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Synthesis of the Adenovirus-Coded DNA Binding Protein in Infected Cells

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Synthesis of the 75K (75K indicates a molecular weight of 70,000 to 75,000) DNA binding protein, an early virus-coded protein in adenovirus 2-infected KB cells, and its regulation were studied by using a radioimmune precipitation inhibition assay. The protein was first detected at 4 h postinfection and accumulated at an exponential rate. An arrest of further synthesis (accumulation) was observed at 10 to 11 h postinfection, coinciding with the onset of synthesis of late virion proteins. In contrast, when the infected cells were treated with 25 μg of arabinosyl cytosine per ml to block viral DNA replication, the synthesis of 75K protein did not cease but continued for up to 36 h postinfection. The synthesis of 75K protein in cells after release from a cycloheximide block (2 to 9 h postinfection) was analyzed. Increased amounts of early adenovirus-specific mRNA accumulate in infected cells during a cycloheximide block (Parsons and Green, 1971). However, cycloheximide treatment did not produce increased levels of 75K protein, and an abrupt arrest of 75K protein formation was again observed at the time of synthesis of late virion proteins. Partition of the 75K protein between the nuclear and cytoplasmic fractions during the course of infection was studied. The 75K protein appeared first in the cytoplasm and then in the nucleus after a slight lag. Accumulation of the 75K protein continued both in the cytoplasm and nucleus, with higher levels being found in the cytoplasm.

Several virus-specific polypeptides that are not viron components are synthesized in human cells infected with adenovirus 2 or 5 (Ad2, Ad5). These new polypeptides were identified by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels of extracts of infected cells pulse labeled at early times after infection (1, 2, 9, 14, 15, 20). They range in size from 75,000 to 10,000 daltons, based on their migration in gels. Only one of these early polypeptides with a molecular weight of 70,000 to 75,000 (referred to as 75K protein) has been purified to date. It binds to single-stranded but not double-stranded DNA and has been termed the "DNA binding protein" (17, 18). The 75K proteins coded by Ad2 and Ad5 have similar properties and molecular weights (13). Use of Ad5 temperature-sensitive mutants has indicated that the 75K protein is virus coded and is necessary for viral DNA replication (19). The viral gene coding for it has been mapped, by the use of temperature-sensitive mutants, in the *Eco*R1 B fragment of the adenovirus genome (T. Grodzicker, C. W. Anderson, and J. Sambrook, personal communication). Another polypeptide of molecular weight 45,000 elutes from DNA-cellulose columns together with the 75K protein and appears to be a degradation product of the 75K protein (13).

We have recently shown by radioimmune precipitation and radioimmune precipitation inhibition (RIPI) that the Ad2 75K protein is virus specific (5). The method involved the use of purified ^{3}H-labeled 75K protein and its homologous antibody present in sera from hamsters immunized with extracts of Ad2-simian virus 40-induced tumors (4, 5). We have now developed the RIPI method for the accurate quantitation of the 75K protein in order to study its synthesis. The present communication describes our findings on the synthesis of this protein during infection and its modulation by interference with DNA or protein syntheses.

MATERIALS AND METHODS

Isolation of labeled 75K protein. Labeled 75K protein was isolated from Ad2-infected KB cells and purified by DNA-cellulose chromatography as previously described (5, 17) with two modifications. We found that the trailing edge of the 0.6 M peak eluting from the DNA-cellulose column contained only 75K protein, whereas the leading edge of the peak contained also the 45K protein. Fractions from the trailing edge of the peak only were pooled and used as the source of 75K protein for the RIPI assay. In

addition, labeling was performed with [^{35}S]methionine instead of [^{3}H]leucine, yielding preparations with higher specific radioactivity. Infected cells were incubated with 5 μCi of [^{35}S]methionine per ml (210 to 466 Ci/mmol) in methionine-free Eagle minimal essential medium containing 5% horse serum. ^{35}S-labeled 75K protein was prepared monthly to minimize radioactive decay.

