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Detection of Adenovirus Type 2-Induced Early Polypeptides Using Cycloheximide Pretreatment to Enhance Viral Protein Synthesis

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[^{35}S]methionine-labeled polypeptides synthesized by adenovirus type 2-infected cells have been analyzed by polyacrylamide gradient gel electrophoresis and autoradiography. Cycloheximide (CH) was added to infected cultures to accumulate early viral mRNA relative to host cell mRNA. This allowed viral proteins to be synthesized in increased amounts relative to host proteins after removal of CH and pulse-labeling with [^{35}S]methionine. During the labeling period arabinosyl cytosine was added to prevent the synthesis of late viral proteins. This procedure facilitated the detection of six early viral-induced polypeptides, designated EP1 through EP6 (early protein), with apparent molecular weights of 75,000 (75K), 42K, 21K, 18K, 15K, and 11K. Supportive data were obtained by coelectrophoresis of [^{35}S]- and [^{3}H]methionine-labeled polypeptides from infected and uninfected cells, respectively. Three of these early polypeptides have not been previously reported. CH pretreatment enhanced the rates of synthesis of EP4 and EP6 20- to 30-fold and enhanced that of the others approximately twofold. The maximal rates of synthesis of the virus-induced proteins varied, in a different manner, with time postinfection and CH pretreatment. Since CH pretreatment appears to increase the levels of early viral proteins, it may be a useful procedure to assist their isolation and functional characterization.

Adenovirus (Ad) type 2 infection of KB cells proceeds in two temporal stages of gene expression, early (before viral DNA synthesis commences) and late (17). Only a limited fraction of the viral genome is expressed early. Early viral proteins are important because they are involved in viral DNA replication, cell transformation, the switch from early to late gene expression, and inhibition of host DNA synthesis (W. S. M. Wold, W. Büttner, and M. Green, *in* D. P. Nayak [ed.], *Molecular Biology of Animal Viruses*, in press). The number of early Ad2 genes is not known. One approach to estimate the number of early viral genes is to determine the number of virus-specific proteins synthesized early after infection. Early viral proteins have been studied by labeling infected cells with [^{35}S]methionine (met), followed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and autoradiography. Walter and Maizel (20) identified three early Ad2 proteins, E_1, E_2, E_3, with molecular weights of 70,000 (70K), 19K, and 10K, respectively. The E_2 protein is glycosylated. The detection of early proteins by in vivo labeling is hindered by the large background of host cell protein synthesis. Bablanian and Russell (2) overcame this problem somewhat by coinfecting cells with Ad5 (closely related to Ad2, reference 9) and poliovirus in the presence of guanidine, a procedure that minimizes host cell protein synthesis. Four early Ad5 protein candidates were detected: ICSP-3 (64K), core 1 (46K), p-core 2 (20K), and ICSP-5 (16K); the functions of these Ad5 proteins and their correlations with Ad2 proteins are not clear.

We have approached the host cell background problem in a different manner. Cycloheximide (CH) has been shown to increase the content of Ad2 mRNA relative to cell mRNA (3, 5, 12). Assuming that an increase in viral mRNA relative to cell mRNA would lead to an increase in synthesis of viral proteins relative to cell proteins, we have studied the polypeptides synthesized by Ad2-infected KB cells after release from CH inhibition. During the labeling period, arabinosyl cytosine (AraC) was used to block viral DNA replication and thus the synthesis of late viral proteins (9; Wold et al., in press). By this procedure, we were able to detect six early viral-induced polypeptides in Ad2-infected KB cells. After completion of these studies, we learned that Chin and Maizel (personal communication) have detected six

proteins with similar mobilities by gel electrophoresis of fractionated extracts of HeLa cells infected by Ad2 and that Lewis et al. (Cell, in press) have observed six similar polypeptides after cell-free translation of Ad2 early mRNA.

MATERIALS AND METHODS

Chemicals. L-[^{35}S]met (100 to 200 Ci/mmol), L-[^{3}H]met (3 to 5 Ci/mmol), and Aquasol were purchased from New England Nuclear Corp., CH and AraC were from Sigma Chemical Co., and acrylamide, bisacrylamide, and SDS were from Bio-Rad Laboratories. All other materials were of analytical grade.

Virus infection and preparation of extracts for polyacrylamide gel electrophoresis. Suspension cultures of KB cells were grown in Eagle minimal essential medium containing 5% horse serum. For infection, cells were centrifuged and resuspended at 4×10^6 cells/ml in Eagle minimal essential medium without serum, and Ad2 (strain 38-2, plaque 4, free of adenovirus-associated viruses) was added at an input multiplicity of 100 (PFU/cell (14). After 1 h of adsorption at 37 C, cells were diluted to a density of 3×10^5 cell/ml with met-free Eagle minimal essential medium containing 5% horse serum. The cell culture was then divided into two portions, one-half was treated with CH (25 μg/ml), and both suspensions were incubated at 37 C. At 4 h postinfection (p.i.) (i.e., time of virus addition), AraC (20 μg/ml) was added to both cultures. After various periods of incubation, CH was removed by washing the cells three times at 37 C with met-free Eagle minimal essential medium plus AraC. Infected cells were then resuspended in fresh met-free Eagle minimal essential medium containing 20 μg of AraC per ml. After approximately 10 min of incubation at 37 C, cells were labeled with [^{35}S]met (2.5 to 5 μCi/ml) for 1 h. At the end of the labeling period, cells were centrifuged, washed twice with cold phosphate-buffered saline, and suspended in cold 10% trichloroacetic acid for 30 min. The precipitate was washed twice with cold 5% trichloroacetic acid twice with 0.05% trichloroacetic acid and solubilized in SDS sample buffer (1% SDS, 1% β-mercaptoethanol, 50 mM Tris-hydrochloride (pH 6.8), 10% glycerol, and 0.003% bromophenol blue). Mock-infected cells were treated as described above for infected cells, except that virus was not added.

