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Characterization of the Major Immediate-Early Polypeptides Encoded by Murine Cytomegalovirus

GÜNTHER M. KEIL, MATHIAS R. FIBI, AND ULRICH H. KOSZINOWSKI*

Federal Research Centre for Virus Diseases of Animals, D-7400 Tübingen, Federal Republic of Germany

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The immediate-early (IE) infected cell proteins induced by the murine cytomegalovirus (Smith strain) were studied. These polypeptides were identified as IE proteins by their synthesis in the presence of actinomycin D after removal from a protein synthesis block mediated by cycloheximide. By using a murine antiserum against murine cytomegalovirus, three abundant polypeptides of 89, 84, and 76 kilodaltons (kd) were immunoprecipitated. The three major proteins are phosphorylated but not glycosylated and share antigenic determinants recognized by monoclonal antibodies. The 84 and 76-kd polypeptides represent post-translational modification products of the 89-kd protein. Accordingly, in vitro translation of IE infected cell RNA revealed only the 89-kd polypeptide. The viral origin of the RNA species directing the synthesis of the major 89-kd IE polypeptide was verified by hybrid selection of IE RNA with DNA fragments representing the region from 0.769 to 0.815 map units of the murine cytomegalovirus genome. IE polypeptides were found to be located in the nuclei and the cytoplasm of infected cells. Studies on the kinetics of IE polypeptide synthesis revealed negative regulatory effects on IE gene expression correlated with the synthesis of early proteins.

The genome of murine cytomegalovirus (MCMV) is a linear double-stranded DNA molecule and contains, similar to human cytomegalovirus, ca. 235 kilobase pairs. Unlike human cytomegalovirus, however, it consists of a long unique sequence and lacks large terminal or internal repeat regions (3, 16). Similar to other herpesviruses, the transcription and translation of MCMV is coordinately regulated. The expression of immediate-early (IE) genes can be studied in cells arrested for protein synthesis. The expression of at least one IE gene product is required for the expression of the early-phase genes. The late-phase gene products are synthesized after the onset of viral DNA synthesis. Recently, the genome of MCMV was cloned (3, 16), and the regions encoding IE, early, and late RNA were studied (11, 15). The coding region for abundant IE transcription was defined by hybridizing IE RNA with cloned restriction fragments (11, 15). This region could be located at between 0.769 and 0.817 map units, and six polyadenylated IE RNA species were identified (11).

Recent studies on the cellular immune response to MCMV (19–21) pointed to a new, so far unknown role of MCMV IE gene products. Murine cytolytic T-lymphocytes generated in vivo during acute infection with MCMV recognized and specifically lysed infected fibroblasts after the expression of IE proteins. The antigens detected by T-lymphocytes on MCMV-infected cells were defined as lymphocyte-detected IE antigen(s), and T-lymphocytes recognizing these antigens were found to represent a population separate from those recognizing MCMV structural antigens. T-lymphocytes with specificity for lymphocyte-detected IE antigen(s) represent a dominant fraction of antigen-specific cytolytic T-lymphocytes during acute infection (21).

The role of IE gene products in both the regulation of viral replication and the host response to acute infection prompted us to study the proteins expressed in the IE phase in more detail. We report here on the regulation of MCMV-encoded IE protein synthesis, the antigenic relationship of the proteins, and the investigation of their distribution within infected cells.

MATERIALS AND METHODS

Virus and cell culture. MCMV (mouse salivary gland virus, strain Smith, ATCC VR194) was obtained from the American Type Culture Collection, Rockville, Md., and propagated on BALB/c mouse embryo fibroblasts (MEF). Details of cell culture, virus propagation, and infection procedures were as described (3, 11). Goat fibroblasts were kindly provided by J. Thiel, Tübingen, Federal Republic of Germany. Cells were labeled in suspension with 1.5 mCi of [^{35}S]methionine (Amersham, Braunschweig, Federal Republic of Germany) per ml in methionine-depleted medium. Labeling of phosphoproteins was done in suspension in phosphate-free medium with 2.5mCi of $^{32}P_i$ (Amersham) per ml. Cycloheximide (CH; 50 μg/ml) or actinomycin D (ActD; 2.5 μg/ml) was added for the times indicated below.

Hybrid selection and in vitro translation of RNA. Whole cell RNA was prepared as described (11). Hybridization of RNA to DNA and the subsequent elution of specifically bound RNA (hybrid selection) was carried out according to the method of Esche and Siegmann (5). Briefly, 100 μg of plasmid DNA was sonicated twice for 15 s, boiled at 100°C for 10 min, and added dropwise to 10 nitrocellulose filter squares of 0.09 cm^2 (pore size, 0.22 μm; Schleicher & Schüll, Dassel, Federal Republic of Germany). The dried filters were washed in 6× SSC (SSC is 0.15 M NaCl plus 0.05 M sodium citrate) and baked in a vacuum oven at 80°C for 4 h. The filters were incubated with 75μg of whole cell RNA in hybridization buffer (0.01 M PIPES [piperazine-*N*,*N'*-bis (2-ethanesulfonic acid], 65% formamide, 0.4 M NaCl [pH 6.8]) at 52°C for 3 h and subsequently washed 10 times with 1× SSC–0.5% sodium dodecyl sulfate (SDS) and 3 times with 2 mM EDTA (pH 7.5). Bound RNA was eluted by boiling the filters in distilled water for 2 min. The eluate was adjusted to 0.2 M potassium acetate and precipitated with 2.5 volumes of ethanol in the presence of 50 μg of tRNA per

