixture as a control. Immunoprecipitates were processed and analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography as described previously (14).

Partial N-terminal amino acid sequence analysis of Ad2 i-leader protein. Ad2-infected cells were labeled with [^{3}H]leucine or [^{35}S]methionine from 13 to 16 h postinfection, and extracts were immunoprecipitated with anti-peptide 65 antibody. The eluates from combined precipitates were electrophoresed on an SDS-polyacrylamide gel. The 16K band was disclosed by autoradiography, excised from the dried gel, eluted overnight with water in the presence of 100 nmol of carrier myoglobin, and dialyzed against water containing 0.05% SDS. The labeled preparation was subjected to 11 cycles of Edman degradation at the protein-sequencing laboratory at the Washington University School of Medicine, St. Louis, Mo., using a Beckman model 890 B sequencer.

Cell fractionation. KB cells infected with Ad2 or mock infected were harvested at 22 h postinfection by washing twice in PBS containing 0.1% Trasylol and fractionated essentially as described by Jay et al. (15). Washed cells were suspended in 10 mM Tris hydrochloride (pH 7.4)–10 mM KCl–2 mM $MgCl_2$–2 mM dithiothreitol and were ruptured in a glass tissue grinder with a Teflon pestle until more than 95% of the cells were broken as determined by phase microscopy. Cells were centrifuged at $800 \times g$ for 15 min. The supernatant was designated cytosol and was frozen at −70°C. Membranes and nuclei were separated by the method of Burnette and Till (2), using an aqueous two-phase polymer system; NaCl replaced $ZnCl_2$ in the system. Nuclei and membranes were mixed with dextran 500 and polyethylene glycol 8000 prepared as described previously (2) and centrifuged at $12,000 \times g$ for 10 min. Nuclei were pelleted, and membranes remained at the interphase. Membranes were recovered as a pellet by centrifugation at $600 \times g$ for 15 min after a fourfold dilution with distilled water. Nuclei were suspended in a buffer containing 10 mM Tris hydrochloride (pH 6.8), 150 mM KCl, 5 mM $MgCl_2$, and 1% Triton X-100

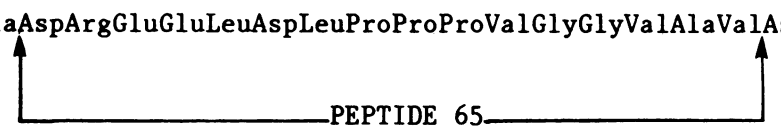

FIG. 1. Sequence of the 16 amino acids present in peptide 65 that is encoded near the N terminus of the Ad2 i-leader protein.

by Dounce homogenization. After 10 min at 4°C, the washed nuclei were centrifuged at 800 × *g* for 15 min. Samples were stored at −70°C until used for immunoblot analysis.

Immunoblot analysis. Samples were electrophoresed on SDS-polyacrylamide gels containing 12.5% acrylamide and 0.12% bisacrylamide. After gel electrophoresis, gels were equilibrated with transfer buffer for 5 min and used for protein transfer analysis as described in detail previously (26). Briefly, electrophoretic transfer of proteins was performed with a Bio-Rad Transblot apparatus at 8 V/cm. Transfers were made onto 0.20-μm-porosity nitrocellulose membranes (BA83; Schleicher & Schuell, Inc., Keene, N.H.) in 20 mM Tris–154 mM glycine–20% methanol for 23 h. Membranes were blocked by gentle shaking with 0.25% gelatin (E1A grade; Bio-Rad Laboratories, Richmond, Calif.) in TBS (50 mM Tris hydrochloride [pH 7.4], 200 mM NaCl) for 1 h. The membranes were then incubated with rocking with a 1 to 25 to 1 to 50 dilution of antiserum in TBS containing 0.05% Tween 20 (TBS-T) for 3 h at room temperature and then overnight at 4°C. After three 5-min washes with 250 ml of TBS-T, the membranes were incubated with rocking in 40 ml of TBS-T containing 10 μCi of ^{125}I-labeled *Staphylococcus* protein A (8 to 9 μCi/μg; New England Nuclear Corp.) for 2 h at room temperature. The membranes were washed three times with TBS-T, blotted, covered with Saran Wrap, and exposed to X-ray film for 4 to 24 h. In some cases, transfers were reprobed with a second antipeptide antiserum as follows. Membranes were reblocked in 0.25% gelatin in TBS for 1 h at room temperature and then incubated with a different antiserum followed by ^{125}I-labeled protein A. Autoradiography showed the presence of newly labeled bands in addition to the original.

Indirect immunofluorescence. KB cells were grown on cover slips for 1 day before infection with 200 PFU of Ad2 per cell. At desired times postinfection, cells were rinsed with cold PBS and cold methanol and fixed with methanol for 10 min at −20°C. Cells were stained with a 1 to 5 dilution of anti-peptide 65 antiserum in PBS for 1 h at room temperature, washed with PBS, and incubated in the dark with a 1 to 10 dilution of fluorescein-conjugated goat anti-rabbit immunoglobulin G (Pel-Freez Biologicals) for 1 h before mounting as described previously (20). Cells were observed with a Nikon Diaphot-TMD microscope with epifluorescence attachment. Photographs were taken at a magnification of ×50 on Kodak Tri-X pan film.