Polyacrylamide gel electrophoresis and determination of molecular weights of labeled polypeptides. Protein samples from infected and uninfected cells were analyzed by SDS-polyacrylamide slab gel electrophoresis (11). Gradient gels of 5 to 22% polyacrylamide containing SDS were formed as slabs 1.3 mm thick and 130 mm long between glass plates. Solubilized proteins in SDS sample buffer were heated at 100 C for 2 min, and 100,000 to 200,000 counts/min was applied to each well. Electrophresis was performed at a constant current of 9 mA. After electrophoresis, slab gels were dried under vacuum and exposed to Kodak single-coated panoramic dental X-ray film (DF-85) for 2 to 7 days.

Molecular weights of labeled polypeptides were determined by electrophoresis in slab gels with protein standards in adjacent wells. Proteins used as molecular weight standards (5 to 10 μg) included P31 (31K) derived from the Moloney murine leukemia virus (16), Ad2 hexon, Ad2 fiber, bovine serum albumin, ovalbumin, γ-globulin (heavy chain), γ-globulin (light chain), chymotrypsinogen, and cytochrome *c*. Moloney murine leukemia virus and Ad2 proteins were purified as described previously (15). After electrophoresis, proteins were stained for 3 h with 0.06% Coomassie blue in methanol-water-acetic acid (5:4:1). The gels were destained for 6 h in methanol-water-acetic acid, soaked for 30 min in 5% glycerol, and dried under vacuum, and autoradiograms were prepared as described above. The migration distance of each protein relative to that of cytochrome *c* was determined (7).

Coelectrophoresis in cylindrical gels of labeled polypeptides from Ad2- and mock-infected cells. Polypeptides from Ad2- and mock-infected cells were labeled with [^{35}S]met (2.5 to 5 μg/ml) and [^{3}H]met (5.5 μCi/ml), respectively, and coelectrophoresed in cylindrical gels. Proteins were precipitated from cell extracts with 20% trichloroacetic acid and washed twice with 5% trichloroacetic acid and then with 0.05% trichloroacetic acid. The precipitate was then dissolved in SDS sample buffer, heated at 100 C for 2 min, and electrophoresed at 2 mA/gel for 3 h in cylindrical gels (0.6 by 10 cm) containing 10% polyacrylamide and 0.26% bisacrylamide according to Laemmli (11). Gels were fractionated into 1-mm slices with a Gilson gel fractionator and counted in 10 ml of Aquasol in a Beckman liquid scintillation counter. The spillover of ^{35}S into the ^{3}H channel (5 to 6%) was calculated using appropriate standards, and the ^{3}H radioactivity was corrected accordingly. Protein markers of known molecular weights were electrophoresed on parallel gels, and molecular weights of polypeptides were determined by the method of Weber and Osborn (21).

RESULTS

Effect of cyloheximide pretreatment on the polypeptides synthesized by Ad2-infected KB cells. To examine the polypeptides induced by Ad2 infection and to test the effect of CH pretreatment, Ad2- and mock-infected cells were labeled for 1 h with [^{35}S]met at 9 to 10 h p.i., and the polypeptides were resolved by polyacrylamide slab gel electrophoresis as described in Materials and Methods. The autoradiograms are shown in Fig. 1. Comparison of polypeptides synthesized by mock-infected (Fig. 1D) and infected (Fig. 1C) cells pretreated with CH revealed the presence of six polypeptides specific for infected cells. These are designated EP1 through EP6 (early protein) in inverse order of their migration rate. EP1, EP3, EP4, and EP6 were easily identified as polypeptides unique to infected cells. EP2 was more difficult to visualize because of the host protein background. As described below, the maximum rates of synthesis of the different early proteins

