Immediate-Early Genes of Murine Cytomegalovirus: Location, Transcripts, and Translation Products

GÜNTHER M. KEIL, ANGELIKA EBELING-KEIL, AND ULRICH H. KOSZINOWSKI*

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

Cloned genomic fragments from the region (0.769 to 0.818 map units) coding for immediate-early (IE) transcripts of murine cytomegalovirus (MCMV) were used to analyze the physical organization of this region, the direction of transcription, and the proteins synthesized in vitro. Three IE transcription units could be identified. From IE coding region 1 (ie1; 0.781 to 0.796 map units) a dominant 2.75-kilobase (kb) RNA was transcribed from right to left on the prototype arrangement of the MCMV genome which directed the snythesis of an 89,000-molecular-weight polypeptide (89K polypeptide), the major IE protein. This phosphoprotein (pp89) has been shown to be active in the regulation of transcription. Upstream of ie1 and separated by the MCMV enhancer sequence was a second IE coding region, ie2, which was mapped at 0.803 to 0.817 map units. From ie2 a 1.75-kb RNA of moderate abundance was transcribed in the direction opposite to that of the ie1 RNA. After hybrid selection of the ie2 transcript, a 43,000-molecular-weight translation product was detected. A third coding region, ie3, was located directly downstream of ie1 (0.773 to 0.781 map units). The series of RNAs with low abundance, terminating in ie3, probably used the ie1 transcription start site and ranged from 1.0 to 5.1 kb in size. The 5.1-kb RNA apparently represents the nonspliced transcript from both coding regions ie1 and ie3. A 15K polypeptide was translated in vitro from RNA that was hybrid selected by ie3 sequences. Immunoprecipitation with monoclonal antibody revealed that 31K to 67K polypeptides were related to pp89. Some of these proteins were translated from RNAs that were smaller than 2.75 kb. Polypeptides related to pp89 were also synthesized in vivo. Because polypeptides unrelated to pp89 that were translated from RNA that was selected by ie2 and ie3 sequences were not immunoprecipitated by murine antisera, we assumed that the amount of these proteins synthesized in vivo during infection was probably very low.

Human cytomegalovirus (HCMV) infection is often associated with congenital and acquired immunodeficiency and represents the most frequent viral cause of morbidity and mortality in patients receiving immunosuppressive therapy after bone marrow transplantation (2). Cytolytic T lymphocytes (CTLs) (14) represent a prominent defense mechanism to HCMV, while antiviral humoral immunity is probably of secondary importance (5a).

Cytomegalovirus (CMV) infection is species-specific and is restricted to only a few cell types, even in the natural host. The infection of the mouse with murine CMV (MCMV) provides an experimental model to analyze the immune mechanisms that control CMV infection (21). During acute infection a specific CTL response is generated (15, 18), and adoptive transfer of CTLs into lethally MCMV-infected recipients can limit viral dissemination and prevent mortality (21). MCMV-specific CTL precursors that are present in lymphoid tissues of acutely and latently infected mice can be isolated, quantitated, and tested for functional activity in the absence of stimulating viral antigens (18, 19). The viral antigens recognized by anti-CMV CTLs are largely undefined. By testing the murine effector cell populations for viral antigen specificity, it was found that a high proportion of CTLs are specific for antigens, with an appearance that is correlated with the expression of viral immediate-early (IE) genes (19, 20). The analysis of IE antigen expression with an IE-specific CTL clone (16) revealed a strict temporal correlation between IE gene expression and target antigen recognition (17).

IE genes of herpesviruses are the first viral genes that are expressed after infection of cells. They code for one or more proteins, of which at least one has a regulatory function during viral replication. The number of IE genes and their physical arrangement in the genome differ among herpesviruses. In MCMV abundant IE transcription is restricted to a region between map units 0.769 and 0.817 of the viral genome (7, 11). Antiserum and several monoclonal antibodies revealed one major IE protein with an apparent molecular weight of 89,000 (89K protein). This phosphoprotein, designated pp89 (8), promotes transcriptional activation of unrelated genes (9).

Thus, IE gene products are of interest for two reasons. They regulate the controlled expression of dominant viral genes and at the same time serve as antigens for the immune system of the host. To identify additional IE genes and proteins for subsequent analysis, we studied the organization and expression of MCMV IE genes in more detail.

MATERIALS AND METHODS

Virus and cell culture. MCMV (mouse salivary gland virus, strain Smith, ATCC VR-194) was propagated on BALB/c mouse embryo fibroblasts (MEFs) as described previously (4).

Isolation of IE RNA. MEFs were infected with MCMV at a multiplicity of 20 to 40 PFU per cell in the presence of cycloheximide (50 μg/ml) (7). The preparation of whole cell RNA and the selection of poly(A)$^+$ RNA by oligo(dT)-cellulose chromatography was carried out as described previously (7).