RESULTS

Preparation and characterization of antipeptide antibody targeted to an amino acid sequence encoded near the N terminus of Ad2 i-leader protein. To develop a sensitive and specific immunological reagent to detect and analyze the expression of the i-leader protein in Ad2-infected cells, we prepared antipeptide antibodies targeted against peptide 65 (Fig. 1). Peptide 65 comprises a 16-amino acid sequence of high proline and acidic amino acid content which is located between amino acid residues 4 and 20 of the i-leader protein, as deduced from DNA sequence analysis, mRNA mapping studies (27), and analysis of in vitro-translated protein (17). Peptide 65 was synthesized by the Merrifield solid-phase procedure, coupled to keyhole limpet hemocyanin by the glutaraldehyde procedure, and used to generate antipeptide antibodies in rabbits as previously described (11). Antibodies directed against peptide 65 were detected by enzyme-linked immunosorbent assay after 3 months and reached very high titers by 6 months after the initial rabbit inoculation.

We investigated first whether anti-peptide 65 antibody is able to recognize the Ad2 i-leader protein in a cell-free translate expected to contain the authentic protein. Cloned Ad2 *Hin*dIII-B DNA (17.0 to 31.5 m.u.) was used to select i-leader-specific RNA by hybridization with cytoplasmic RNA prepared from KB cells at 21 h postinfection. Hybridization-selected RNA was translated in the rabbit reticulocyte system with [^{35}S]methionine as labeled precursor and analyzed by SDS-polyacrylamide gel electrophoresis. Multiple protein species were detected in the translate programmed by the Ad2 *Hin*dIII-B-selected mRNA (Fig. 2A, lane 2) that were absent in the translate programmed by endogenous mRNA (Fig. 2A, lane 1); these include numerous late proteins in addition to the putative i-leader protein with an expected M_r of 16,000 (27). Incubation of the translate with anti-peptide 65 antibody immunoprecipitated a 16,000-M_r protein (Fig. 2B), thus providing evidence that the antipeptide antibody is specific for the i-leader protein. Immunoprecipitation of the 16K protein was blocked by peptide 65 (data not shown).

We investigated next whether the 16K i-leader protein is synthesized in Ad2-infected KB cells. Extracts were prepared from [^{35}S]methionine-labeled cells at 15 h postinfection and incubated with anti-peptide 65 antibody. A major 16K protein that comigrated with the 16K protein present in the cell-free translate was immunoprecipitated from the infected-cell extract (Fig. 2C, lane 1). The precipitation of the 16K protein was blocked completely by peptide 65 (Fig. 2C, lane 2) but not by a heterologous peptide (Fig. 2C, lane 3), thus providing further evidence that the 16K protein is the i-leader protein.

The Ad5 i-leader amino acid sequence, based on the DNA sequence of the Ad5 *Hin*dIII C fragment (5), differed from the Ad2 i-leader sequence in only two amino acid residues. Valine at residues 14 and 19 in the Ad2 protein was replaced with isoleucine in the Ad5 protein. Immunoprecipitation analysis of extracts of Ad2- and Ad5-infected cells revealed that the Ad5 i-leader protein migrated with a slightly greater mobility on an SDS-polyacrylamide gel than did the Ad2 i-leader protein (Fig. 3). This difference constitutes additional evidence that the 16K protein is the viral-coded i-leader protein and not a cell protein nonspecifically precipitated by antibody. It is interesting that the Ad5 i-leader is recognized by anti-peptide 65 antibody even though the Ad5 N-terminal sequence differs in two amino acid residues from peptide 65.

Partial N-terminal sequence analysis of Ad2 i-leader protein immunoprecipitated by anti-peptide 65 antibody. We performed partial N-terminal sequence analysis of the 16K protein immunoprecipitated with anti-peptide 65 antibody