* Corresponding author.

ml from calf liver (Boehringer, Mannheim, Federal Republic of Germany). The precipitate was washed twice with 70% ethanol and resuspended in distilled water. In vitro translation of whole cell RNA and of hybrid selected RNA from infected cells was carried out in a cell-free rabbit reticulocyte lysate prepared according to the method of Pelham and Jackson (18).

Antisera and monoclonal antibodies. Antisera were produced in BALB/c mice as described (20). For the generation of monoclonal antibodies directed against IE antigens, BALB/c mice were injected three times with 10^7 MEF, which were infected in the presence of CH for 3 h, followed by a 4-h treatment with ActD, and therefore expressed selectively IE proteins of MCMV. For the first subcutaneous injections infected cells were suspended in Freunds complete adjuvant. The second subcutaneous injections in incomplete Freunds adjuvant were carried out at day 6. The final injection of infected cells suspended in phosphate-buffered saline (PBS) was given at day 9 by the intraperitoneal route. Animals were sacrificed at day 12, and the spleen cells were fused with the Sp2/0 Ag 14 myeloma cells. After selection in hypoxanthine-aminopterin-thymidine medium, the antibody-producing hybridomas were identified by an enzyme-linked immunosorbent assay by using sonicated IE infected cells (3 μg of protein per well) as antigen. Microtiter plates coated with sonicated noninfected cells provided the controls. Bound antibody was detected with peroxidase-labeled $F(ab')_2$ fragments of goat anti-mouse immunoglobulin G (Tago, Inc, Burlingame, Calif.). Bound enzyme was visualized with *p*-phenylene-diamine (1 μg/ml) and H_2O_2. Positive hybridomas were cloned twice by limiting dilution.

Immunoprecipitation. Radiolabeled MEF were washed with PBS, and 2×10^6 cells were lysed in 0.3 ml of lysis buffer (10 mM Tris, 1% Nonidet P-40 [NP-40], 1% sodium deoxycholate, 0.1% SDS, 1 mg of ovalbumin per ml, 0.2% NaN_3, 1 mM methionine [pH 7.6], and 1 mM phenylmethylsulfonyl fluoride, [Serva, Heidelberg, Federal Republic of Germany]). In some experiments the protease inhibitors *p*-chloromercuribenzoic acid (Serva) and *N*-ethylmaleimide (Serva) were used. The cell lysates were clarified by centrifugation at 39,000 rpm for 1 h, and samples of lysates containing 2×10^6 to 4×10^6 cpm were incubated with 100 μl of hybridoma supernatant or 10 μl of serum for 60 min at room temperature and 60 min on ice. Fifty microliters of a 10% suspension of fixed *Staphylococcus aureus*, prepared according to the method of Kessler (13), was added and incubated on ice for 30 min. Immunoprecipitates were pelleted by centrifugation, washed three times with lysis buffer, and resuspended in sample buffer for SDS-polyacrylamide gel electrophoresis (PAGE). Gradient gels, (7.5 to 20%), 10% SDS-polyacrylamide gels, and the visualization of protein bands by autoradiography or fluorography were performed as described (4, 12).

To separate the nuclear and cytoplasmic fraction, 2×10^6 cells were suspended in 100 μl of ice cold NP-40 buffer (0.5% NP-40, 10 mM Tris [pH 7.6], 0.15 M NaCl, 1.5 mM $MgCl_2$), vortexed for 10 s, and stored on ice for 5 min. Thereafter, the disrupted cells were layered on a sucrose cushion (24% sucrose in 1% NP-40–10 mM Tris [pH 7.6]–0.15 M NaCl–1.5 mM $MgCl_2$) and centrifuged at $4,000 \times g$ for 20 min (14). To the cytoplasmic fraction, represented by the upper phase, 200 μl of lysis buffer was added for immunoprecipitation. The sucrose phase was removed, and the nuclei in the pellet were washed twice in NP-40 buffer and then resuspended in 300 μl of lysis buffer. Immunoprecipitation of proteins from subcellular fractions was carried out as described above.

Western blotting. After lysis in the sample buffer, followed by 5 min of boiling, the proteins from infected MEF were separated by 10% SDS-PAGE. The gel was renatured (25), and proteins were transferred electrophoretically to nitrocellulose filters (pore size, 0.22 μm; Schleicher & Schüll). After preincubation for 4 h with 1% bovine serum albumin in PBS, the filters were incubated overnight at 4°C with antiserum or ascites fluid-derived monoclonal antibody diluted 1:10 in PBS containing 1% albumin. Filters were washed with PBS containing 0.1% Triton X-100 and incubated with peroxidase-labeled $F(ab')_2$ fragments of goat anti-mouse immunoglobulin G (Tago). Bound enzyme was visualized with chloronaphtol (0.3 mg/ml) and H_2O_2.