Labeling of nucleic acids and blot hybridization techniques. Purification of plasmids, electroelution of DNA fragments

---

* Corresponding author.

from agarose gels, and labeling by nick repair have been reported previously (4, 7). ^{32}P-labeled cRNA was transcribed with SP6 RNA polymerase by the method of Melton et al. (12). Briefly, 3.0-μg plasmid templates were linearized with appropriate restriction enzymes and purified by phenol extraction. cRNA was transcribed in a 50-μl reaction volume containing 40 mM Tris hydrochloride (pH 7.6); 6 mM MgCl$_2$; 4 mM spermidine; 10 mM dithiothreitol; 400 μM each of ATP, CTP, and GTP; 12.5 μM UTP; 50 μCi of [α-^{32}P]UTP (800 Ci/mmol; Amersham, Braunschweig, Federal Republic of Germany); 50 U of human placental RNase inhibitor; 1 mg of bovine serum albumin per ml; and 9 U of SP6 RNA polymerase. After 60 min at 37°C vanadyl ribonucleoside complex was added to a final concentration of 10 mM, and plasmid DNA was digested with DNase I (20 μg/ml) for 10 min at 37°C. The RNA was extracted with phenol, and free nucleotide triphosphates were separated from the cRNA by chromatography on Sephadex G50 columns.

The synthesis of oligo(dT)-primed and randomly primed ^{32}P-labeled cDNA and blot hybridization techniques for DNA and RNA were carried out as described previously (4, 7). Hybridization with the ^{32}P-labeled cRNA probe was performed at 72°C. After the third wash, the filters were incubated with 20 μg of RNase A per ml in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.1% sodium dodecyl sulfate (SDS) for 20 min at 72°C to remove nonspecifically bound cRNA.

Sucrose gradient centrifugation of RNA. Whole cell IE RNA was size fractionated on linear 15 to 30% (wt/vol) sucrose gradients in 5 mM Tris hydrochloride (pH 7.5)–0.1 M NaCl. RNA (150 μg) was incubated for 2 min at 95°C and laid on top of the gradient. After 15 h at 30,000 rpm in a rotor (SW41; Beckman Instruments, Inc., Fullerton, Calif.), frac-

tions of 320 μl were collected from the bottom. The RNA was precipitated with ethanol, washed twice with 70% ethanol, and suspended in 20 μl of distilled water for further analysis.

Hybrid selection and in vitro translation of RNA. Hybridization of whole cell RNA to viral DNA fragments was carried out by the method of Esche and Siegmann (5), as reported previously in detail (8). Whole cell RNA, size-fractionated RNA, and hybrid-selected RNA were translated into polypeptides in a cell-free rabbit reticulocyte lysate prepared by the method of Pelham and Jackson (13).

Immunoprecipitation and analysis of polypeptides. In vitro-translated proteins were immunoprecipitated after the samples were adjusted to 10 mM Tris hydrochloride (pH 7.6), 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 1 mM methionine, and 1 μg of ovalbumin per ml. These lysates were vortexed for 5 s, left on ice for 30 min, vortexed again for 5 s, and cleared by centrifugation for 30 min at 15,000 × g. Immunoprecipitation with serum or monoclonal antibody and SDS-polyacrylamide gel electrophoresis was carried out as described previously (8).

Enzyme reactions and cloning procedures. All enzymes were purchased from Boehringer, Mannheim, Federal Republic of Germany. Enzyme reactions were performed under conditions recommended by the supplier. Recombinant plasmids were prepared by following published procedures (4, 10) using the plasmid vectors pACYC177, pACYC184, and pSP62. The subclones of pAMB25 were obtained from fragments that were electroeluted from agarose gels after digestion of pAMB25 with the respective enzymes. The plasmid pAMB10 was prepared after digestion of pHindIII-L (4) with HindIII and PstI followed by electroelution of the respective fragment from agarose gels.