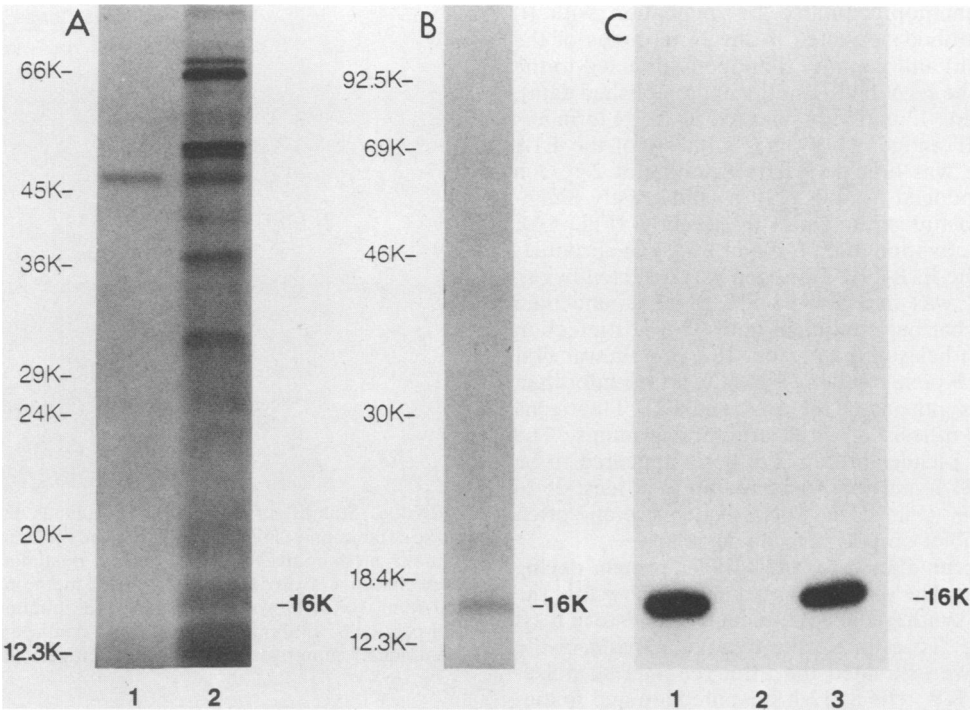

FIG. 2. In vitro translation of Ad2 i-leader protein and immunoprecipitation from translate and from Ad2-infected KB cells with anti-peptide 65 antibody. Cytoplasmic RNA selected on *Hin*dIII-B (17.0 to 31.5 m.u.) was used to direct protein synthesis in a rabbit reticulocyte system in the presence of [^{35}S]methionine. The products were separated on a 12.5% polyacrylamide gel and visualized by autoradiography. (A) Lane 1 shows endogeneous protein translate; lane 2 shows protein synthesized with *Hin*dIII-B mRNA. Molecular size markers were visualized by staining and are shown in kilodaltons (K). (B) Protein precipitated from the *Hin*dIII-B translate with anti-peptide 65 serum targeted to a portion of the i-leader sequence. ^{14}C-labeled marker proteins were used for calibration as shown. (C) Immunoprecipitation from Ad2 15-h infected KB cell extracts. Lanes: 1, anti-peptide 65 antiserum; 2, the inclusion of 5 μg of peptide 65 per ml with anti-peptide 65 serum; 3, the inclusion of an unrelated peptide.

from Ad2-infected cells labeled with [^{3}H]leucine and [^{35}S]methionine. Methionine is present at residue 1 and leucine is present at residues 8 and 10 (Fig. 4), as expected from the amino acid sequence predicted from the DNA sequence of the i-leader protein (Fig. 1). These data provide definitive evidence that the 16K protein immunoprecipitated by the anti-peptide 65 antibody is indeed the i-leader protein.

Time course of synthesis of Ad2 i-leader protein during the infectious cycle and comparison with that of E1A 289R T antigen and E1B-19K (175R) T antigen. To determine the time of synthesis of the i-leader protein, Ad2-infected KB cells were labeled for 1-h periods with [^{35}S]methionine at intervals from 2 to 25 h postinfection. Cell extracts were

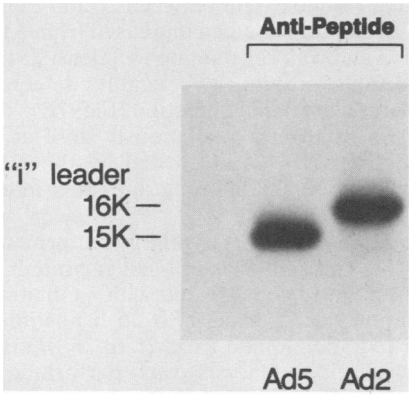

FIG. 3. Immunoprecipitation of i-leader protein from Ad2- and Ad5-infected cell extracts. Extracts of KB cells infected with Ad2 or with Ad5 and labeled with [^{35}S]methionine from 15 to 16 h postinfection were immunoprecipitated with anti-peptide 65 antibody and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography as described in Materials and Methods. K, Kilodaltons.

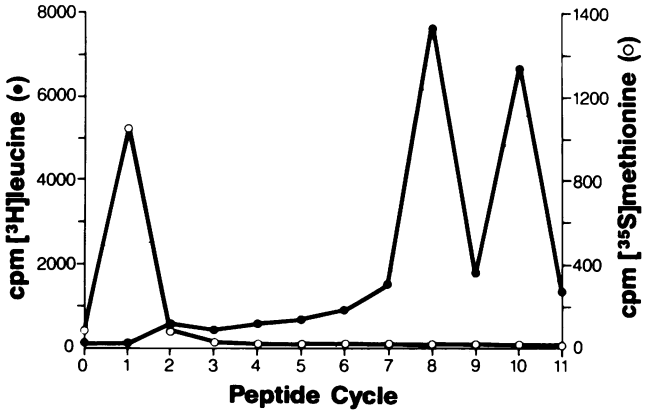

FIG. 4. Partial N-terminal amino acid sequence analysis of the i-leader 16K protein immunoprecipitated by anti-peptide 65 antibody from Ad2-infected KB cells labeled with [^{3}H]leucine or [^{35}S]methionine. Protein was purified by gel electrophoresis. Approximately 50,000 [^{3}H] and 10,000 [^{35}S] cpm of protein were subjected to automated sequential Edman degradation for 11 cycles.

prepared and immunoprecipitated by incubation with (i) anti-peptide 65 antibody directed to the N terminus of the i-leader protein, (ii) anti-peptide 34 antibody directed to the unique region of the E1A 289R T antigen (unpublished data), and (iii) anti-peptide 12 antibody directed to the N terminus of the E1B-19K T antigen (11). The synthesis of the E1A T-antigen species was first detected beginning at 2 to 3 h (faint), was pronounced from 4 to 10 h, and greatly diminished at 15 h postinfection and subsequently (Fig. 5A). Consistent with activation of E1B by an E1A gene product, the synthesis of the E1B-19K T antigen was detected beginning at 4 to 5 h, was maximal from 8 to 15 h, and then decreased somewhat but continued until 25 h postinfection (Fig. 5B). The synthesis of the i-leader 16K protein was first detected at 8 to 9 h postinfection (Fig. 5C), a time later than the beginning of synthesis of the E1A and E1B T antigens but before the synthesis of viral structural proteins. The maximum rate of i-leader protein synthesis appeared to be reached at 10 to 11 h and was sustained up to at least 25 to 26 h postinfection (Fig. 5C). The i-leader protein often migrated as a doublet on polyacrylamide gels.