RESULTS

Identification of IE MCMV-infected cell proteins with antisera and monoclonal antibodies. To achieve the enhanced and selective expression of IE MCMV genes, murine and caprine fibroblasts were infected in the presence of CH to prevent synthesis of IE proteins and the subsequent transcription of early viral RNA. For the selective synthesis of IE polypeptides CH was removed 3 h postinfection (p.i.), and ActD was added to inhibit early viral RNA transcription. A murine antiserum to MCMV was used to precipitate the IE infected cell proteins. In the lysates of the infected cells of both species, additional polypeptides with identical migration patterns indicative for the viral origin of these proteins were detected (Fig. 1A). A dominant protein of 89 kilodaltons (kd) and two less abundant polypeptides of 84 and 76 kd were seen. The 84 kd protein was sometimes detectable (Fig. 1A) and sometimes absent (Fig. 1B) in lysates. Two additional minor IE infected cell proteins of ca. 31 and 15 kd were seen in lysates (Fig. 1A, arrowheads) and after prolonged exposure were seen also in the immunoprecipitates (not visible in Fig. 1A). The small concentration of these proteins precluded their further analysis. The three major IE infected cell proteins incorporated [^{35}S]methionine, [^{3}H]leucine, [^{3}H]arginine, and [^{3}H]lysine but could not be labled with [^{3}H]glucosamine (data for ^{3}H-labled amino acids and for [^{3}H]glucosamine not shown). They were also detected after labeling with $^{32}P_i$ (Fig. 1C) and therefore designated as phosphoproteins pp89, pp84, and pp76.

Relation between pp89 and pp84. Because MCMV replicates poorly in established cell lines, MEF had to be used in this study. We observed that the concentration of pp84 varied in lysates of different cell batches, but was always detectable after immunoprecipitation with native antiserum (Fig. 2, lane b). After heat inactivation of the antiserum (56°C, 30 min), pp84 was absent (lane c). A monoclonal antibody, MCMV-6/20/1, also precipitated only the 89- and the 76-kd proteins (lane d). Addition of native mouse control serum during immunoprecipitation with the monoclonal antibody (lane e) led to the reappearance of the 84-kd protein. The heat-sensitive factor present in native mouse serum, most probably a protease, was resistant to 1 mM phenylmethylsulfonyl fluoride and to 2 mM *p*-chloromercuribenzoic acid, but could be inhibited by 15 mM *N*-ethylmalleimide (data not shown). It should be noted, however, that in several experiments pp84 could also be seen in lysates of infected cells and immunoprecipitates carried out with heat-inactivated antiserum (see also Fig. 5 through 7), which indicates that the protease represents a variable constituent not only of murine serum but also of murine embryonic cells.

Relation between pp89 and pp76. The monoclonal antibody MCMV-6/20/1 (and also several other monoclonal antibodies

which are not presented here) precipitated both pp89 and pp76. If the 76-kd protein was precipitated due to complex formation of an unrelated protein with pp89, the antibody should bind only to one of both proteins after separation of proteins in SDS-polyacrylamide gel and transfer to nitrocellulose filters. To identify the IE proteins on the filters, samples of radiolabeled immunoprecipitates were added to the lysates before electrophoresis and transfer to nitrocellulose (Fig. 3, lower part). The immunoblot (Fig. 3, upper part) shows clearly that in lysates of infected cells both proteins are visualized by antiserum to MCMV, which indicates that pp76 represents a virus-induced protein (lane b). The reactivity of the monoclonal antibody (lane e) with both proteins provided the evidence that pp89 and pp76 share antigenic determinants.