Experimental infection of KB cells and preparation of cell extracts. Suspension cultures of KB cells were infected with purified Ad2 at a multiplicity of 200 PFU/cell as previously described (17). Infected cells were maintained at a concentration of 3×10^5 cells/ml in Eagle minimal essential medium containing 5% horse serum. To prepare whole-cell extracts for assay of 75K protein, 9×10^6 cells (30 ml) were withdrawn at specified times and washed with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS). The cells were resuspended in 1 ml of PBS containing 1% Triton X-100 and stored at −5 C. Before assay, suspensions were disrupted for 3 min at full power in a Raytheon DF-101 sonic oscillator and clarified at 2,000 × *g* for 10 min at 4 C. The supernatant fractions were collected for assay. For the preparation of cytoplasmic and nuclear extracts, washed samples of 9×10^6 cells were resuspended in 1 ml of PBS containing 1% Triton X-100 at room temperature, and after 5 min nuclei and cytoplasm were separated by centrifugation at 800 × *g* for 10 min. The nuclei were sonicated and clarified as described for whole-cell extracts.

To prepare extracts for complement fixation tests, 10-ml samples of infected cells (3×10^6 cells) were washed with PBS, resuspended in 0.5 ml of veronal buffer (16), sonicated for 3 min, and clarified at 2,000 × *g* for 10 min. The supernatant fractions were collected for assay.

RIPI. The double-antibody method for radioimmune precipitation of labeled purified 75K protein and the source of antisera have been described (5). In the RIPI assay, 10-μg aliquots of immune hamster immunoglobulin G (IgG) (supplemented with 70 μg of "carrier" normal hamster IgG) were preincubated with various amounts of extracts of unlabeled infected cells sampled at various times postinfection to block antibody activity. Preincubation was for 1 h at 37 C in PBS containing 1% Triton X-100 (vol/vol) and 1% sodium deoxycholate (wt/vol) in conical 0.75-ml centrifuge tubes (Sarstedt, Princeton, N.J.). The final reaction volume was 200 μl. Labeled 75K protein (10,000 counts/min) was then added, and incubation was continued for 1 h. Finally, 30 μl of goat serum antihamster IgG was added, and the precipitates formed after 2 h of incubation at 37 C were centrifuged, washed, and counted as previously described (5). Background precipitation values (obtained with 80 μg of normal hamster IgG) were subtracted in each determination. The degree of inhibition observed with extracts of unlabeled infected cells was calculated as percentage of control by comparison with reactions substituting PBS for cell extracts.

Titration of virion proteins in extracts of infected cells by the complement fixation. The micro-complement fixation test of Sever (16) was used for the titration of virion proteins as previously described (6). Eight units of goat antibody to purified Ad2 virions was used with 2 U of complement.

RESULTS

Quantitation of 75K protein in extracts of infected cells by the RIPI assay. The RIPI assay is one of the few methods available to accurately measure early proteins present in relatively low concentrations in infected cells. We have developed a RIPI assay for quantitating intracellular 75K protein using ^{35}S-labeled 75K protein and its homologous antibody from hamsters immunized with extracts of tumors induced by an Ad2-simian virus 40 hybrid. To standardize the assay, a precipitation reaction was performed first with increasing amounts of immune hamster IgG and 10,000 counts/min of ^{35}S-labeled 75K protein (Fig. 1A). Ten micrograms of immune IgG, which precipitated about one-half of the 10,000-counts/min input (Fig. 1A), was used subsequently in the RIPI assays as follows. Aliquots of 10 μg of immune IgG, supplemented with 70 μg of normal IgG, were preincubated with increasing volumes of unlabeled infected cell extract for 1 h at 37 C. ^{35}S-labeled 75K protein (10,000 counts/min) was then added, the reaction mixtures were further incubated, and the assay was continued as described in Materials and Methods. A representative inhibition curve obtained with different volumes of a 20-h infected cell extract is shown in Fig. 1B. In this instance, 50% inhibition of radioimmune precipitation was obtained with 1.03 μl of cell extract; the volume of extract causing 50% inhibition is designated V_{50}. Quantitation of the 75K protein content in cell extracts is based upon the determination of V_{50} from curves obtained for the individual extracts, as described in Fig. 1B, using different extract volumes. Since the amount of 75K protein present in the extract is inversely related to V_{50}, the 75K protein titer is therefore expressed in inhibitory units as $1/V_{50} \times 100$; multiplication by 100 is used to avoid fractional values.