Stability and accumulation of Ad2 i-leader protein during the infectious cycle. The data presented above show that the i-leader protein is synthesized continuously from 8 to at least 25 h postinfection. To estimate the relative stability of the i-leader protein, we estimated the turnover time by pulse-chase labeling of KB cells at 12 h postinfection and immunoprecipitation with anti-peptide 65 antibody. After a 30-min pulse with [^{35}S]methionine, a pronounced 16,000-M_r doublet was immunoprecipitated (Fig. 6B, 0 min of chase). No decrease in the amount of labeled 16,000-M_r was detected after 60 to 180 min of chase with cold methionine (Fig. 6B). Thus, the i-leader protein is a very stable protein that possesses a low turnover rate. For comparison, the turnover time of the E1A T-antigen species encoded by the 13S mRNA was measured. Spindler and Berk (25) have reported that the half-life of the E1A T antigens is 30 to 80 min in productively infected cells. The half-life of the E1A 289R T-antigen species was found to be approximately 30 min, as estimated by immunoprecipitation with anti-peptide 34 antibody, using the same labeled preparations that were used for analysis of the i-leader protein (Fig. 6A).

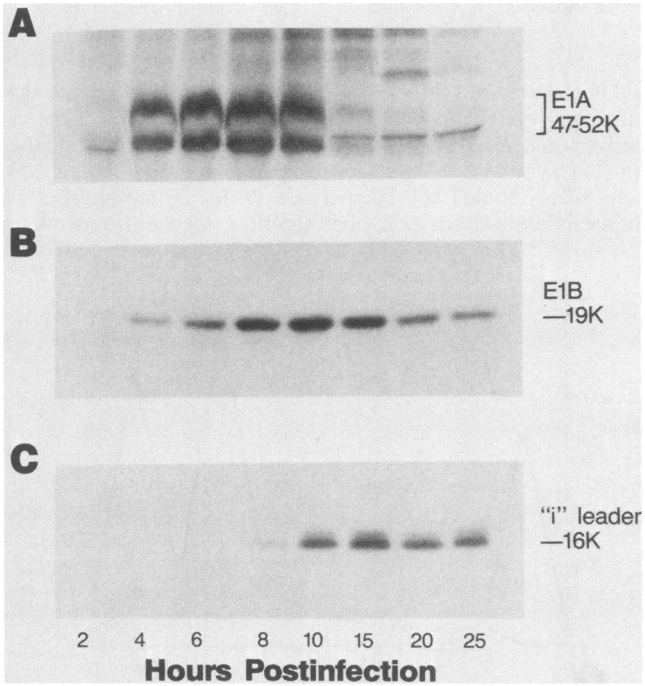

FIG. 5. Time course of synthesis of Ad2 i-leader 16K protein, Ad2 E1A T antigens, and Ad2 E1B-19K (175R) T antigen. Extracts of KB cells infected with Ad2 and labeled with [^{35}S]methionine for a 1-h period at 2, 4, 6, 8, 10, 15, 20, and 25 h postinfection were immunoprecipitated with antipeptide antibodies specific for the i-leader protein, the Ad2 E1A T antigens, and the Ad2 E1B-19K protein as described in Materials and Methods. K, Kilodaltons.

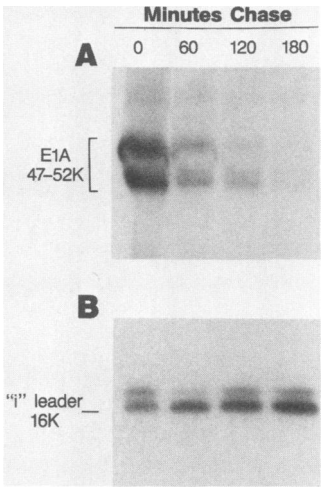

FIG. 6. Stability of Ad2 i-leader protein as determined by pulse-chase experiments. KB cells were labeled for 30 min with [^{35}S]methionine in methionine-free MEM at 12 h postinfection, and portions were chased with an excess of unlabeled methionine for 60, 120, and 180 min. Extracts were prepared and immunoprecipitated with anti-peptide 65 antibody to the i-leader or anti-peptide 34 antibody to the unique region of the E1A 289R T antigen. K, Kilodaltons.

The relative concentration of the i-leader protein was estimated from 2 to 25 h postinfection by immunoblot analysis. For comparison, the levels of E1A 289R and E1B-19K T antigens were estimated by immunoblot analysis with the same cell extracts (Fig. 7). The amount of E1A 289R T antigen was relatively constant between 4 and 10 h postinfection, decreased dramatically between 10 and 15 h, and was virtually nonexistent between 20 and 25 h (Fig. 7B). The level of E1B-19K T antigen increased from 6 to about 15 h postinfection and was constant up to at least 25 h (Fig. 7A). The 16K i-leader protein was readily detected by immunoblot analysis at 8 h postinfection (Fig. 7B). The level of i-leader protein increased continuously until at least 25 h postinfection (Fig. 7B), as illustrated by the densitometer scan of the immunoblot at different times after infection (Fig. 7C).

Effect of inhibition of DNA synthesis on formation of Ad2 i-leader protein. The synthesis of i-leader protein was determined in Ad2-infected cells treated with an inhibitor of viral DNA replication, araC, from 1 to 26 h postinfection and labeled with [^{35}S]methionine from 25 to 26 h postinfection. Immunoprecipitation analysis was performed with the antipeptide antibodies specific for the i-leader protein as well as for the E1A 289R and E1B-19K T antigens. araC greatly stimulated the synthesis of E1A T antigen, as previously reported by Gaynor et al. (8), but had no appreciable effect on the level of E1B-19K (Fig. 8). High levels of the 16K protein were synthesized in the presence of araC, although