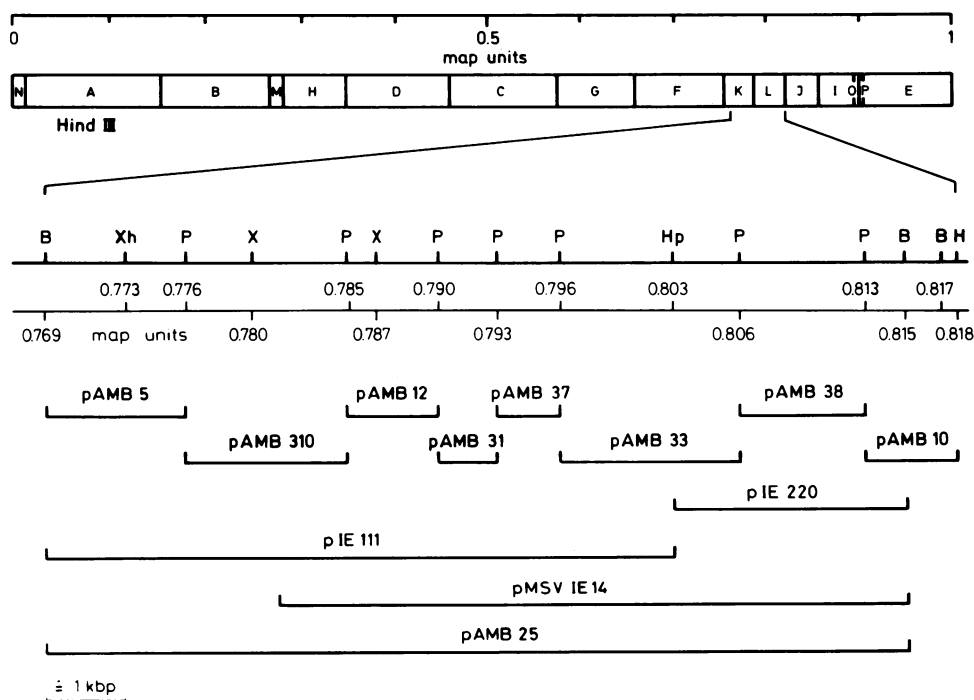

FIG. 1. HindIII cleavage map of the MCMV (Smith) genome. The region between map units 0.769 and 0.818 is expanded. Of the plasmids shown below pIE111, pIE220, pMSVIE14, and pAMB25 have been described previously (7, 9). Viral DNA fragments in pAMB5, -310, -12, -31, -37, -33, -38, and -10 were cloned in pACYC184 and pACYC177. Sequences of pAMB33 were also cloned in pSP62. Only cleavage sites used in this study are shown. Enzymes: B, BamHI; H, HindIII; Hp, HpaI; P, PstI; X, XbaI; Xh, XhoI. kbp, Kilobase pairs.

RESULTS

Coding regions and direction of transcription of abundant IE RNA species. Abundant IE transcription after infection in the presence of cycloheximide originates from a region of approximately 11 kilobase pairs (kbp) (0.769 to 0.817 map units) (4, 7). The map positions of some restriction enzyme cleavage sites and plasmid-cloned DNA fragments of this region are shown in Fig. 1. By using the PstI subclones for hybridization to detect poly(A)$^+$ IE RNA from infected cells, a 5.1-kbp RNA, a dominant 2.75-kbp RNA, and additional 1.0- to 2.75-kbp RNAs that were present in low abundance (7) hybridized to the cloned fragments in pAMB5, -310, -12, -31, -37, and -33. With pAMB33 a minor 1.75-kbp RNA hybridized as well (7), and the plasmids pAMB38 and pAMB10 hybridized selectively to the 1.75-kbp RNA (data not shown).

These results indicate the existence of at least two transcription units that are located separately within the IE coding region. Northern blot hybridizations of IE RNA with strand-specific, ^{32}P-labeled RNA transcribed in vitro by the SP6 RNA polymerase revealed the direction of transcription. In Fig. 2 (lane 1) it is shown that RNA transcribed in vitro from map units 0.769 to 0.803 hybridizes to the 5.1-kbp RNA, the 2.75-kbp RNA, and to the 1.0- to 2.75-kbp RNA smear. For all these RNAs, the direction of transcription in the MCMV genome is from right to left on the prototype arrangement of the viral genome. Because the 2.75-kbp transcript represents the most abundant IE mRNA, the transcription unit coding for this RNA has been referred to as IE coding region 1 (ie1) (9), according to the designation used for clusters of transcription within the IE region of HCMV (25). The 1.75-kbp RNA is transcribed from the separate IE coding region 2 (ie2). Hybridization to RNA transcribed in vitro from map units 0.815 to 0.803 (Fig. 2, lanes 3 and 4) revealed transcription of the 1.75-kbp RNA from left to right on the complementary strand. The transcription start sites of both the 2.75- and 1.75-kbp RNAs were located within the pAMB33 fragment (Fig. 2, lanes 5 and 6). Prolonged exposure of lane 5 (Fig. 2, lane 5') revealed that the start site of the less-abundant 5.1-kbp RNA also maps to pAMB33. The start site for the 2.75-kbp RNA from ie1 was located by S1 nuclease mapping 50 nucleotides to the right of the PstI site at map unit 0.796 (3) (Fig. 1). Because this region contains no additional start, the 5.1- and 2.75-kbp transcripts have a common start site. The transcription start of the 1.0- to 2.75-kbp RNAs in pAMB33 could not be unequivocally demonstrated. In conclusion, at IE times only one strand of each coding region is transcribed into stable transcripts, and coding regions ie1 and ie2 are transcribed in opposite directions.