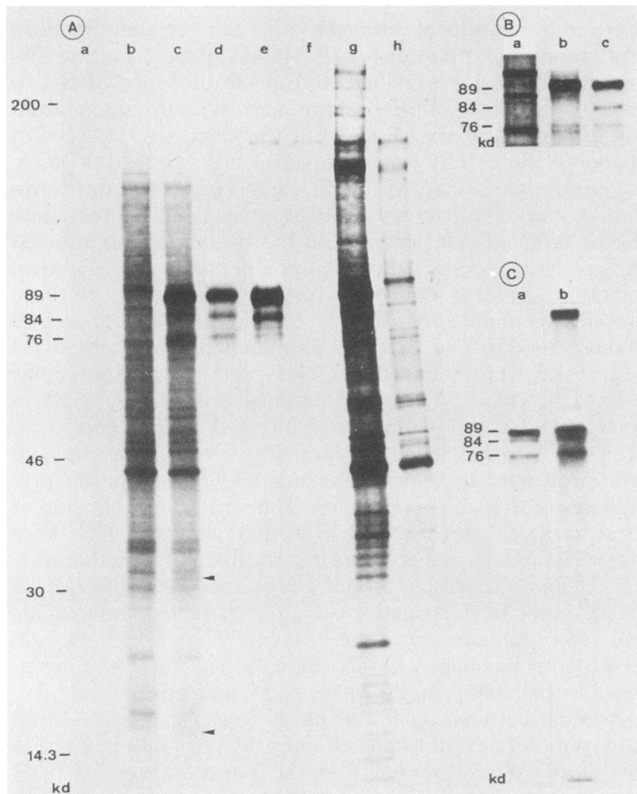

FIG. 1. Fluorograms of IE infected cell proteins synthesized in fibroblasts after infection with MCMV. MEF and goat fibroblasts were infected in the presence of CH. At 3 h p.i. CH was replaced by ActD, and synthesized proteins were labeled for 3 h with [^{35}S]methionine or $^{32}P_i$. Cell lysates and proteins obtained after immunoprecipitation with native murine antiserum to MCMV were analyzed in 7.5 to 20% SDS-polyacrylamide gradient gels (A and B) or in 10% SDS-polyacrylamide gels (C). (A) Immunoprecipitates from noninfected murine (lane a) and caprine (lane f) fibroblasts, lysates of noninfected murine (lane b) and caprine (lane h) fibroblasts, lysates of IE infected murine (lane c) and caprine (lane g) fibroblasts, and immunoprecipitates from IE infected murine (lane d) and caprine (lane e) fibroblasts. (B) Lysates from noninfected (lane a) and IE infected (lane b) MEF and immunoprecipitates from IE infected MEF (lane c). The experimental conditions were identical to those of (A), but this was a separate experiment. (C) Immunoprecipitates from IE infected MEF labeled with [^{35}S]methionine (lane a) or $^{32}P_i$ (lane b). The molecular masses of marker proteins and the apparent molecular masses of the three major virus-induced infected cell proteins are designated. Minor proteins are indicated by arrowheads.

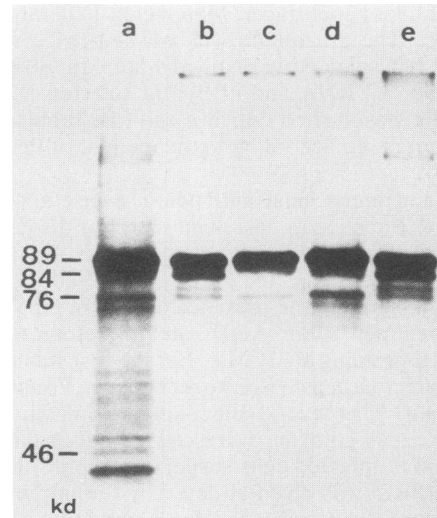

FIG. 2. Fluorograms of IE infected cell proteins after degradation in vitro by heat-labile serum factors. MEF were infected as described in the legend to Fig. 1. Native and heat-inactivated (30 min, 56°C) murine antiserum and control serum were tested for differential effects on IE infected cell proteins during the immunoprecipitation procedure. The cell lysate (lane a) and the immunoprecipitated proteins (lanes b through e) were analyzed in 10% gels. Immunoprecipitation with 10 μl of native murine antiserum (lane b), 10 μl of inactivated antiserum (lane c), 100 μl of monoclonal antibody MCMV-6/20/1 derived from cell culture supernatants (lane d), and 100 μl of monoclonal antibody MCMV-6/20/1 plus 10 μl of native control serum (lane e). The molecular masses of IE proteins and the position of the 46-kd standard are indicated.

To study the kinetics of the generation of the 76-kd polypeptide, pulse-chase experiments were carried out. IE proteins were pulse-labeled with [^{35}S]methionine for 15 min, and then, after removal of the radiolabeled methionine, they were chased for different periods of time. The smaller polypeptide was already detectable in cell lysates and immunoprecipitates after the 15-min pulse. During the whole chase period the relative concentration of pp89 and pp76 was not found changed (data not shown).

Viral origin of pp89. The smaller phosphoprotein pp76 could be generated during cell-free translation of IE infected cell RNA, if it is translated from a RNA species separate from the RNA directing the synthesis of pp89. After cell-free translation of IE RNA the translation product migrated at an apparent molecular mass of 89 kd (Fig. 4A, lane b) and was precipitated by specific antiserum (lane c). The absence of pp76 indicated that pp76 also represents a processed product derived from pp89.

Abundant IE transcription occurs from the genomic region between 0.769 and 0.817 map units of MCMV (11). Since this region is contained within the plasmid clone pAMB25 (0.769 to 0.815 map units), this MCMV DNA fragment was used to select IE mRNA for in vitro translation. In Fig. 4B it is shown that DNA from MCMV-pAMB25 selects RNA, which after elution directs the synthesis of a protein migrating at an apparent molecular mass of 89 kd (lane c). DNA from a region which is not transcribed at IE times (MCMV *Hin*dIII-D map units 0.35 to 0.46) failed to select IE RNA that could direct specific IE protein synthesis (lane d). Thus, pp89 is encoded by the IE region of MCMV. As expected, pp84 and also pp76 were not detected. Additional smaller polypeptides of 69 and 51 kd were seen after in vitro translation of RNA selected with MCMV-pAMB25.