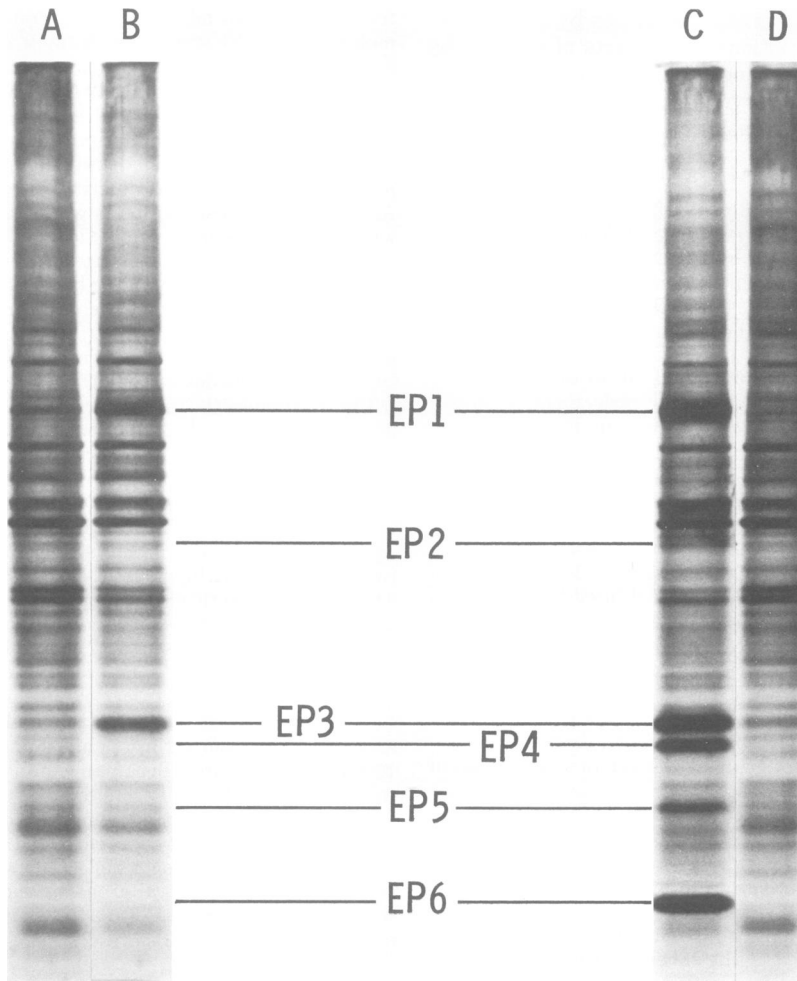


FIG. 1. *Comparison of [^{35}S]met-labeled polypeptides synthesized by Ad2- and mock-infected KB cells, with and without CH pretreatment. Infected and mock-infected cell cultures were split into two portions; one was treated with CH (25 μg/ml) at 1 to 8.5 h p.i., and the second received no CH. At 4 h p.i. AraC (20 μg/ml) was added to both CH-treated and untreated cultures. After washing the cells at 8.5 h to remove CH, the cells were pulse-labeled at 9 to 10 h in medium containing [^{35}S]met (2.5 μCi/ml) and AraC. Samples were electrophoresed on gradient gels and autoradiographed as described in the text. (A) Mock-infected cells; (B) Ad2-infected cells; (C) Ad2-infected cells treated with CH; and (D) mock-infected cells treated with CH.*

varied with time after infection. EP2 is clearly apparent in Fig. 2.

Comparison of Fig. 1C (CH pretreated) with Fig. 1B (not treated with CH) shows that pretreatment with CH greatly enhanced the synthesis of EP4 and EP6 at 9 to 10 h, as well as that of EP1, -2, -3, and -5, although not as dramatically. CH pretreatment did not perceptibly affect the number of bands found in mock-infected cells (Fig. 1A and D). The combination of CH pretreatment and virus infection did not influence the synthesis of most host polypeptides, although a few very large host proteins (about 100K) did seem to be synthesized in reduced amounts in infected CH-pretreated cells.

Although EP2 was apparent in infected cells pretreated with CH and labeled at 9 to 10 h p.i. (Fig. 1), larger amounts of EP2 can be seen by optimizing the period of CH treatment and labeling. A series of experiments was performed to investigate the time of maximal synthesis of each early protein in Ad2-infected cells, as discussed in a later section. These experiments showed that the synthesis of EP2, after removal of CH, was maximal at 4.5 to 5.5 h, whereas that of the other EPs was greater at 9 to 10 h p.i. One such experiment is illustrated in Fig.

2, which shows autoradiograms and corresponding densitometer tracings of electrophoresed Ad2 polypeptides labeled 4.5 to 5.5 and 9 to 10 h p.i. Figure 2 also shows the electrophoretic profile of mock-infected polypeptides labeled 9 to 10 h p.i. A distinct peak of EP2, clearly absent from mock-infected cells (Fig. 2B), is visible with infected cells labeled at 5.5 h p.i. (Fig. 2C). The EP2 peak is less apparent at 10 h p.i. (Fig. 2A). The EP4 and EP5 peaks are barely apparent at 5.5 h (Fig. 2C) but are readily discernible at 10 h p.i. (Fig. 2A). These experiments have been repeated several times with consistent results.