Identification of a third coding region, ie3. To study the functional activity of the isolated ie1 gene product we constructed plasmid pMSVIE14 (0.781 to 0.815 map units). After transfection of this plasmid into L cells, the authentic pp89 could be immunoprecipitated from several transfected L-cell clones, and its regulatory function was demonstrated. Hybridization of RNA from L-cell clone 45/1 to the BamHI fragment of plasmid pAMB25 revealed a single 2.75-kbp mRNA (9). Consistent with these data the 2.75-kbp transcript from the transfectant hybridized to DNA from map units 0.796 to 0.803 (Fig. 3, lane 2) but not to DNA from map units 0.773 to 0.780 (Fig. 3, lane 4). RNA from infected cells, however, hybridized to both probes (Fig. 3, lanes 1 and 3) and in both hybridizations a 5.1-kbp RNA, a 2.75-kbp RNA, and the 1.0- to 2.75-kbp RNA smear was seen. This suggests
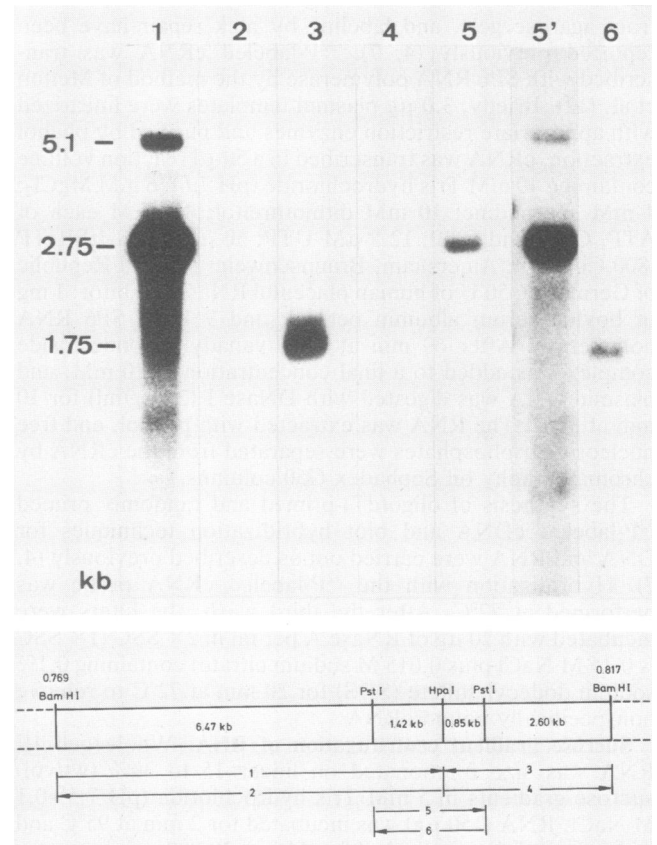
FIG. 2. Autoradiograph of a Northern blot hybridization of ^{32}P-labeled cRNA. A total of 1 μg of IE poly(A)$^+$ RNA per lane was size fractionated in denaturing agarose gels. cRNA was transcribed by SP6 RNA polymerase and labeled with [α-^{32}P]UTP. The direction of cRNA transcription is indicated by arrows in the diagram, and the numbers of the cRNAs used for hybridization correspond to the lanes. cRNA was transcribed from pIE111 (lanes 1 and 2) from pIE 220 (lanes 3 and 4) and from pAMB33 (lanes 5, 5', and 6) (for map locations, see Fig. 1). Abbreviations for restriction enzymes are as given in the legend to Fig. 1. Hybridization was performed as described in the text, and the autoradiographs were exposed for the same period of time, with the exception of lane 5', which shows a prolonged exposure of the hybridization in lane 5.

the existence of two separate 2.75-kbp RNA species: the ie1 transcript translated into pp89 and another 2.75-kbp RNA that hybridizes to sequences from map units 0.769 to 0.780, to which the 5.1-kbp RNA and the 1.0- to 2.75-kbp RNAs hybridized as well.

To confirm the existence of different termination sites, the 3' ends of IE transcripts were analyzed by oligo(dT)-primed, ^{32}P-labeled cDNA from poly(A)$^+$ IE RNA (average size, about 700 base pairs [bp], as determined by gel electrophoresis) (Fig. 4). No hybridization to the 950-bp BamHI-XhoI fragment of pAMB5 could be detected (Fig. 4, lane 1). The moderate hybridization to the adjoining 740-bp XhoI-PstI fragment (Fig. 4, lane 1) and hybridization to the following 860-bp PstI-XbaI fragment of pAMB310 (Fig. 4, lane 2) defined the region of termination for RNA species of low abundance which were shown to hybridize to the XhoI-XbaI fragment (0.773 to 0.780 map units) (Fig. 2, lane 3). The intense hybridization of the 1,210-bp XbaI-PstI fragment of pAMB310 indicated the termination of a dominant transcript(s). This was in accordance with the termination of the 2.75-kbp mRNA to the right of map unit 0.781, as shown
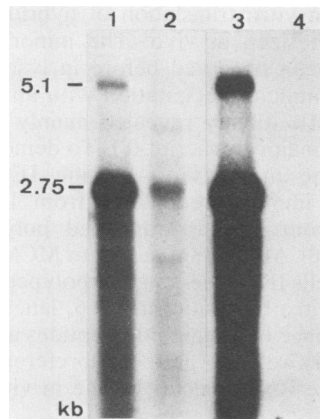
FIG. 3. Autoradiographs of Northern blot hybridizations of ^{32}P-labeled viral DNA fragments to whole cell IE RNA from MEFs and whole cell RNA from 45/1 cells. A total of 0.5 μg of IE RNA from infected MEFs (lanes 1 and 3) and 5 μg of RNA from 45/1 cells (lanes 2 and 4) were size-fractionated in denaturing agarose gels. Virus-specific RNA was visualized after hybridization to the ^{32}P-labeled *Xba*I subfragment of pIE111, representing map units 0.780 to 0.787 (lanes 1 and 2), and to the ^{32}P-labeled *Xho*I-*Xba*I subfragments of pIE111, representing map units 0.773 to 0.780 (lanes 3 and 4). Lanes 3 and 4 were exposed for a prolonged period of time.

with expression from plasmid pMSVIE14, but was incompatible with the hypothesis that the authentic 2.75-kbp RNA from infected cells terminates further downstream. Transcription termination of the major ie1 mRNA to the right of map unit 0.781 and 600 bp downstream of the *Pst*I site (map position 0.785) was further confirmed by S1 nuclease analysis and sequencing of the ie1 gene (G. Keil, A. Ebeling-Keil, and U. H. Koszinowski, manuscript in preparation). The faint hybridization to the adjacent pAMB12 fragment (Fig. 4, lane 3) was therefore also attributed to the ie1 transcript. No termination of poly(A)$^+$ RNA was seen in fragments pAMB31 (Fig. 4, lane 4), -37 (Fig. 4, lane 5), -33 (Fig. 4, lane 6), and -38 (Fig. 4, lane 7).