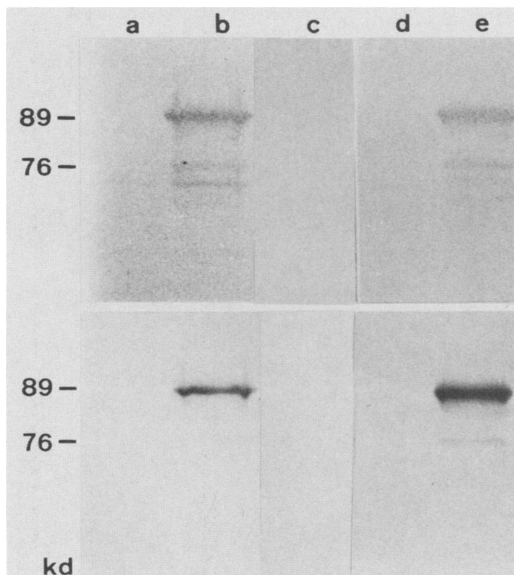

FIG. 3. Analysis of IE polypeptides by Western blotting. Upper part, Proteins were separated by 10% SDS-PAGE by using lysates equivalent to 6×10^5 cells of infected MEF (lanes b, c, and e) and control cells (lanes a and d), followed by transfer to nitrocellulose filters. The binding of inactivated murine antiserum (lanes a and b), inactivated control serum (lane c), or monoclonal antibody MCMV-6/20/1 (lanes d and e) to nitrocellulose-bound proteins was visualized by the enzymatic activity of peroxidase-labeled $F(ab')_2$ fragments of goat anti-mouse immunoglobulin G bound to the murine immunoglobulin G. Lower part, To identify the position of the IE proteins on the filters, samples of precipitates (10^4 cpm per lane) from lysates of [^{35}S]methionine-labeled IE infected MEF (lanes b, c, and e) and control cells (lanes a and d) immunoprecipitated with inactivated antiserum (lanes a and b), inactivated control serum (lane c), or monoclonal antibody MCMV-6/20/1 (lanes d and e) were added to the cell lysates before SDS-PAGE. After photography of the enzymatic reaction, the filters were subjected to autoradiography.

Intracellular distribution of IE proteins. The specific functions of cytomegalovirus (CMV) IE polypeptides in the switch from limited to extensive transcription of the genome would suggest that the functionally active polypeptide should reside within the nucleus. To analyze the intracellular distribution of the IE proteins, cells were infected in the presence of CH. CH was removed 3 h later, and synthesized proteins were labeled in the presence of ActD, followed by an 1-h chase to allow intracellular transport of labeled proteins. After the chase the cells were lysed, and the lysates of whole cells, the cytoplasmic and the nuclear fractions as well as the respective immunoprecipitates, were analyzed. IE proteins were found in both the cytoplasmic and the nuclear fractions (Fig. 5). Similar data were also obtained when the experiments were carried out in absence of protein synthesis inhibitors (data not shown).

Production kinetics and regulation of expression of IE infected cell proteins. The expression of IE genes during MCMV replication was investigated. To study the kinetics of IE RNA transcription, MEF were infected in the presence of CH to prevent synthesis of IE proteins. At various times later ActD was added to infected cells to limit the period of IE RNA transcription. Three hours after infection CH was removed, and under the continuous presence of ActD the IE RNA, which was transcribed previous to the ActD block, was allowed to direct protein synthesis. Faint protein bands could be already detected when transcription was inhibited at 60 min p.i. (Fig. 6A, lane b; not visible in the photography). The kinetics of IE RNA accumulation revealed, however, that at least 90 to 180 min of transcription was required to generate abundant concentrations of IE proteins (lanes c, d, and e).

MCMV IE viral proteins are required for early gene expression, which in turn may regulate the expression of MCMV IE gene functions. The appearance of early proteins was studied by addition of CH and ActD to cells at different times after infection. The pattern of precipitated proteins remained constant during the first 120 min and identical to that of those proteins translated from RNA transcribed in the continuous presence of CH (Fig. 6B, lanes a through c). After 120 min, however, and clearly demonstrable after 230 min of infection, additional proteins with apparent molecular masses of 65 and 46 kd were precipitated which require translation of IE RNA and therefore by definition represent