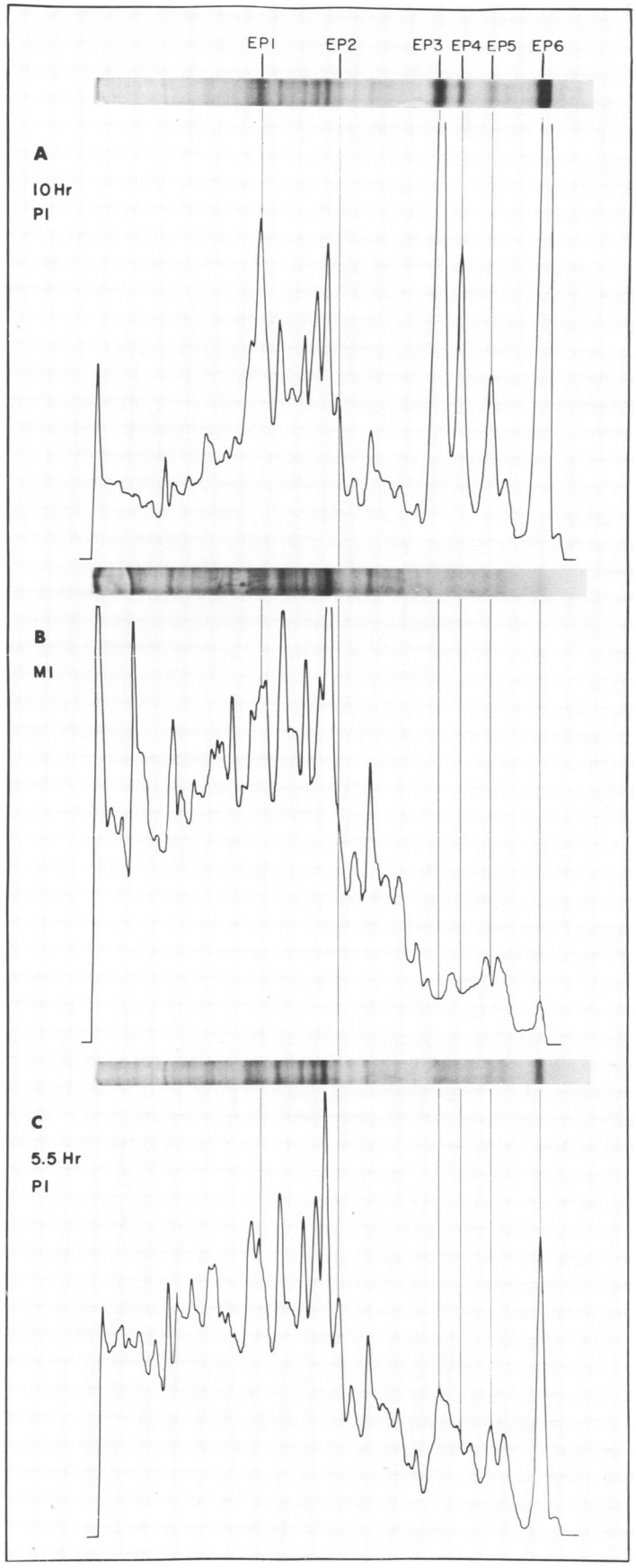
To exclude the possibility that late viral proteins are synthesized under our labeling conditions in the presence of AraC, we compared the pattern of proteins synthesized in the presence of AraC after release from CH block with proteins synthesized at late stages of infection. Figure 3 indicates that well-defined late proteins were not detectably synthesized in the presence of AraC. Since some labeled polypeptides have a mobility similar to EP5, we cannot exclude the possibility that EP5 is a late protein synthesized in spite of AraC. However, this is unlikely, since AraC did prevent the synthesis of other late proteins.

Molecular weight estimates of early proteins. The apparent molecular weights of EP1 through EP6 were estimated, based on their mobilities in SDS gels compared to 10 marker proteins ranging in size from 11K to 120K. Figure 4 shows the determination of EP1 and EP3 through EP6 molecular weights, using a cell extract labeled between 8 and 9 h p.i. EP2 was determined in a separate experiments using a cell extract labeled 4.5 to 5.5 h p.i., the period of maximal synthesis of EP2. The molecular weight standards migrated as a linear function of the logarithm of the molecular weight (Fig. 4). From these data we conclude that the molecular weights of EP1 through EP6 are, respectively: 75K, 42K, 21K, 18K, 15K, and 11K (see Table 1).

Rate of synthesis of early polypeptides in Ad2-infected cells after different periods of treatment with CH. The rate of synthesis of these early polypeptides, with and without CH pretreatment, was analyzed at various times p.i. CH was added to one-half the cultures at 1 h p.i., and AraC was added to all cultures except A and B at 4 h p.i. CH was removed from the pretreated cultures at 90-min intervals, and proteins were labeled for 1 h with [^{35}S]met in the presence of AraC and analyzed by gel electrophoresis and autoradiography. Figure 5 presents autoradiograms from a representative experiment. The numbers at the bottom are the times p.i. when the pulse was completed. Gels B, D, F, H, and J display polypeptides synthesized by Ad2-infected cells treated with CH, whereas A, C, E, G, and I show those synthesized by Ad2-infected cells not treated with CH. To quantitate the relative amounts of early polypeptides, densitometric tracings of each gel autoradiogram were obtained, and the area under each peak was measured.

The results of these densitometric quantitations are summarized in Fig. 6, which shows the relative amounts of EP1 through EP4 and EP6 synthesized at different times p.i. with and without pretreatment with CH. EP5 was difficult to quantitate because of closely migrating cellular polypeptides and is not shown in Fig. 6. However, EP5 can be detected in the 1-h pulse-labeled samples 7 h p.i. and reached a maximum rate of synthesis at about 10 h p.i. (see Fig. 1 and 2). The synthesis of EP1 was apparent in both CH-treated and untreated cultures. In untreated cultures a plateau was reached at 7 h p.i. The rate of synthesis of EP1 was higher in CH-treated cells and, under our conditions, reached a plateau by 7 h p.i. In most experiments, the appearance of EP1 changed from a sharp, well-defined band into a somewhat diffuse band at 10 h (although this is not shown clearly in Fig. 5); a similar phenomenon has been reported for the Ad2-induced 75K (72K in reference 1) polypeptide (1). It was difficult to detect EP2 without CH pretreatment. With CH treatment, EP2 synthesis was maximal at 4 to 5.5 h p.i. (see Fig. 2) and decreased at 8.5 to 10 h p.i. CH treatment surprisingly inhibited the rate of EP3 synthesis at 4 and 5.5 h. The synthesis of EP3 was readily apparent at 4 h p.i. in untreated cells and at 5.5 h in CH-treated cells, with maximal rates at 5.5 to 7 h p.i. in untreated cells and 7 to 8.5 h p.i. in CH-treated cells. The synthesis of EP4 and EP6 was barely detectable in untreated cells but was markedly enhanced in CH-treated cells. Synthesis was maximum at 7 h, with a slight decrease occurring subsequently. Pulse-chase experiments (Y. Jeng et al., unpublished data) suggest that the half-lives of the EPs under study are long and do not differ grossly, indicating the EP turnover was not a significant factor in these experiments.