Thus, IE RNAs of low abundance terminate downstream of ie1 within pAMB5 and the adjoining 1,210-bp *Xba*I-*Pst*I subfragment of pAMB310 (map coordinates 0.773 to 0.781). This region of about 2 kbp is too short to completely encode all the 1.0- to 2.75-kbp RNAs and the 5.1-kbp transcript. For reasons discussed below, this region was defined as transcription unit 3 (ie3).

In the same experiment, the region of transcription termination of the 1.75-kbp RNA from ie2 was shown by the selective hybridization of the cDNA to the 520-bp *Bam*HI subfragment of pAMB10 (Fig. 4, lane 8). Because, as has been shown previously (7), the 1.75-kbp RNA does not hybridize to the neighboring *Bam*HI-*Hin*dIII subfragment of pAMB10, the site of transcription termination in ie2 could be placed to the left of map unit 0.817.

Proteins encoded by ie1, ie2, and ie3. For the assignment of in vitro-translated proteins to the three IE coding regions, hybrid selection experiments were carried out. After translation of RNA that was hybrid selected by the DNA of pAMB5, which represents ie3 sequences (Fig. 5a, lane 1), a protein of about 15K was faintly detectable. Neither antiserum to MCMV proteins nor monoclonal antibody 6/20/1 precipitated this protein (Fig. 5b and c, lanes 1).

DNA from plasmids pAMB310, pAMB12, pAMB31, and pAMB37 selected RNA that directed the in vitro synthesis of

different patterns of proteins (Fig. 5a to c, lanes 2 to 5). DNA corresponding to map coordinates 0.777 to 0.786 (clone pAMB310), representing both ie3 and ie1 sequences, selected RNA that was translated into several polypeptides ranging in molecular weight from 15,000 to 89,000 (Fig. 5a, lane 2). With the exception of a 15K protein, all prominent in vitro translation products were precipitated by both antiserum and monoclonal antibody 6/20/1 (Fig. 5 b and c, lane 2). The 76K protein derivative of pp89 was not detected because processing requires a cellular protease (8). The presence of common epitopes has been confirmed by Western blotting for the 89K and the 76K proteins (8). The low amount of in vitro-synthesized proteins precluded this analysis for the smaller peptides. From RNA selected by the adjacent sequences of pAMB12, similar polypeptides could be translated and immunoprecipitated. Only the RNA encoding a 15K protein could not be selected (Fig. 5a, b, and c, lanes 3). This RNA was selected again by the neighboring DNA fragments in pAMB31 and pAMB37 (Fig. 5a, b, and c, lanes 4 and 5, respectively). If the 15K protein obtained after in vitro translation of RNA that was hybrid selected with different sequences of the MCMV IE region was indeed one and the same, the RNA species directing the synthesis of this polypeptide is probably spliced.

DNA from plasmid pAMB33, which contains only 50 nucleotides of the ie1 coding region and also only a few coding sequences of ie2, was inefficient to select RNA translated into protein (Fig. 5a, b, and c, lanes 6). From pAMB38 and pAMB10, however, RNA was selected which
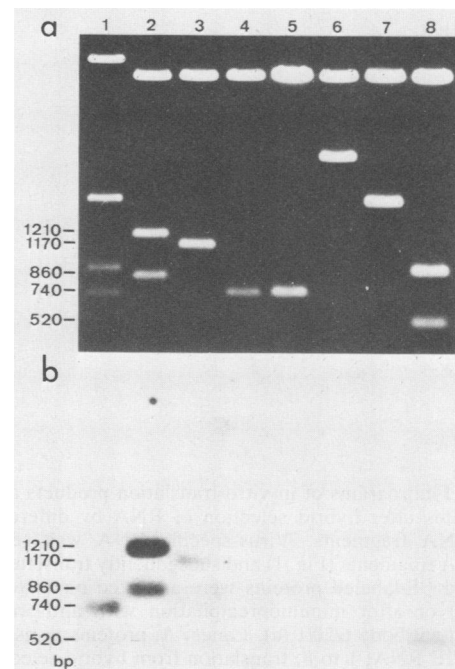