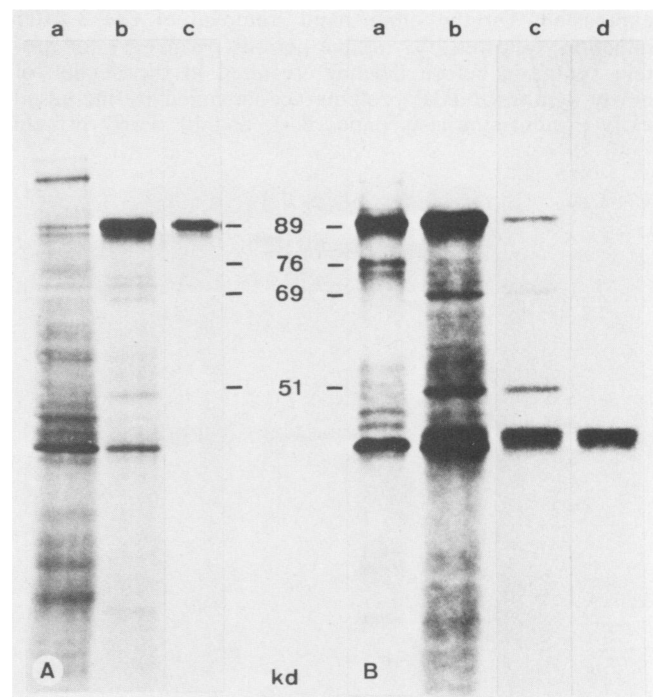

FIG. 4. Hybrid selection and in vitro translation of IE RNA and analysis of translated products. (A) Fluorograms of IE polypeptides synthesized in vitro. IE proteins were synthesized in a rabbit reticulocyte lysate translation system directed by whole-cell RNA from IE infected cells. Lysates and immunoprecipitates were analyzed in 10% polyacrylamide gels. Shown are lysates of cell-free [^{35}S]methionine-labeled translation products directed by RNA from noninfected (lane a) and IE infected (lane b) cells. Lane c, Proteins precipitated with inactivated antiserum after in vitro translation of IE RNA. (B) In vitro translation of hybrid-selected IE RNA Nitrocellulose-bound fragments of clone MCMV DNA from genomic regions transcriptionally active (pAMB25) or silent (*Hin*dIII-D fragment) at IE times were used for selection. Bound RNA was eluted and translated in vitro, and the translation products were analyzed by 10% SDS-PAGE and fluorography. Lanes: a, lysates of [^{35}S]methionine-labeled proteins synthesized in IE infected MEF; b, proteins translated in vitro from whole-cell IE RNA; c, proteins translated from RNA hybrid selected with pAMB25; d, proteins translated from RNA hybrid selected with the *Hin*dIII-D fragment. Samples from (A) and (B) were run on different gels. Exposure times were 2 days for (A) and 14 days for (B).

early infected cell proteins (lanes d and e). In accordance with the appearance of these early proteins the amount of the IE proteins was found diminished (lane e).

The finding that transcription of IE RNA ceases at early times of infection (11) and the new observation that the translation of IE infected cell proteins was reduced already after 230 min of infection led to the question of whether IE gene products exert self-regulatory effects on the stability of IE RNA or whether early gene products are involved in the regulation of IE gene expression, or both. In cells infected and maintained in the presence of CH, the transcribed IE RNA was still able to direct protein synthesis after removal of CH several hours later (Fig. 7, lanes a, c, e, and g). Similarly, IE RNA transcribed in cells in the presence of CH, which were then maintained in presence of ActD after removal of CH, was active in IE protein synthesis during the period of at least 11 h (lane b). The same result was obtained when the presence of ActD was extended to 24 h before labeling of newly synthesized proteins (data not shown), indicating the stability of IE RNA in absence of early gene expression. On the other hand, removal of CH 3 after infection, followed by variable periods permissive for protein synthesis before labeling, resulted in the decline of newly synthesized IE proteins accompanied by increased early protein synthesis (lanes d, f, and h). Early protein synthesis is therefore associated with negative regulative effects on IE protein synthesis.

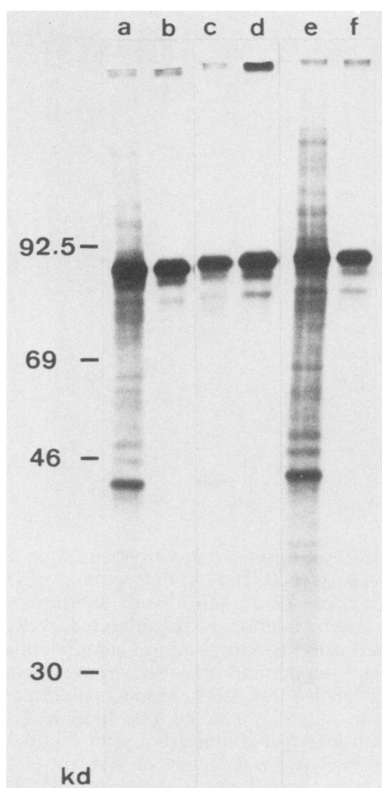

FIG. 5. Intracellular localization of IE proteins. MEF were infected in the presence of CH, and a 3 h p.i. CH was exchanged for ActD. Infected cells were labeled for 3 h with [^{35}S]methionine, followed by a 1-h chase. Shown are the whole cell lysate (lane a) and immunoprecipitation with inactivated antiserum (lane b), the lysate of the nuclear fraction (lane c) and immunoprecipitated proteins (lane d), and the lysate of the cytoplasmic fraction (lane e) and the respective immunoprecipitation (lane f). Samples were run on the same gel and exposed to film for the same period of time. Standard molecular weight markers are indicated.