Coelectrophoresis of polypeptides synthesized by mock-infected and Ad2-infected cells pretreated with CH. The autoradiograms and densitometric tracings illustrated in Fig. 1 through 6 indicate that Ad2-infected cells contain six unique polypeptides. To obtain additional evidence for this, ^{35}S- and ^{3}H-labeled


EP1
EP2
EP3
EP4
EP5
EP6
A
10 Hr
PI
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C
5.5 Hr
PI

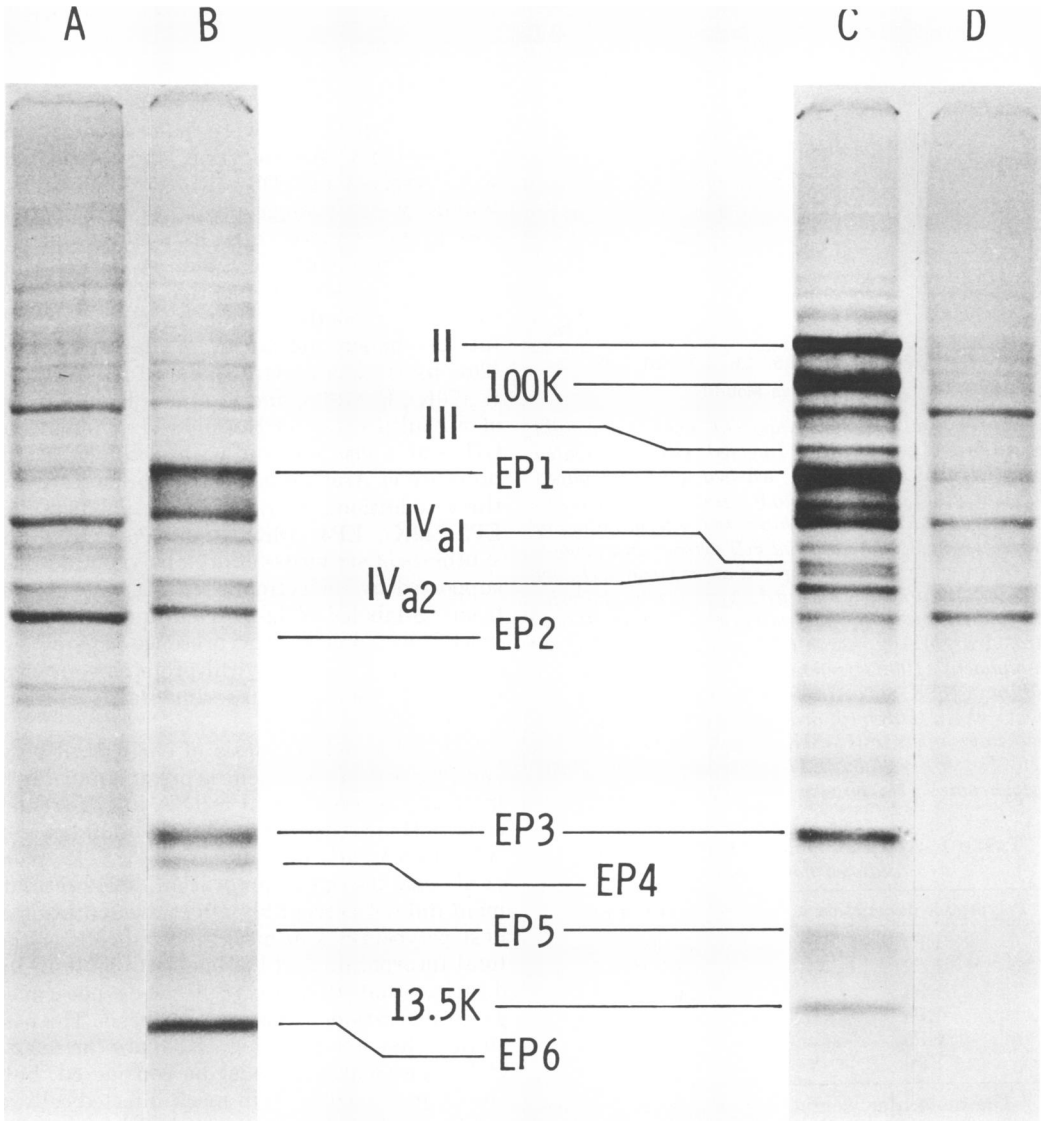

FIG. 3. *Comparison of early Ad2 polypeptides synthesized in the presence of AraC after CH pretreatment with Ad2 polypeptides synthesized in the absence of AraC (no CH pretreatment). Cultures of Ad2-infected and mock-infected cells were prepared. At 1 h p.i., each culture was split equally, and CH (25 μg/ml) was added to one portion. At 4 h p.i., AraC (20 μg/ml) was added to the CH-treated cultures. At 8.5 h p.i., cultures containing CH and AraC were washed and resuspended in Eagle miminal essential medium containing AraC, and all cultures were labeled at 9 to 10 h p.i. with [^{35}S]met. Samples were processed as described in the text. (A) Untreated mock-infected cells; (B) Ad2-infected cells pretreated with CH; (C) untreated Ad2-infected cells; (D) mock-infected cells treated with CH. Several virus-induced late polypeptides, e.g., II, III, 100K, IVa1, IVa2, 13.5K, are designated in Fig. 3C, as deduced from the results of Anderson et al. (1).*