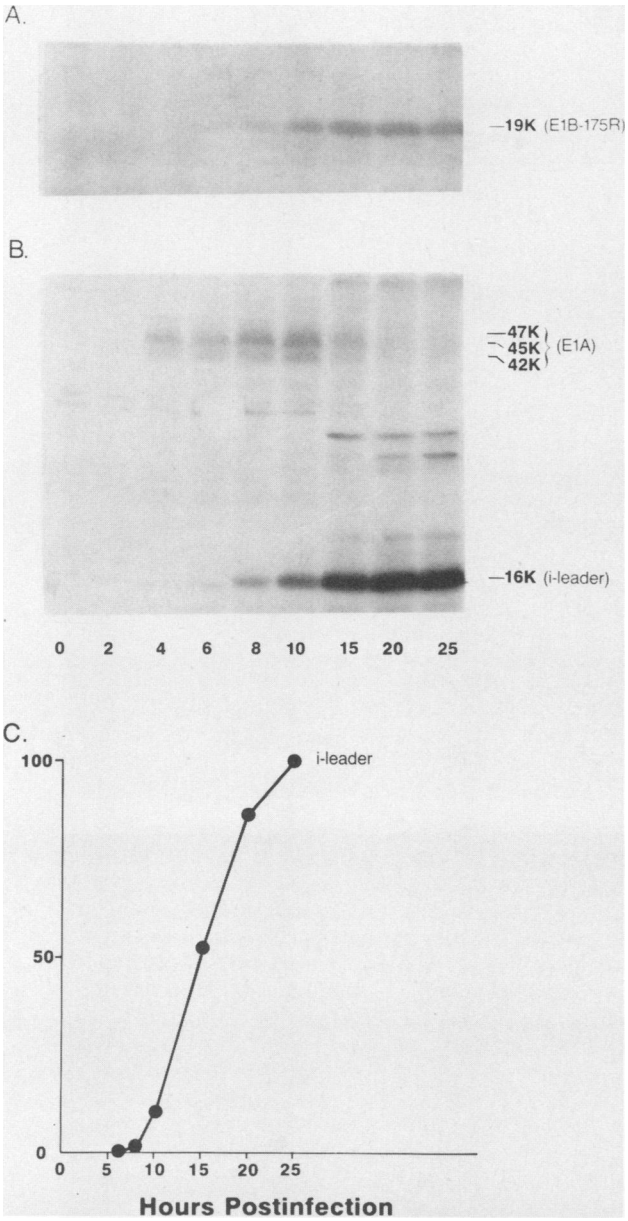

FIG. 7. Time course of accumulation of Ad2 i-leader protein, E1A T antigens, and E1B-19K T antigen in KB cells as determined by immunoblot analysis. Extracts of Ad2-infected cells were electrophoresed on a 12.5% polyacrylamide gel, transferred to nitrocellulose, and treated with antipeptide serum followed by ^{125}I-labeled protein and autoradiography as described in Materials and Methods. (A) Antipeptide antibody to the Ad2 E1B-19K protein. (B) The blot was first probed with anti-peptide 65 antibody to the i-leader protein. After autoradiography, the blot was reprobed with antipeptide antibody to the Ad2 E1A 289R T antigen. Numbers refer to apparent molecular weights (K, $\times 10^3$) as determined by marker proteins transferred simultaneously and detected by Coomassie blue staining. (C) Densitometer scan of i-leader portion of autoradiographic film shown in panel B. Vertical axis represents percent of total i-leader protein found at various times postinfection; 25-h value normalized to 100%.

they were somewhat less than those of 16K protein synthesized in the absence of araC (Fig. 8). These data suggest that viral DNA replication is not required for the synthesis of the i-leader protein.

Intracellular localization of i-leader protein in Ad2-infected cells. The function of the i-leader is not known. Knowledge of the intracellular location of the i-leader protein may provide clues as to its role in virus replication. In view of the discrepancies that are often associated with different methods used to localize viral proteins, both immunofluorescence microscopy and biochemical fractionation were used to study the intracellular distribution of the i-leader protein. For immunofluorescence analysis, cover slip preparations of KB cells infected with Ad2 were stained with anti-peptide 65 antibody. Comparison of the distribution of fluorescence at 15 h postinfection when abundant i-leader protein is present within the cell (Fig. 9A) with that at 2 h postinfection, used as control for nonspecific staining since no i-leader protein has been synthesized as yet (Fig. 9C), suggests that the i-leader protein is concentrated in the perinuclear region. Figures 9B and D are phase-contrast pictures of the same cells.

The intracellular distribution of i-leader protein was studied further by subcellular fractionation and immunoblot analysis. KB cells infected with Ad2 were harvested at 22 h postinfection, disrupted with hypotonic buffer, and fractionated as described in Materials and Methods. The equivalent of 5×10^5 cells of each fraction was electrophoresed, transferred to nitrocellulose, and probed with anti-peptide 65 antibody and ^{125}I-labeled protein A. The results (Fig. 10) indicate that with the fractionation conditions that were used, most of the i-leader protein is present in the cytoplasmic fraction. However, some i-leader protein remains associated with the nuclear fraction even after a wash with 1% Triton X-100 which removes an appreciable amount. A very small amount is found in the membrane fraction. These results are in contrast to the distribution of L1-encoded 52,000- and 55,000-M_r proteins found primarily in the nuclear wash (Lucher, Brackmann, Symington, and Green, J. Virol., in press).

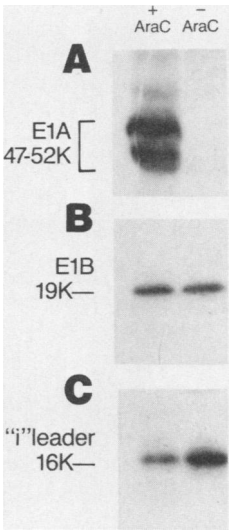

FIG. 8. Synthesis of Ad2 i-leader protein, Ad2 E1B-19K T antigen, and Ad2 E1A T antigens in the presence or absence of ara-C. Ad2-infected cultures maintained in the absence or the presence of 20 μg of araC per ml from 1 h postinfection were labeled with [^{35}S]methionine for 1 h at 24 h postinfection. Extracts were prepared and immunoprecipitated with antipeptide antibodies to the Ad2 i-leader protein, the Ad2 E1B-19K T antigen, and the Ad2 E1A 289R T antigen, as described in Materials and Methods. k, kilodaltons.