The fidelity of the RIPI assay was tested by reconstruction reactions with known amounts of unlabeled 75K protein. The 0.6 M eluate, from a DNA-cellulose column, which is the source for labeled 75K protein, contains also unlabeled host components (Sugawara, Gilead, and Green, unpublished data). Unlabeled 75K protein was therefore purified from the 0.6 M eluate to homogeneity (Sugawara, Gilead, and Green, manuscript in preparation). Known amounts of pure 75K protein were mixed with

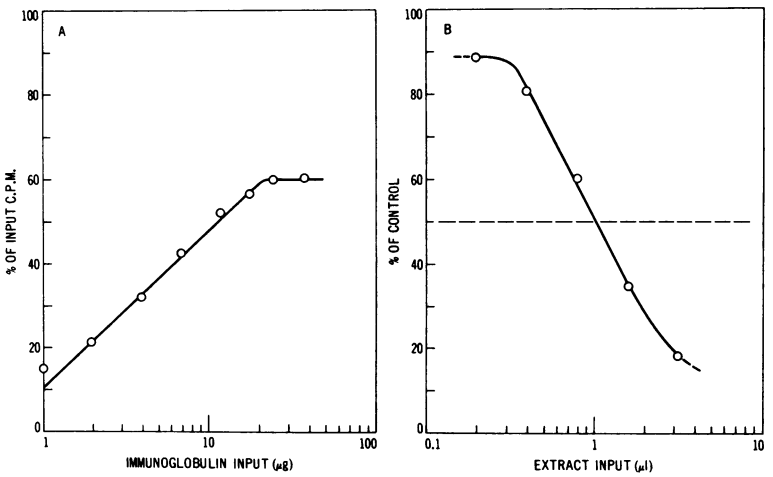

FIG. 1. *Quantitation of the RIPI assay. (A) Standard precipitation curve of ^{35}S-labeled 75K protein. Aliquots of 75K protein (10,000 counts/min) were mixed with increasing amounts of immunoglobulin from immunized hamsters (immune IgG), supplemented with IgG from normal hamsters (normal IgG) to give 80 μg of total IgG, in reaction mixtures containing PBS, 1% Triton X-100, and 1% sodium deoxycholate. After 1 h of incubation at 37 C, 30 μl of goat serum anti-hamster IgG was added and precipitates formed after 2 h of incubation at 37 C were centrifuged, washed twice with PBS containing 1% Triton X-100 and 1% sodium deoxycholate, dissolved in 0.25 M acetic acid, and counted in a scintillation spectrometer. Background radioactivity due to nonspecific precipitation, measured with 80 μg of normal IgG alone, was subtracted. (B) The RIPI assay. Increasing volumes of a 20-h infected cell extract were preincubated with 70 μg of "carrier" normal IgG and 10 μg of immune IgG, shown in (A) to precipitate 50% of the 10,000-counts/min input of ^{35}S-labeled 75K protein, for 1 h at 37 C in the buffer described in (A). ^{35}S-labeled 75K protein (10,000 counts/min) was added, and incubation was continued for an additional 1 h at 37 C. Goat anti-hamster IgG (30 μl) was added, and the precipitates formed after 2 h at 37 C were treated was described in (A). The background counts per minute precipitated with 80 μg of normal IgG was subtracted, and the percentage of control was calculated by comparison with a reaction mixture in which PBS was substituted for the infected-cell extract.*

extracts of uninfected KB cells (9×10^6 cells/ml) prepared as described for infected cells. A plot of the concentration of 75K protein in the extracts versus the inhibitory titer measured in the RIPI test ($1/V_{50} \times 100$) was linear (Fig. 2), even at low 75K protein concentrations. The smallest detectable titer of 75K protein in infected cell extract is 0.5 inhibitory units. One unit corresponds to a concentration of 1.4 μg of 75K protein per ml of extract, according to the plot in Fig. 2. However, we have not used this plot to convert the relative inhibitory titers into actual 75K protein concentrations in this paper since the reconstruction mixtures are not identical to extracts of infected cells. Purified 75K protein shows a tendency to aggregate and may be more prone to inactivation in the reconstructed extracts than intracellular 75K protein in extracts of infected cells.