FIG. 4. Southern blot hybridization of ^{32}P-labeled oligo(dT)-primed cDNA from IE poly(A)$^+$ RNA to cloned fragments. (a) Ethidium bromide fluorescence of enzyme-digested DNA fragments after agarose gel separation. (b) Hybridizations of cDNA synthesized with reverse transcriptase to nitrocellulose-bound DNA fragments. Lanes: 1, pAMB5 cleaved with *Bam*HI, *Pst*I, and *Xho*I; 2, pAMB310 cleaved with *Pst*I and *Xba*I; 3, pAMB12 cleaved with *Pst*I; 4, pAMB31 cleaved with *Pst*I; 5, pAMB37 cleaved with *Pst*I; 6, pAMB33 cleaved with *Pst*I; 7, pAMB38 cleaved with *Pst*I; 8, pAMB10 cleaved with *Bam*HI and *Hin*dIII. Some DNA fragment sizes are indicated to the left of the gels in base pairs.
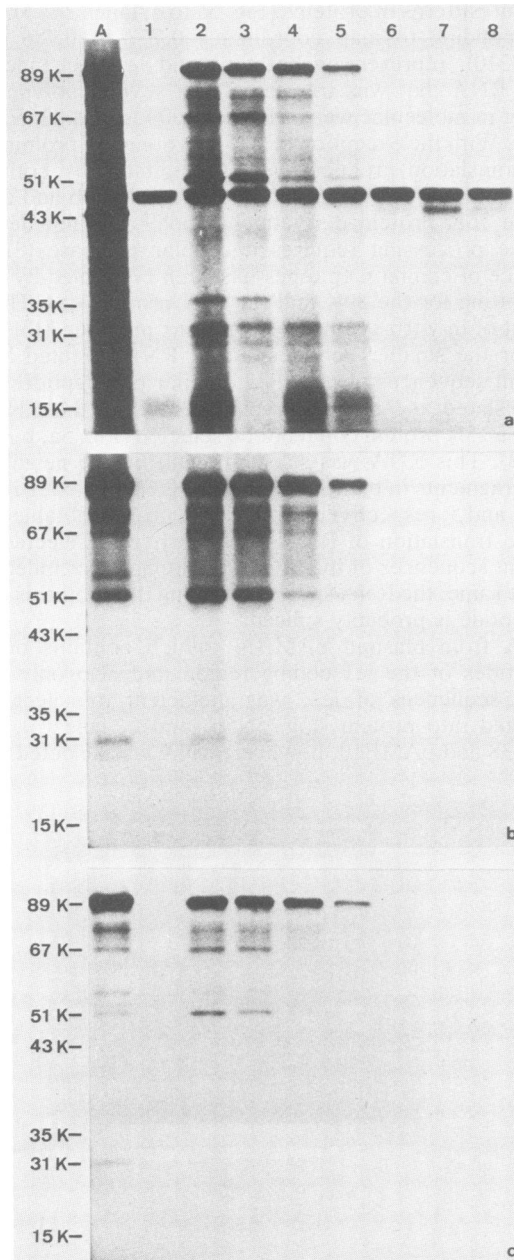
FIG. 5. Fluorograms of in vitro translation products and immunoprecipitates after hybrid selection of RNA by different cloned MCMV DNA fragments. Virus-specific RNA was selected by cloned DNA fragments (Fig. 1) and subsequently translated in vitro. Synthesized ^{35}S-labeled proteins were analyzed on 10% SDS gels directly (a) or after immunoprecipitation with antiserum (b) or monoclonal antibody 6/20/1 (c). Lanes: A, proteins translated from whole cell IE RNA; 1 to 8, translation from hybrid-selected RNA. Cloned fragments used for selection were pAMB5 DNA (lane 1), pAMB310 DNA (lane 2), pAMB12 DNA (lane 3), pAMB31 DNA (lane 4), pAMB37 DNA (lane 5), pAMB33 DNA (lane 6), pAMB38 DNA (lane 7), and pAMB10 DNA (lane 8).

directed the synthesis of a 43K protein (Fig. 5a, b, and c, lanes 7 and 8). It was concluded that the 1.75-kbp RNA from ie2 gives rise to this polypeptide. This protein was not immunoprecipitated by murine antiserum.

It was further tested whether the various polypeptides

detected after in vitro translation of hybrid-selected RNA were also synthesized in vivo. The minor 31K and 15K proteins have been observed before in lysates of infected cells, whereas immunoprecipitation with antiserum or specific monoclonal antibody revealed mainly the translation products of the major transcript (8). To demonstrate in vivo synthesis of the minor virus-specific IE polypeptides, fluorograms of immunoprecipitates from IE-infected cell proteins and from in vitro-translated polypeptides were compared (Fig. 6). Murine antiserum to MCMV precipitated from infected cells the same range of polypeptides that were found after in vitro translation (Fig. 6, lanes 2 and 4). The different abundance of certain polypeptides after in vivo and in vitro synthesis was interpreted as preferential translation of the respective RNA species by the in vitro reticulocyte system.