FIG. 2. *Densitometer tracings of autoradiograms after electrophoresis of [^{35}S]met-labeled polypeptides from Ad2- and mock-infected cells pretreated with CH. At 4 and 8.5 h p.i., cultures of Ad2- and mock-infected cells, maintained in the presence of CH from 1 h p.i., were washed and pulse labeled for 1 h in the presence of AraC. Samples were processed and electrophoresed, and autoradiograms were prepared as described in the text. (A) Ad2-infected cells pretreated with CH and labeled between 9 and 10 h p.i.; (B) mock-infected cells pretreated with CH and labeled between 9 and 10 h p.i. (an identical pattern was obtained with mock-infected cells labeled between 4.5 and 5.5 h p.i.); (C) Ad2-infected cells pretreated with CH and labeled between 4.5 and 5.5 h p.i.*

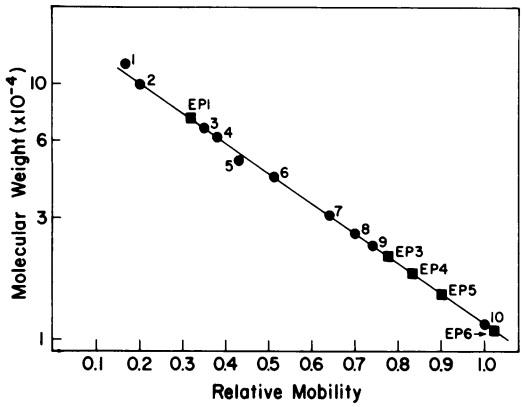

FIG. 4. *Estimation of apparent molecular weight of Ad2 early polypeptides. Infected cells were treated with CH at 1 to 7.5 h p.i., washed, and then pulse-labeled with [^{35}S]met at 8 to 9 h p.i. The culture was treated with AraC from 4 h p.i. to the termination of the experiment. The labeled cell extract and protein standards were electrophoresed on separate wells on a 5 to 22% gradient slab gel. After electrophoresis, the gel was processed as described in the text. Standards are: (1) Ad2, hexon (120K); (2) Ad2 nonvirion component (100K) (reference 1); (3) bovine serum albumin (68K); (4) Ad2 fiber (62K); (5) γ-globulin, heavy chain (50K); (6) ovalbumin (43K); (7) murine leukemia virus P31 (31K); (8) chymotrypsinogen (25.7K); (9) γ-globulin, light chain (23.5K); (10) cytochrome c (11.7K).*

TABLE 1. *Apparent molecular weights of Ad2-induced polypeptides*[a]

Polypeptide designation	Apparent mol wt
EP1	75,000
EP2	42,000[b]
EP3	21,000
EP4	18,000
EP5	15,000
EP6	11,000

[a] The molecular weight values were determined as described in the legend to Fig. 4.

[b] Determined from mobility of EP2, using extracts of CH-treated cells labeled 4.5 to 5.5 h p.i. based on the calculated values of EP1, EP3, EP4, EP5, and EP6.

polypeptides from Ad2- and mock-infected cells, respectively, were mixed and coelectrophoresed in cylindrical gels. Figure 7 illustrates the distribution of radioactivity in each gel fraction and the ratio of ^{35}S to ^{3}H radioactivity. Any increase in the ^{35}S/^{3}H ratio is indicative of viral-induced polypeptides. As can be seen, this ratio increased in the areas of EP1 (75K), EP3 (21K), EP4 (18K), and EP6 (11K). Slight increases were also observed in the areas of EP2 (42K) and EP5 (15K). These data are consistent with the above-mentioned results that detected six early polypeptides.

DISCUSSION

Ad2-infected cells were pulse-labeled with [^{35}S]met early after infection, and polypeptides were resolved by SDS-gel electrophoresis followed by autoradiography. Two polypeptides specific for infected cells, EP1 (75K) and EP3 (21K), were readily detected, as well as trace amounts of a third polypeptide, EP6 (11K). Using similar methods, but fractionating cells into cytoplasm and nuclei, Walter and Maizel (20) observed three viral-induced polypeptides, E_1 (70K), E_2 (19K), and E_3 (10K). Pretreatment of our cultures with CH, followed by removal of CH and pulse-labeling with [^{35}S]met in the presence of AraC, has allowed the detection of three additional viral-induced polypeptides, EP2 (42K), EP4 (18K), and EP5 (15K). The synthesis of six virus-induced polypeptides was supported by coelectrophoresis of polypeptides from ^{35}S-labeled, Ad2-infected and ^{3}H-labeled, mock-infected cells. The presence of AraC ensured that only early viral proteins were synthesized under our experimental conditions (see Fig. 3).

The enhanced synthesis of early viral polypeptides by CH treatment is presumably due to increased levels of viral mRNA in CH-treated cells. CH increases the relative abundance of Ad2 mRNA early after infection (3, 5, 12; Wold et al., manuscript in prepration). CH pretreatment did not perceptibly affect the synthesis of host polypeptides in mock-infected cells or the total incorporation of [^{35}S]met into protein, indicating that this procedure does not cause gross aberrations in protein synthesis. The possibility that some EPs (eg., EP5) are "artifacts" of CH pretreatment must be considered, but, since Eps are not seen in mock-infected cells, a combination of CH pretreatment plus virus infection is required for their production. Pulse-chase experiments suggested that all EPs are stable polypeptides and not cleavage products of larger polypeptides (Y. Jeng, M. Cartas, M. L. Harter, W. S. M. Wold, and M. Green, submitted for publication). These observations strongly suggest that the EPs we have described are bona fide Ad2-induced polypeptides and not artifacts of CH pretreatment. Therefore, this procedure may be used to increase the quantity of early Ad2-induced proteins and should facilitate not only analytical studies, but also the isolation of sufficient amounts of these polypeptides to study their biological and chemical properties.