Kinetics of synthesis of 75K protein in infected cells in the absence and presence of Ara C. The 75K protein is an early protein essential for viral DNA replication (19). It is synthesized before the onset of viral DNA replication, and also when viral DNA synthesis is inhibited with arabinosyl cytosine (Ara C) (17, 18). The kinetics of accumulation of the 75K protein in infected KB cells were examined (Fig. 3). It was first detected at 4 h postinfection, and its synthesis (accumulation) was exponential up to 11 h postinfection, at which time it stopped. There was a slow decline in the amount of 75K protein after 11 h, perhaps due to lysis of some cells late during productive infection or to inactivation of 75K protein molecules in vivo. In contrast, the initial rate of accumulation of 75K protein in Ara C-treated cells was much slower, but did not exhibit the arrest observed in uninhibited cultures (Fig. 3). The synthesis of 75K protein continued up to 36 h postinfection, at which time Ara C-treated cells accumulated four times more 75K protein than the highest level observed in uninhibited cultures.

Synthesis of 75K protein in cells after release from a cycloheximide block and effect of Ara C. Cells treated early after infection with cycloheximide do not synthesize viral DNA (18) and accumulate up to five times as much early viral mRNA as untreated infected cells (12). It was of interest to determine whether increased levels of viral mRNA would result in an accelerated accumulation of 75K protein after release from inhibition. Two pairs of cultures, A and B, were infected. One A

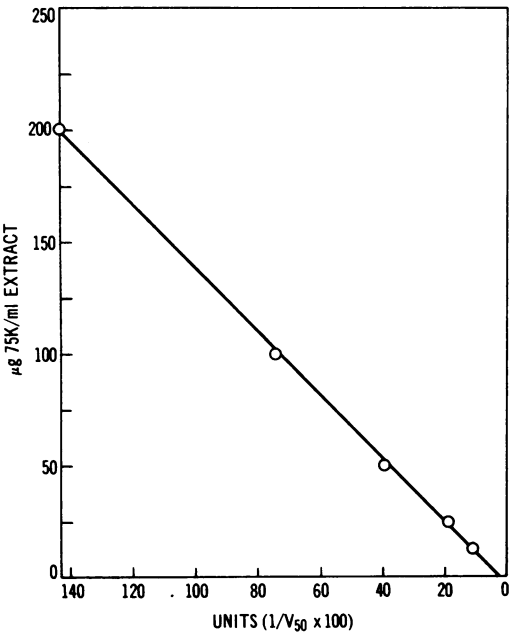

FIG. 2. *Linearity of RIPI assay. A series of extracts containing known concentrations of 75K protein was prepared by adding different amounts of purified unlabeled 75K protein to an extract of uninfected KB cells (9 × 10⁶ cells per ml of extract). The reconstructed extracts were assayed by the RIPI assay. The inhibitory titer measured in the assay ($1/V_{50} \times 100$) was plotted against 75K protein concentration (micrograms per milliliter of extract).*

culture was treated with cycloheximide (25 μg/ml) at 2 h postinfection, and the other A culture served as control. At 9 h postinfection, both A cultures were washed twice with warm medium and incubated in fresh medium lacking inhibitor. One B culture received 25 μg of cycloheximide per ml at 2 h postinfection, while the other B culture received 25 μg of Ara C per ml at 1 h postinfection. Both B cultures were washed twice at 9 h postinfection with warm medium containing 25 μg of Ara C per ml and incubated in medium containing 25 μg of Ara C per ml. Samples of 30 ml (9 × 10⁶ cells) were removed from A and B culture pairs throughout the experiment for analysis of 75K protein. The A cultures (no Ara C) were sampled also for assay of late virion proteins by the complement fixation test. The kinetics of accumulation of 75K protein in A cultures are presented in Fig. 4A. The synthesis of 75K protein in the control A culture (not treated with cycloheximide) exhibited again the arrest of 75K protein synthesis. After release from inhibition, the cycloheximide-treated A culture exhibited a more rapid rate of accumulation of the 75K protein as compared with the control A culture. However, a somewhat faster onset of arrest was observed, resulting in a lower final titer. The time of arrest in both A cultures coincided with the onset of synthesis of late virion proteins, measured by CF. In contrast, no arrest in synthesis of the 75K protein was observed in the B culture released from cycloheximide and then treated with Ara C immediately after release. Again, the released culture exhibited a somewhat faster initial rate of accumulation as compared with the parallel B culture that was not treated with cycloheximide.