Polypeptides synthesized in vitro by size-fractionated RNA. Proteins that are antigenically related to pp89 could indicate premature termination of the ie1 transcript or could appear following processing of pp89, degradation or both. Furthermore, the 1.0- to 2.75-kbp RNA smear could represent the breakdown of the dominant 2.75-kbp mRNA. To associate the different RNA species with translation products, whole cell IE RNA was size fractionated by sucrose gradient centrifugation. Fractionated RNA was analyzed by Northern blotting (Fig. 7a and b), in vitro translation (Fig. 7c), and immunoprecipitation (Fig. 7d). Figure 7a shows the hybridization of the fractionated RNAs originating from ie1 and ie3 to pAMB310, and Fig. 7b shows the hybridization of ie2 transcripts to pAMB38. Most of the 2.75-kbp mRNA was found in fractions 14 to 20, and the 1.75-kbp RNA accumu-
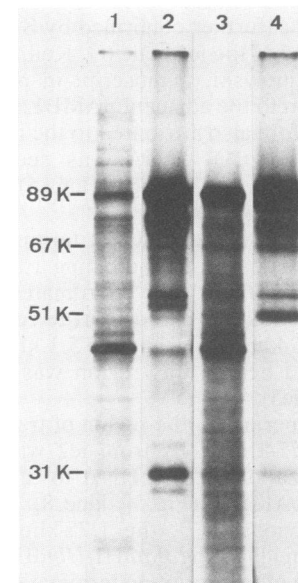
FIG. 6. Fluorograms of IE-infected cell proteins and proteins synthesized in vitro from IE RNA after immunoprecipitation with murine antiserum to MCMV. MEFs were infected in the presence of cycloheximide, and at 3 h postinfection cycloheximide was exchanged for actinomycin D. Proteins were labeled for 3 h with [^{35}S]methionine. In the figure are shown lysates and the polypeptides that were immunoprecipitated with antiserum from IE-infected MEFs (lanes 1 and 2) and proteins synthesized in vitro (lanes 3 and 4). Proteins were visualized by fluorography after separation in 10% SDS-polyacrylamide gels. The film was exposed for 3 days (lanes 1 and 3) and 21 days (lanes 2 and 4) to demonstrate minor IE proteins.

lated in fractions 17 to 22. The 5.1-kbp RNA was detectable in fractions 11 to 14 after longer exposure times (data not shown). In vitro translation products of fractionated RNAs were separated in 10% SDS-polyacrylamide gels (Fig. 7c) and were also subjected separately to immunoprecipitation by murine antiserum (Fig. 7d) or monoclonal antibody. The results of the immunoprecipitation with the monoclonal antibody are not shown because precipitation with antiserum was more effective (Fig. 5).

The proteins translated from RNA in fractions 11 to 14, which contained the 5.1-kbp RNA, could be attributed to the smaller viral RNA species. From RNA in fractions 14 to 20,

FIG. 8. Summary of the proposed organization of the MCMV IE region. Map units indicate the limits of the coding regions of ie1, ie2, and ie3. The arrow indicates the direction of transcription and the relative abundance of RNA. The RNA size classes are given below each arrow in kilobases, and the apparent molecular weights (10^3) of the polypeptides are indicated above. The RNA that terminates within ie3 contains several size classes.

which contained the bulk of the 2.75-kbp mRNA, the 89K protein was translated. Fractions 16 to 20 also gave rise to the 31K polypeptide. Therefore, this polypeptide is either a processed product of pp89 or a translation product of an RNA species that is very similar in size. The 2.75-kbp RNA from ie3, which terminated in pAMB5, could not be associated with a translation product. If RNA species smaller than 2.75 kbp represented degradation, this should affect especially larger RNAs and result in a smear of small polypeptides. The 67K and 51K proteins, however, were efficiently translated from RNA fractions (Fig. 7, lanes 21 to 23) which contained only small amounts of the 2.75-kbp mRNA. By using the 1.75-kbp RNA from ie2 as size marker, the 51K polypeptide appeared to be derived from an RNA of about 1.7 kbp. A 15K polypeptide was translated from an RNA of small size. Because this protein could not be immunoprecipitated, its relation to the 15K translation product of hybrid-selected ie3 RNA (Fig. 5, lane 1) could not be established. Precipitation of smaller in vitro-translated proteins by monoclonal antibody from fractions that contained little or no pp89 excluded immunoprecipitation as a result of complex formation with pp89. For reasons mentioned above, the 43K translation product of the 1.75-kbp RNA from ie2 could not be immunoprecipitated. A scheme of the proposed organization and expression of the MCMV IE region is depicted in Fig. 8.

DISCUSSION

Products of IE genes of MCMV appear to be dominant antigens that are recognized by the cellular immune system of the host (20). One of the interesting aspects of herpesvirus IE genes is the interaction between virus and host cell that leads to the expression of IE gene products which perform tasks that lead to the expression of subsequent genes. In HCMV and MCMV a dominant IE gene product was identified, and it was shown that one of the functions of the major IE protein is to regulate transcription of other genes. Although the major IE protein of MCMV pp89 is by far the most abundant IE gene product, other MCMV IE genes exist (7, 9). In this study the organization of this region was studied in more detail.

We demonstrated that IE RNA is transcribed from three transcription units, ie1, ie2, and ie3. ie2 is located at the far right in the IE region and is separated from ie1 by the MCMV enhancer. ie1 is located to the left of the enhancer, and ie3 is defined by sequences downstream of the termination site of ie1 transcription.