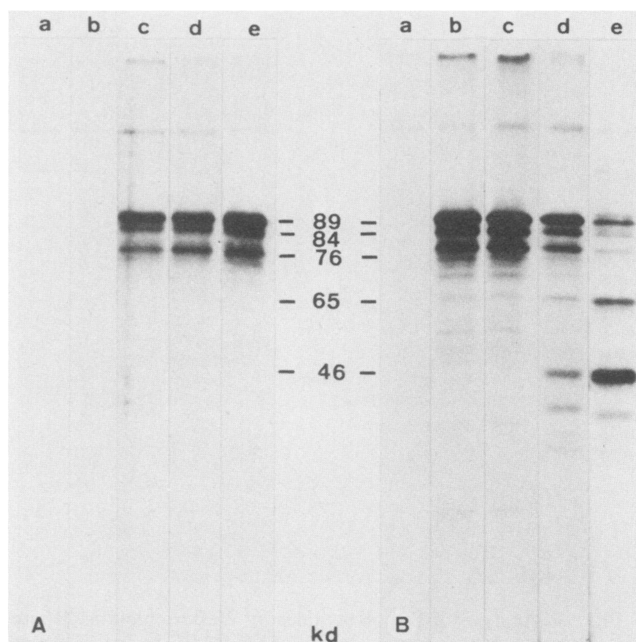

FIG. 6. Kinetics of IE and early gene expression. (A) Minimal time of RNA synthesis required to detect IE polypeptides MEF were infected in the presence of CH, and at different times p.i. ActD was added. At 3 h p.i. CH was removed. In the continuous presence of ActD the synthesized proteins were labeled for 3 h, and immunoprecipitates were prepared with antiserum and separated on 10% gels. ActD addition p.i.: 0 min (lane a), 60 min (lane b), 90 min (lane c), 120 min (lane d), 180 min (lane e). (B) Switch from IE to early gene expression. MEF were infected, and at different times p.i. CH was added. CH was removed at 4 h p.i., synthesized proteins were labeled for 3 h in the presence of ActD, and immunoprecipitates were prepared with antiserum. CH addition p.i.: mock-infected cells, 60 min (lane a); infected cells, 60 min (lane b), 90 min (lane c), 120 min (lane d), and 230 min (lane e). Panels A and B represent separate experiments. In each experiment all samples were run on the same gel and exposed to film for the same period of time. Molecular masses of IE and early polypeptides are indicated.

DISCUSSION

At least one viral protein encoded by the IE mRNA of herpesviruses is required for the expression of later gene functions. IE polypeptides are those viral proteins which are synthesized immediately after reversal of a protein synthesis block. Here we report on the biochemical properties and intracellular distribution, as well as on the regulation of synthesis, of the IE MCMV proteins.

After separation in polyacrylamide gels IE proteins migrated as an abundant band of 89-kd apparent molecular mass, two less prominent and sometimes diffuse bands of variable abundance at 84 and 76 kd, and two faint bands at 31 and 15 kd. The three major proteins are phosphorylated (pp89, pp84, and pp76). The number and the estimated sizes of the three abundant IE polypeptides coincide with earlier molecular mass determinations (of 86.5, 74, and 69 kd) for the IE nonstructural MCMV proteins (2). In recent studies the same laboratory reported estimations of 105, 98 and 88 kd. (D. G. Walker and J. B. Hudson, personal communication).

The 84-kd protein is a processed product derived from pp89. Processing can occur to a variable extent before or