Partition of the 75K protein between cytoplasm and nucleus. The cytoplasmic fraction from Ara C-treated infected cells harvested at 24 h postinfection is routinely used for the purification of the 75K protein. Unlike the nuclear fraction, chromatography of the cytoplasmic fraction on a DNA-cellulose column yields almost pure labeled 75K protein (17). It was of interest to determine the kinetics of synthesis and the partition of 75K protein in the cytoplasmic and nuclear fractions. Infected cells were sampled at various times after infection, and

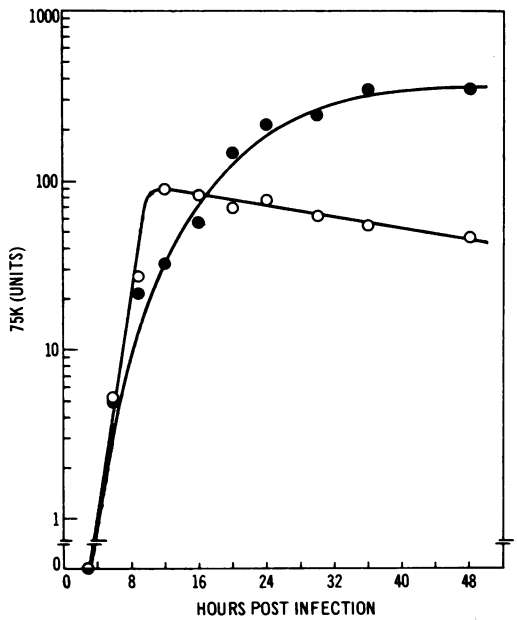

FIG. 3. *Synthesis of 75K protein in Ad2-infected KB cells in the presence and absence of Ara C. A suspension culture of KB cells was infected with 200 PFU of Ad2 per cell, divided into two portions, and maintained at 300,000 cells/ml in Eagle minimal essential medium containing 5% horse serum. Ara C was added to final concentration of 25 μg/ml at 1 h postinfection to one culture. Samples (30 ml) were removed at the indicated times, centrifuged, washed with PBS, and resuspended in 1 ml of PBS containing 1% Triton X-100. Cell suspensions were sonicated, clarified, and assayed for 75K protein by the RIPI assay. Symbols: ○, infected culture, no drug; ●, infected culture treated with Ara C.*

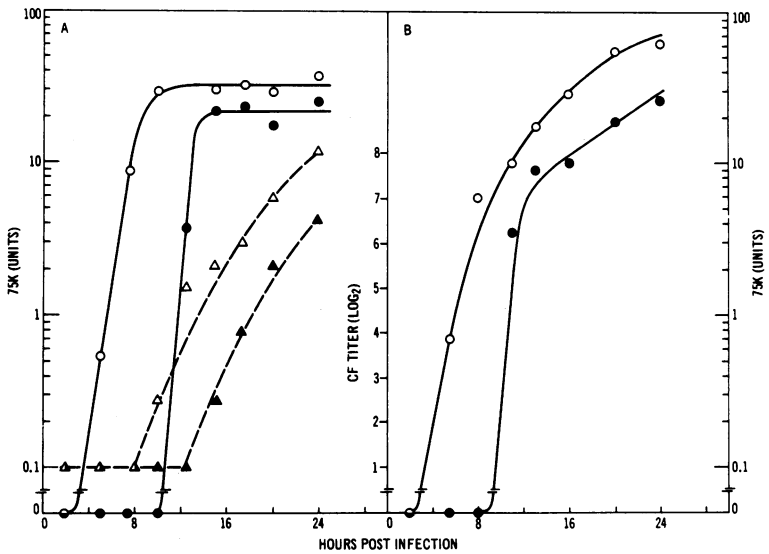

FIG. 4. *Synthesis of 75K protein and virion proteins in Ad2-infected KB cells after release from a cycloheximide block in the absence (A) and the presence (B) of Ara C. (A) A suspension culture of KB cells was infected and divided into two. One culture received 25 μg of cycloheximide per ml at 2 h postinfection, and the other culture served as control. Both cultures were centrifuged at 9 h postinfection, washed twice with warm medium, and further incubated at 37 C. Samples (30 ml) were removed from each culture at the indicated times, and extracts were prepared and assayed for 75K protein content by the RIPI assay. Ten-milliliter samples were removed also for virion protein assay by the complement fixation test. (B) A suspension culture of KB cells was infected and divided into two portions. One culture received 25 μg of Ara C per ml at 1 h postinfection, and the second received 25 μg of cycloheximide per ml at 2 h postinfection. Both cultures were centrifuged at 9 h postinfection, washed twice with warm medium containing 25 μg of Ara C per ml, and further incubated in medium containing 25 μg of Ara C per ml. Samples (30 ml) were removed and extracts were prepared and assayed for 75K protein by the RIPI assay. 75K protein titer: (A) ○, No drug; ●, cycloheximide treated (2 to 9 h). (B) ○, Ara C (1 to 24 h); ●, cycloheximide (2 to 9 h), Ara C (9 to 24 h). Virion protein titer: △, No drugs; ▲, cycloheximide treated.*