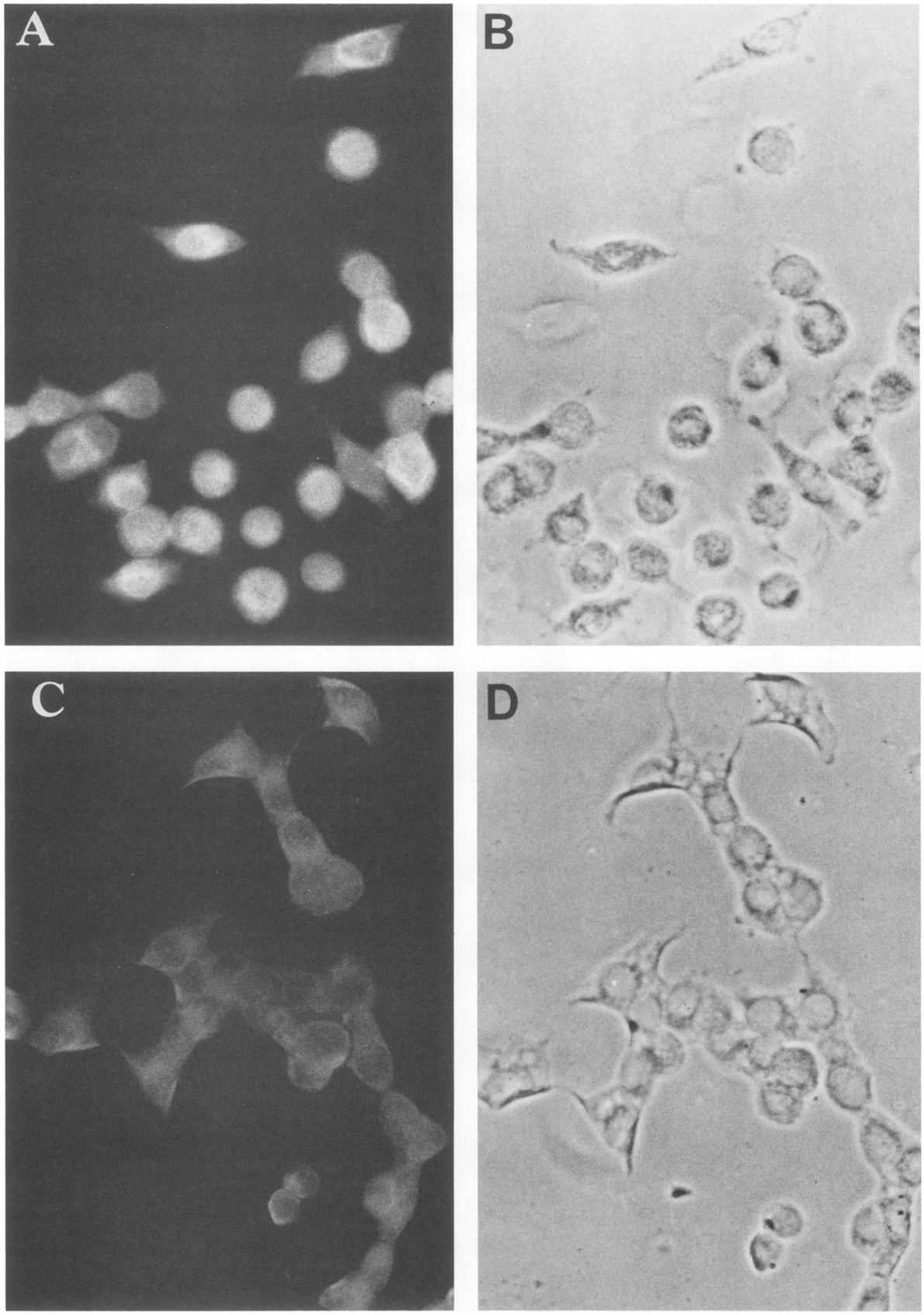

FIG. 9. Indirect immunofluorescent staining of Ad2 i-leader protein in Ad2-infected KB cells by anti-peptide 65 antibody. (A) Cells at 15 h postinfection showing i-leader location. (B) Phase-constrast picture of cells in panel A. (C) Cells at 2 h postinfection used as control for nonspecific background stain. (D) Phase-contrast picture of cells in panel C.

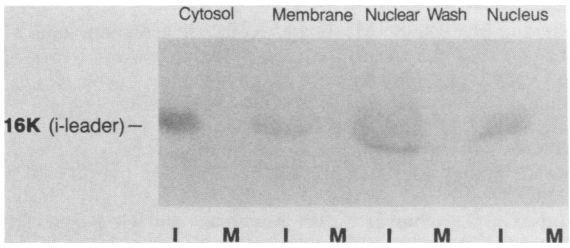

FIG. 10. Localization of Ad2 i-leader protein in Ad2-infected KB cells by subcellular fractionation and immunoblot analysis. KB cells infected with Ad2 (lanes I) or mock infected (lanes M) were harvested at 22 h postinfection and fractionated as described in Materials and Methods. The equivalent of 5×10^5 cells of each fraction was separated on a 12.5% polyacrylamide gel, transferred to nitrocellulose, and probed with anti-peptide 65 antibody followed by ^{125}I-labeled protein A. I, Define; M, define.

DISCUSSION

We show in this paper that anti-peptide 65 antibody, targeted to a 16-amino acid sequence located near the N terminus of the hypothetical i-leader protein, immunoprecipitates a 16K protein from Ad2 productively infected KB cells that comigrates with a 16K protein immunoprecipitated by the same antibody from in vitro translates programmed with i-leader-containing RNA. The close relationship between the in vitro- and in vivo-synthesized 16K proteins is supported further by the specific inhibition by peptide 65 of the immunoprecipitation of each protein.