Kinetic studies showed different rates of syn-

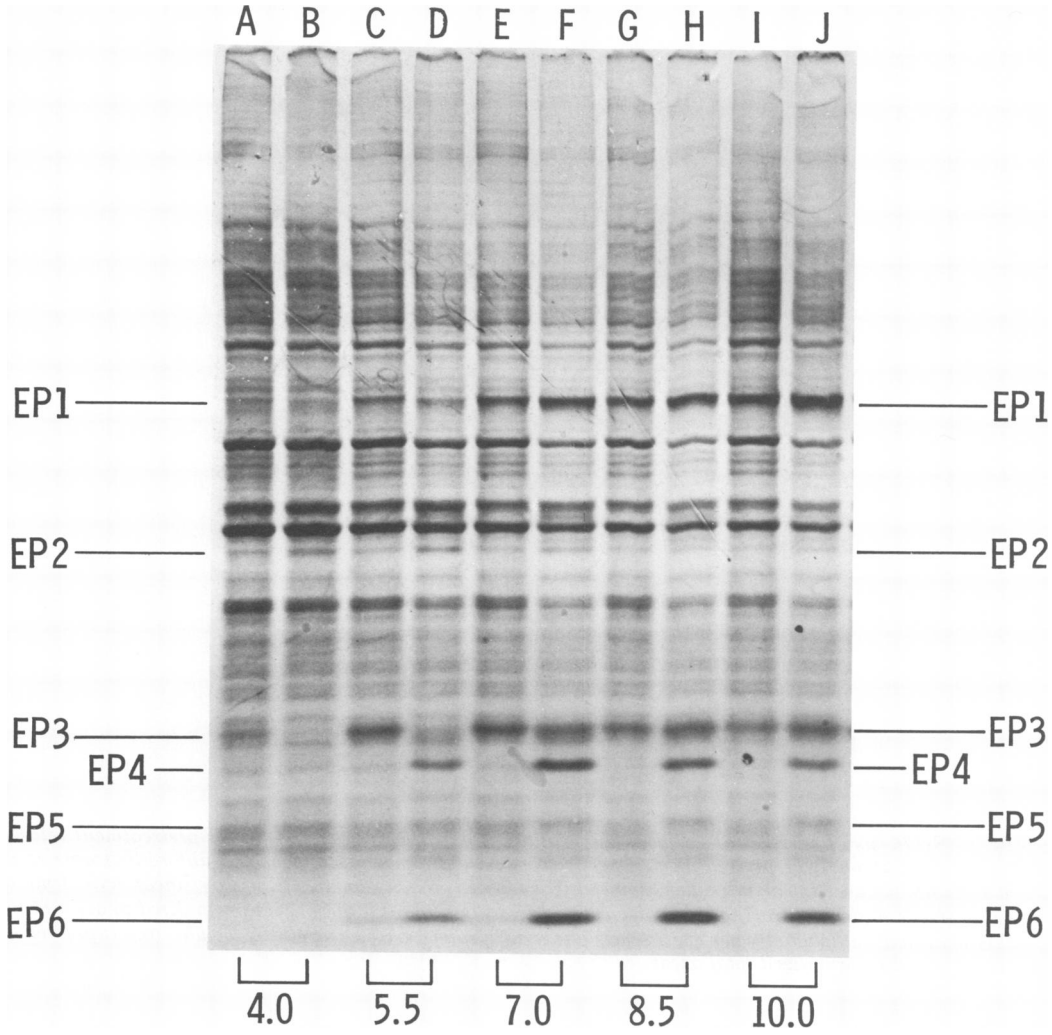

FIG. 5. *Comparison of polypeptides from Ad2-infected KB cells labeled at different times after infection with and without pretreatment with CH. KB cells were treated with CH from 1 h after infection to 30 min prior to the beginning of a 60-min pulse with [^{35}S]met. All cultures (except A and B) were treated with AraC from 4 h p.i. to the termination of the experiment. Cell extracts were prepared as described in the text. About 100,000 counts/min was analyzed on 5 to 22% gradient gels. A, D, E, G, and I are polypeptides from cells not treated with CH; B, D, F, H, and J are those from cells treated with CH. The time after infection, when the 1-h pulse was completed, is shown at the bottom of the figure.*

thesis of the early polypeptides at various times after infection and differential effects of CH pretreatment. Because these cells were incubated in the presence of CH and AraC, we do not wish to speculate on the significance of these kinetics. However, the inhibition by CH pretreatment of the rate of EP3 synthesis by CH early after infection is interesting. The huge enhancement by CH pretreatment of the rates of synthesis of EP4 and EP6 is also interesting.

Our EP1 (75K) appears to be identical to E_1 (70K) described by Walter and Maizel (20). This is the single-stranded, DNA-binding protein described by van der Vliet and Levine (18) and Shanmugam et al. (15), which has been shown to be an early viral-coded protein (19). Our EP6 (11K) is probably identical to E_3 (10K) observed by Walter and Maizel (20). Although we cannot be certain, our EP3, which was readily apparent in untreated cells, is probably E_2 (19K) of Walter and Maizel (20). Since E_2 is glycosylated (10), this can be checked by determining if EP3 is glycosylated. We believe that EP2, EP4, and