the cytoplasmic and nuclear fractions were prepared as described in Materials and Methods. The accumulation of 75K protein in cytoplasmic and nuclear fractions of untreated infected cells (Fig. 5A) and Ara C-treated cells (Fig. 5B) showed the same overall kinetics observed for whole cells (Fig. 3). There was some lag in the appearance of the 75K protein in the nucleus as compared with the cytoplasm, and the amount of 75K in the cytoplasm always exceeded that present in the nuclear fraction, except for late times in Ara C-treated cultures.

DISCUSSION

Several investigations have identified early and late adenovirus-specific polypeptides in infected human cells by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis of extracts of infected cells labeled with [^{35}S]methionine (1, 2, 9, 14, 15, 20). Early virus-specific protein synthesis was detected starting at 2.5 to 4 h postinfection, and maximum rates of synthesis occurred at 6 to 12 h postinfection. A gradual cessation was observed at later times, and a "switch-over" to late virion protein synthesis occurred at 12 to 16 h postinfection. The electrophoretic study of 75K protein, termed $ICSP_3$ or E_1 by Russel et al. (14, 15) or Maizel et al. (9, 20), respectively, was complicated by the presence of a host protein band that migrated close to the 75K protein band (9, 20). The present study, measuring 75K protein synthesis by a different method, is consistent with the general pattern of early adenovirus protein synthesis that was previously observed by gel analyses. The method used in our study has the advantage that it accurately measures the net accumulation of immunologically active protein molecules and is not influenced by possible variations in the equilibration and concentration of radioactive amino acid pools and by the background of similarly migrating host proteins. By immunological assay, we observed an early synthesis of 75K protein that is apparently influenced by later events in the viral replication cycle. The synthesis of 75K protein occurred from 4 to 11 h postinfection, with an arrest at 11 h that coincided with the onset of virion protein synthesis. However, the synthesis of the 75K protein continued without arrest

if the synthesis of viral DNA and consequently late proteins was inhibited. Some investigators have observed by gel electrophoresis a residual low level of synthesis of 75K protein at late times postinfection. This synthesis could be due to a certain degree of asynchrony in infection. Alternatively, since the RIPI test measures net accumulation of immunologically reactive molecules rather than the rate of synthesis, we cannot completely rule out the existence of a residual level of synthesis. The arrest in accumulation of 75K protein observed in the present study can, therefore, reflect a steady-state level whereby residual synthesis is masked by the inactivation of some existing 75K protein.

Progeny viral DNA synthesis is blocked in Ad2-infected cells treated with cycloheximide early after infection (8), leading to the accumulation of five times as much early viral mRNA in uninhibited cells (12). We do not know whether the viral mRNA that codes for the 75K protein is increased in amount by this procedure. Release from a cycloheximide block produces an overall decrease in the rate of synthesis of host polypeptides and increase in the synthesis of several early adenovirus-specific polypeptides, as measured by gel electrophoresis of extracts of cells pulse labeled immediately after release from inhibition (M. Harter, G. Shanmugam, and M. Green, in preparation). By the RIPI assay, which measures the actual quantity of 75K protein, we observed an initially accelerated accumulation of 75K molecules after release from cycloheximide, but the accelerated rate was not maintained even in cells treated with Ara C to prevent arrest.

The gene coding for the 75K protein is located in *Eco*RI restriction fragment B (Grodzicker, Anderson, and Sambrook, personal communication). A 19 to 20S early mRNA mapped in this region (3) that is H-strand specific (W. Buettner, S. Veres-Molnar, and M. Green, manuscript in preparation) has the appropriate coding capacity for a 75,000-dalton polypeptide and is a class I early mRNA (3) by the nomenclature of Lucas and Ginsberg (10); i.e., it is absent or greatly reduced in amount at 18 h postinfection.