The partial amino acid sequence analysis of the 16K in vivo-synthesized protein which we present here shows agreement as to positions of methionine and leucine within the first 11 N-terminal amino acids with positions predicted from the i-leader DNA sequence. It is also consistent with results of partial sequence analysis of the 13.6K (probably corresponding) protein produced by in vitro synthesis from mRNA hybridized to the i-leader region (17). Our studies are consistent with the conclusion that the 16K protein is the i-leader protein and that the i-leader protein represents a major virus-coded protein synthesized during productive infection. The i-leader protein often migrates as a doublet on polyacrylamide gels. It is possible that the doublet represents two different forms of i-leader protein that contain the same 5′ terminus but different 3′ termini (7). Alternatively, one of the bands could represent a posttranslational product of the i-leader protein.

The regulation of i-leader protein synthesis, especially by other viral gene products, is of interest. As described in this report, the synthesis of i-leader protein begins at 8 h postinfection, toward the end of the early phase and the beginning of the late phase of Ad2 productive infection (10, 12) and subsequent to the initiation of E1A T-antigen synthesis at 2 to 3 h and E1B-19K T-antigen synthesis at 4 to 5 h. The synthesis of i-leader protein occurs in the presence of an inhibitor of viral DNA replication, ara-C, just as does the synthesis of early viral gene products. These properties suggest the possibility that the i-leader gene is regulated in the same manner as are viral early genes that are expressed toward the end of the early phase of infection.

The i-leader protein accumulates up to at least 25 h postinfection, and the concentration of i-leader protein appears to exceed that of the E1A and E1B-19K T antigens, as estimated by immunoblot analysis of unlabeled protein. The immunoprecipitation analysis in Fig. 5 shows similar levels of synthesis of i-leader protein and E1B-19K T antigen. However, immunoprecipitation of [^{35}S]methionine-labeled proteins probably underestimates the relative amount of i-leader protein (Fig. 5), because the i-leader contains only two methionine residues (7, 27). The high levels of accumulation, the intermediate time of synthesis, and the results of inhibitor studies discussed above suggest that the i-leader gene is regulated in a manner similar to the gene that encodes virion protein IVa (4). However, the i-leader protein was not detected by immunoprecipitation analysis of [^{35}S]methionine-labeled virions (data not shown) and thus does not appear to be a viral structural protein.

After we had completed the investigations reported in this paper, Lewis et al. (18) reported studies which included findings on the i-leader protein. Their studies are in general agreement with our studies. They found that antibody to a synthetic peptide comprising the N-terminal 9 amino acids of the i-leader sequence recognized a 13.6K protein in Ad2-infected cells. The 13.6K protein is probably the same protein as the 16K protein translated in vitro found by Virtanen et al. (27) and the 16K i-leader protein described in the present paper. We reproducibly obtain M_r values of 16,000 to 17,000 for the i-leader protein with several different commercial protein standards; the lower value described by Lewis et al. (18) may be due to the use of different marker proteins. The 13.6K i-leader protein was referred to as a late protein by Lewis et al. (18), inasmuch as it was detected by immunoprecipitation only after 15 h postinfection and by immunoblot analysis only after 26 h (18). In the present study with anti-peptide 65 antibody, the i-leader protein was detected at 8 h postinfection both by immunoprecipitation and by immunoblot analysis. This difference probably reflects a lack of potency of the antipeptide antibody directed to the 9-amino acid peptide (18). It is probable that the 9-amino acid peptide is less immunogenic than the 16-amino acid peptide that we used in thie present study.

The simian virus 40 (SV40) agnoprotein may be analogous to the Ad2 i-leader protein and is encoded in the SV40 late leader region (6, 16). The cellular distribution of the SV40 agnoprotein is the same as that reported here for i-leader protein (22). However, the SV40 agnoprotein differs in several respects from the Ad2 i-leader protein. The SV40 protein is about half the size (61 amino acids) of the Ad2 protein (149 amino acids), is highly basic, and binds to DNA (16); in contrast, the Ad2 protein is acidic. The SV40 protein is made late after infection, and current evidence suggests that it may play a role in virion assembly (21); the SV40 protein is not present in SV40 virions.

There are few clues as to the function(s) of the i-leader protein. Our preliminary immunofluorescence microscopy suggests that the i-leader protein is localized at the periphery of the nuclear membrane, whereas cell fractionation studies detect most of the i-leader protein in the cytoplasm with a lesser amount in the nuclear wash. These results are not necessarily discordant, and they suggest that the i-leader protein is loosely associated with the nuclear membrane. Similar conclusions as to localization of i-leader were reached by Lewis et al. (18). The synthesis of i-leader protein begins just at the time when the dramatic switch from early- to late-gene expression begins (12). It is conceivable that the i-leader protein plays a role in the specific expression of late viral genes.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants CA21824 and CA29561 from the National Institutes of Health. M.G. holds

Research Career Award 5 K06 AI04739 from the National Institute of Allergy and Infectious Diseases. L.A.L. was supported by Postdoctoral Training grant 3T3 CA09222 from the National Cancer Institute.

We thank G. Chinnadurai for reading the manuscript and C. E. Mulhall for editorial assistance.