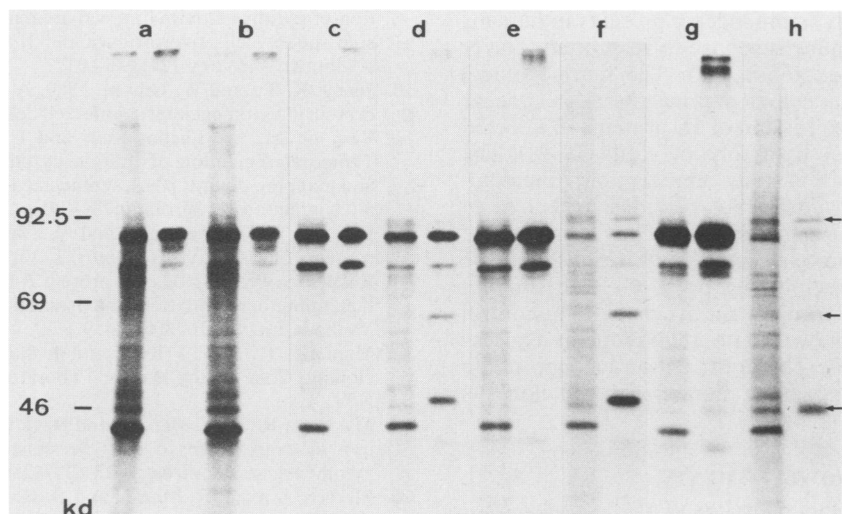

FIG. 7. Effect of early proteins on IE protein synthesis. Shown are fluorograms of IE and early polypeptides synthesized in vivo after inhibitor treatment and immunoprecipitated by inactivated antiserum. In each panel the cell lysate is shown in the left lane and the immunoprecipitate is shown in the right lane. (a) Infection in the presence of CH for 9 h, followed by 2 h of labeling in the presence of ActD after removal of CH; (b) infection in the presence of CH for 3 h. After removal of CH, incubation was in the presence of ActD for 8 h, and labeling was during the last 2 h; (c, e, and g) infection in the presence of CH for 5 h (c), 7 h (e), and 9 h (g), followed by 2 h of protein synthesis and labeling; (d, f, and h) infection in the presence of CH for 3 h; after removal of CH there was further incubation without inhibitors for 4 h (d), 6 h (f), and 8 h (h). Proteins were labeled during the last 2 h. The samples shown in panels a and b were prepared from a different batch of MEF and run on a separate gel. The samples shown in panels c through h were from the same batch of cells and run on the same gel. The samples shown in panels g and h were exposed to film for a longer period of time. Standard molecular mass markers are indicated. Early polypeptides are indicated by arrows.

during lysis of infected cells and is seen after immunoprecipitation due to the activity of a serum protease which is active in presence of 1 mM phenylmethylsulfonyl fluoride and 2 mM *p*-chloromercuribenzoic acid but inhibited by 15 mM *N*-ethylmaleimide. Monoclonal antibodies were prepared to study the relationship between pp89 and pp76. Western blot experiments with the monoclonal antibody MCMV-6/20/1 and other monoclonal antibodies showed that both polypeptides must share antigenically homologous regions.

The genomic region from 0.769 to 0.817 map units contains the information for the IE mRNAs (11). Splicing of human CMV IE mRNA has been reported recently (22, 24). Since several RNAs originate from the IE region of MCMV, two or more RNAs transcribed from the same strand, but differing in splice points or stop codons, or both, could have given rise to antigenically related proteins, which would explain the homology between pp89 and pp76. In vitro translation of IE RNA from infected cells and of an IE RNA hybrid selected by a MCMV DNA fragment representing the IE region (11) resulted mainly in the protein with an apparent molecular mass of 89 kd. These experiments proved the viral origin of pp89 and gave no indication for the existence of a separate RNA encoding pp76. The translation of pp89 from the 2.75-kb RNA was shown by in vitro translation of size-fractionated RNA (G. Keil, manuscript in preparation). In conclusion, only one abundant viral polypeptide is synthesized at IE times.

Proteolysis at specific sites leads to the 76-kd form. The high concentration of pp76 in preparations boiled and separated immediately after lysis excludes the appearance of this protein due to a preparation artifact. Processing to the 76-kd polypeptide occurs immediately after the synthesis of the pp89. The absence of pp76 and also of pp84 in cell-free protein-synthesizing systems reflects the absence of the respective enzymes in the reticulocyte lysate.

Since the 89- and the 76-kd IE proteins are located mainly in the nucleus and the cytoplasm of infected cells, we suggest that pp89 represents the major regulatory protein involved in the transcription of early viral genes. If the post-translational modification of pp89 to pp76 is not accompanied with a loss of function, both polypeptides could exert different regulative activities.

The first IE protein synthesis was detectable between 1 and 1.5 h after infection. In the presence of ActD IE mRNA was still translated after 24 h. A similar stability of IE RNA has also been reported for the transcripts of CMV from other species (9, 10). The gradual decrease of IE RNA under conditions permissive for early protein synthesis (11) explains the observation of decreased de novo IE protein synthesis during the early phase. We suggest that the stability of MCMV IE RNA is controlled by an early function with negative regulatory effects on IE protein synthesis. The possibility that IE polypeptides themselves regulate IE RNA transcription has not been investigated.

The comparison of the situation found in MCMV with IE proteins encoded by the human and simian CMV reveals several homologies. (i) IE proteins are phosphorylated but not glycosylated (6, 9). (ii) Abundant IE proteins migrate in polyacrylamide gels either as single 94-kd bands (7, 10) or multiple bands between 70 and 90 kd in size (1, 17, 23, 24). (iii) Comparison of in vivo- and in vitro-generated polypeptides reveals a shift in migration patterns, and the appearance of bands differing in size indicates a molecular heterogeneity, which is at least in part due to some post-translational modification in vivo (9, 24). (iv) IE proteins are distributed within the nuclear and the cytoplasmic fractions of infected cells (7, 9, 10).

Thus, the IE phosphoproteins of CMV show similarities with regard to their physical and biochemical properties. Similar functional properties can be suggested.

IE proteins are strongly immunogenic proteins in humans (8), and the humoral immune response to IE proteins correlates with active virus excretion (17). The murine model added the information that cell membrane alterations appear in association with the expression of IE proteins in infected cells which are recognized by cytolytic T-cells (19–21). The proteins characterized in this study represent only the dominant IE proteins. The smaller polypeptides generated in vivo and in vitro require further studies. They appear to be related to the 89-kd protein since they react with the monoclonal antibody precipitating pp89 (G. Keil, unpublished data). To prove that pp89 and its derivate(s) control early RNA transcription, cells are required that express selectively these proteins. The construction of appropriate transformants and the characterization of such cell clones is under way.

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