The arrest in 75K synthesis cannot be explained by the expected normal rate of decay of mRNA, but could be due to (i) more effective competition for available ribosomes by abundant late mRNA species or (ii) inhibition by late virion or virus-coded proteins. Inhibition of viral DNA synthesis by Ara C may allow continued transcription of the mRNA for 75K protein and/or the inhibition of synthesis of late virion proteins may prevent competition and/or regulation.

Regulated synthesis of virus-specific early proteins that is linked to DNA synthesis has been described in several other DNA virus systems. For example, the synthesis of phage T_4-

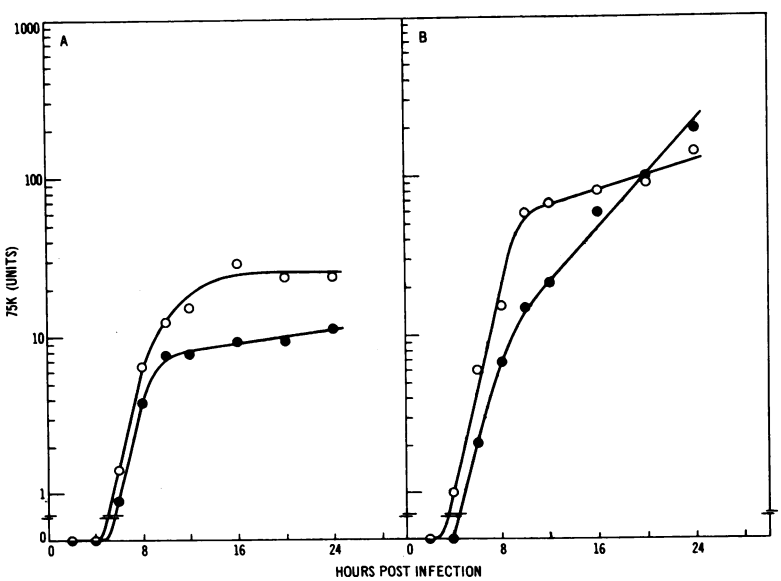

FIG. 5. *Partition of 75K protein in cytoplasmic and nuclear fractions of Ad2-infected cells in the absence (A) and presence (B) of Ara C. A culture of KB cells was infected and divided into two portions. One culture received 25 μg of Ara C per ml at 1 h postinfection, and the second culture was untreated. Samples (30 ml) were removed at the indicated times, washed, and resuspended in PBS containing 1% Triton X-100. Nuclear and cytoplasmic fractions were separated and assayed for 75K protein by the RIPI assay. Symbols: ○, Cytoplasmic fractions; ●, nuclear fraction.*

coded early enzymes ceases at 10 to 12 min after infection. When phage DNA synthesis is inhibited or delayed, such as by infection with phage T_4 amber mutants or UV-irradiated phage, early enzyme synthesis continues well beyond 10 to 12 min. The amount of enzymes at 60 min after infection may reach levels four to five times greater than after infection with wild-type T_4 phage (21). The synthesis of poxvirus-coded thymidine kinase, an "immediate" early protein, is also abruptly inhibited early after infection. Studies using inhibitors have suggested that a viral gene product synthesized after uncoating of virus (perhaps a "delayed" early protein) turns off the synthesis of thymidine kinase (11). The synthesis of herpesvirus type 1-specific polypeptides is coordinately regulated and sequentially ordered. Three classes, α, β, and γ, were described according to their times of synthesis. The synthesis of one or more functional α polypeptides is necessary to "switch on" the synthesis of β or γ polypeptides, and one or more β or γ polypeptides "switch off" the synthesis of α polypeptides (7).

The intracellular localization of virus-specific early proteins is of interest since it may provide clues regarding function. We have shown that the 75K protein is present in both the cytoplasm and nucleus, and the kinetics of accumulation provide guidelines for the isolation of maximum yields for further studies. However, conclusions regarding intracellular localization are subject to the usual cautions concerning leakage from the nucleus during cell fractionation. The lag in appearance of 75K protein in the nucleus and the association of 75K protein with rapidly sedimenting structures in the cytoplasmic and nuclear fractions (Gilead et al., unpublished data) suggest that the intracellular localization deduced from biochemical fractionation may be valid. Immunoflourescence studies with fixed-cell preparations using antibody to purified 75K protein may resolve this important point.

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