LITERATURE CITED

1. **Barany, G., and R. B. Merrifield.** 1980. Solid-phase peptide synthesis, p. 1–284. *In* E. Gross and J. Meienhofer (ed.), The peptides, vol. 2. Academic Press, Inc., New York.
2. **Burnette, D. M., and J. E. Till.** 1971. A rapid method for the isolation of L-cell surface membranes using an aqueous two-phase polymer system. J. Membr. Biol. **5**:215–224.
3. **Chow, L. T., T. R. Broker, and J. B. Lewis.** 1979. Complex splicing patterns of RNAs from the early regions of adenovirus-2. J. Mol. Biol. **134**:265–303.
4. **Chow, L. T., J. B. Lewis, and T. R. Broker.** 1980. RNA transcription and splicing at early and intermediate times after adenovirus-2 infection. Cold Spring Harbor Symp. Quant. Biol. **44**:401–414.
5. **Dekker, B. M. M., and H. van Ormondt.** 1984. The nucleotide sequence of fragment HindIII-C of human adenovirus type 5 DNA (map positions 17.1-31.7). Gene **27**:115–120.
6. **Dhar, R., K. N. Subramanian, J. Pan, and S. M. Weissman.** 1977. Structure of a large segment of the genome of simian virus 40 that does not encode known proteins. Proc. Natl. Acad. Sci. USA **74**:827–831.
7. **Falvey, E., and E. Ziff.** 1983. Sequence arrangement and protein coding capacity of the adenovirus type 2 "i" leader. J. Virol. **45**:185–191.
8. **Gaynor, R. B., A. Tsukamoto, C. Montell, and A. J. Berk.** 1982. Enhanced expression of adenovirus transforming proteins. J. Virol. **44**:276–285.
9. **Gingeras, T. R., D. Sciaky, R. E. Gelinas, J. Bing-Dong, C. E. Yen, M. M. Kelly, P. A. Bullock, B. L. Parsons, K. E. O'Neill, and R. J. Roberts.** 1982. Nucleotide sequences from the adenovirus-2 genome. J. Biol. Chem. **257**:13475–13491.
10. **Green, M.** 1962. Studies on the biosynthesis of viral DNA. Cold Spring Harbor Symp. Quant. Biol. **27**:219–235.
11. **Green, M., K. H. Brackmann, L. A. Lucher, J. S. Symington, and T. Kramer.** 1983. Human adenovirus 2 E1B-19K and E1B-53K tumor antigens: antipeptide antibodies targeted to the NH_2 and COOH termini. J. Virol. **48**:604–615.
12. **Green, M., J. T. Parsons, M. Piña, K. Fujinaga, and I. Landgraf-Leurs.** 1970. Transcription of adenovirus genes in productively infected and in transformed cells. Cold Spring Harbor Symp. Quant. Biol. **35**:803–818.
13. **Green, M., and W. S. M. Wold.** 1979. Preparation of human adenoviruses. Methods Enzymol. **58**:425–435.
14. **Green, M., W. S. M. Wold, K. H. Brackmann, and M. A. Cartas.** 1979. Identification of families of overlapping polypeptides coded by early "transforming" gene region 1 of human adenovirus type 2. Virology **97**:275–286.
15. **Jay, G., F. T. Jay, R. M. Friedman, and A. S. Levine.** 1977. Simian virus 40-specific ribosome-binding proteins induced by a nondefective adenovirus 2-simian virus 40 hybrid. J. Virol. **23**:692–699.
16. **Jay, G., S. Nomura, C. W. Anderson, and G. Khoury.** 1981. Identification of the SV40 agnogene product: a DNA binding protein. Nature (London) **291**:346–349.
17. **Lewis, J. B., and C. W. Anderson.** 1983. Proteins encoded near the adenovirus late messenger RNA leader segments. Virology **127**:112–123.
18. **Lewis, J. B., M. L. Fahnestock, M. M. Hardy, and C. W. Anderson.** 1985. Presence in infected cells of nonvirion proteins encoded by adenovirus messenger RNAs of the major late transcription regions L0 and L1. Virology **143**:452–466.
19. **Lewis, J. B., and M. B. Mathews.** 1980. Control of adenovirus early gene expression: a class of immediate early products. Cell **21**:303–313.
20. **Lucher, L. A., D. Kimelman, J. S. Symington, K. H. Brackmann, M. A. Cartas, H. Thornton, and M. Green.** 1984. Identification of adenovirus 12-encoded E1A tumor antigens synthesized in infected and transformed mammalian cells and in *Escherichia coli*. J. Virol. **52**:136–144.
21. **Ng, S.-H., J. E. Mertz, S. Sanden-Will, and M. Bina.** 1985. Simian virus 40 maturation in cells harboring mutants deleted in the agnogene. J. Biol. Chem. **260**:1127–1132.
22. **Nomura, S., G. Khoury, and G. Jay.** 1983. Subcellular localization of the simian virus 40 agnoprotein. J. Virol. **45**:428–433.
23. **Perricaudet, M., G. Akusjärvi, A. Virtanen, and U. Pettersson.** 1979. Structure of two spliced mRNAs from the transforming region of human subgroup C adenoviruses. Nature (London) **281**:694–696.
24. **Pettersson, U., and G. Akusjärvi.** 1983. Molecular biology of adenovirus transformation, p. 83–131. *In* G. Klein (ed.), Advances in viral oncology, vol. 3. Raven Press, New York.
25. **Spindler, K. R., and A. J. Berk.** 1984. Rapid intracellular turnover of adenovirus 5 early region 1A proteins. J. Virol. **52**:706–710.
26. **Symington, J. S.** 1984. Electrophoretic transfer of proteins from two dimensional gels to sheets and their detection, p. 127–168. *In* J. E. Celis and R. Bravo (ed.), Two dimensional gel electrophoresis: methods and applications. Academic Press, Inc., New York.
27. **Virtanen, A., P. Aleström, H. Persson, M. G. Katze, and U. Pettersson.** 1982. An adenovirus agnogene. Nucleic Acids Res. **10**:2539–2548.