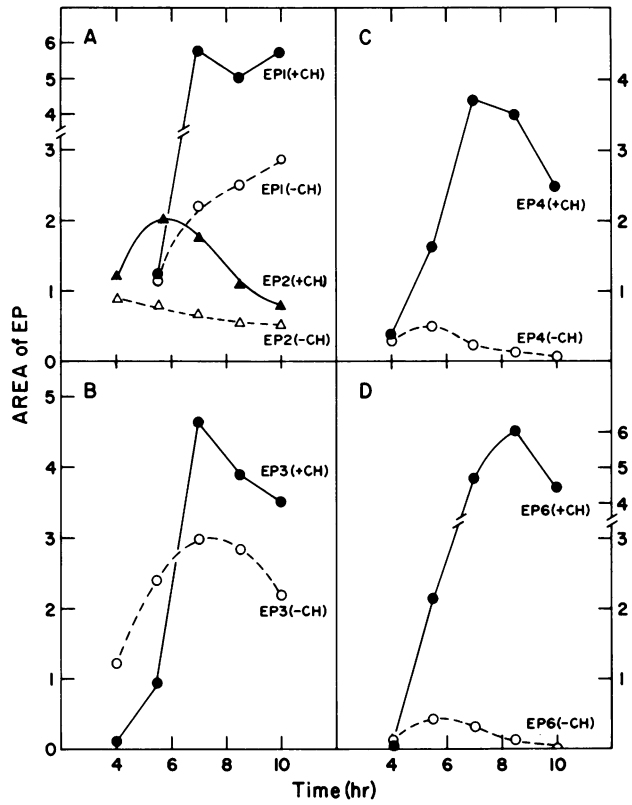

FIG. 6. *Synthesis of early viral-induced polypeptides after different periods of CH pretreatment (closed symbols) and without CH pretreatment (open circles). Densitometer scans of the autoradiograms were prepared with a Joyce-Loebl microdensitometer. The grain density on the film was linear with time of exposure. Areas under the tracings were determined by weighing, and these were normalized to account for variations in total radioactivity per well. Areas under EP peaks were measured (by weight) and expressed as percentages of the normalized total areas.*

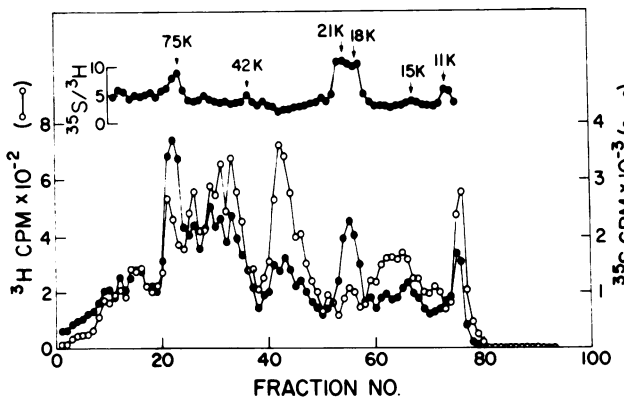

FIG. 7. *Coelectrophoresis of polypeptides from Ad2-infected and mock-infected cells labeled with [^{35}S]met and [^{3}H]met, respectively. Ad2-infected and mock-infected cells were incubated in the presence of CH from 1 to 7.5 h p.i., washed, and pulse-labeled from 8 to 9 h p.i. in medium containing AraC. AraC was present from 4 h p.i. Samples containing 58,700 ^{3}H and 176,000 ^{35}S counts/min were coelectrophoresed on 10% polyacrylamide gels. The arrows indicate the positions in which the polypeptides with the indicated molecular weights are expected to migrate, as determined from a parallel gel containing molecular weight markers.*

EP5 are new proteins induced by Ad2 infection. Comparable proteins were independently discovered by W. Chin and J. V. Maizel (Virology, in press) and by J. B. Lewis et al. (Cell, in press).

EP2 (42K) may not be the degradation product(s) of 70 to 75K DNA-binding protein that has been reported to migrate as 46 to 50K (15, 18, 19), for the following reasons. (i) The synthesis of EP2 in CH-treated cultures was maximal at about 4 to 5.5 h p.i. (Fig. 2 and 6) and declined subsequently, whereas the synthesis of EP1 was visualized beginning only at 5.5 h and was maximal at later times (Fig. 6). This result is inconsistent with a precursor-product relationship between EP1 and EP2. (ii) Lewis et al. (Cell, in press) have synthesized a similar size polypeptide by cell-free translation of viral mRNA purified by hybridization to the Ad2 *Eco*R1-A fragment (and the HpaI-E fragment). The gene for the 75K DNA-binding protein is located in the *Eco*R1-B fragment (Lewis et al., Cell, in press).

Our results do not establish whether these early polypeptides are viral coded or viral induced, i.e., coded by the cell genome and stimulated by infection and CH pretreatment. However, the in vitro translation of early Ad2 mRNA to produce six polypeptides of similar molecular weights (Lewis et al., Cell, in press) raises the possibility that the proteins we are studying are also viral coded. Thus there may be at least six early viral genes. The combined molecular weights of these six early viral proteins is approximtley 200,000, which is about 20% of the coding capacity of the Ad2 genome. It has been estimated that early mRNA originates from 23 to 32% (8; Wold et al., in preparation), or as high as 40% (13), of the viral genome. Thus, there may be additional early polypeptides not detected by our procedures because of the background of host cell protein synthesis. This possibility is supported by hybridization analyses of early mRNA size, fractionated by polyacrylamide gel electrophoresis, which provided evidence for seven to nine distinct early viral mRNA molecules (4, 5). On the other hand, it is not known whether all nucleotide sequences in mRNA are translated into protein or whether early mRNA is processed on polyribosomes from precursor molecules. Therefore, estimates based on mRNA studies may be misleading with regard to the number of early genes and the genome fraction encoding early proteins. If these are additional early proteins, their identification will require techniques of higher resolution, such as immunological selection or two-dimensional electrophoresis.

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