Transcription unit ie1 was defined as the genomic region that encodes the dominant 2.75-kbp RNA. By hybridization
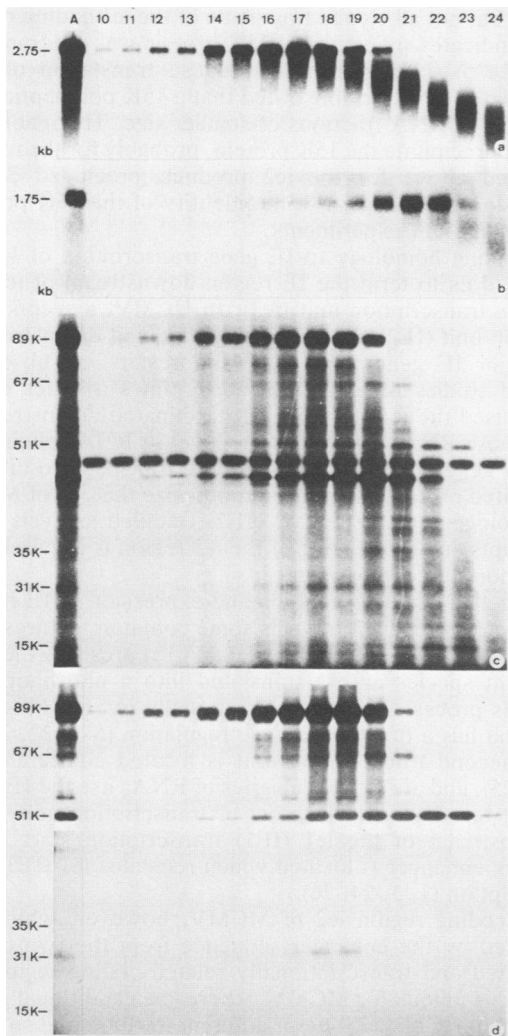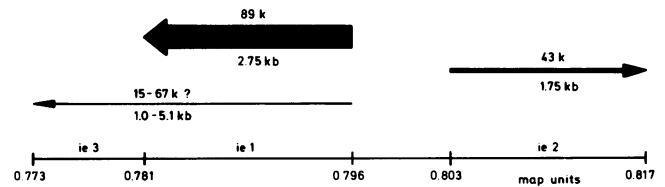

FIG. 7. Autoradiograms of Northern blot hybridizations of size-fractionated IE RNA and fluorograms of in vitro translation products of the fractionated IE RNA. Whole cell IE RNA was size fractionated by sucrose gradient centrifugation; fractions (indicated numerically above the lanes) were collected from the bottom of the gradients, and the RNA was concentrated by ethanol precipitation. RNA from each fraction was analyzed by Northern blot hybridization to nick-translated DNA from pAMB310 (a) and pAMB38 (b). Fractions from each RNA fraction were used for in vitro translation (c), followed by immunoprecipitation with a murine antiserum to MCMV proteins (d). Proteins were analyzed on 10% SDS-polyacrylamide gels. Lane A shows properties of unfractionated IE RNA. The lower part of panel d was overexposed to demonstrate the 31K protein.

to viral DNA (7) and to plasmid-cloned fragments, this RNA was found to be by far the most abundant transcript. The transcription start site was mapped by S1 nuclease analysis to a position 50 nucleotides upstream from a *Pst*I site (map unit 0.796). The region of the 3' terminus of the RNA was mapped by expression studies of cloned fragments and by DNA hybridization of poly(A)$^+$ RNA from infected cells to a position upstream of map unit 0.781. These data were corroborated by in vitro translation of RNA that was selected by different fragments of the IE region. The precise determination of the 3' terminus, the intron-extron structure of the ie1 gene, and the amino acid sequence of the proteins encoded by ie1 will be reported in a subsequent report (Keil et al., in preparation). The 2.75-kbp mRNA from ie1 directed the synthesis of pp89, as shown by selective expression of the transfected gene (9) and by translation of size-fractionated RNA. The pp89, a phosphoprotein (8), is active in transcriptional regulation (9).

The major IE protein was immunoprecipitated with a monoclonal antibody and murine antiserum to MCMV. In addition to the 89K product and its 76K derivative, a product of enzymatic in vivo cleavage (8), a variety of additional smaller proteins were immunoprecipitated by the monoclonal antibody from lysates of infected cells. The same range of smaller proteins occurred after in vitro translation of IE RNA selected by ie1 DNA. The finding that some of the smaller proteins were immunoprecipitated from size-fractionated RNA that did not give rise to pp89 suggests for these peptides, first, that immunoprecipitation was not due to complex formation with pp89 but to antigenic homology; second, that they were not processed derivatives of pp89; and third, that RNA species smaller than the 2.75-kbp RNA directed the synthesis of these proteins. There remained an ambiguity with regard to the precursor product relation to a 31K protein which was translated from RNA that comigrated with the major IE transcript and was also immunoprecipitated with monoclonal antibody.

The transcription unit ie2 is separated from ie1 by the MCMV enhancer sequence (3) and codes for a 1.75-kbp RNA that is transcribed in the opposite direction to ie1. The transcription start site maps to the right of map unit 0.803 because cRNA transcribed from pAMB33 hybridized with the 1.75-kbp RNA, whereas ^{32}P-labeled DNA from the *Pst*I-*Hpa*I subfragment of pAMB33 (0.796 to 0.803 map unit) did not (unpublished data). The 3' end maps between map units 0.815 and 0.817, which is within the 520-bp *Bam*HI subfragment of pAMB10. The 43K translation product of the 1.75-kbp RNA was detectable only after in vitro translation of hybrid-selected RNA because an abundant cellular protein, probably actin, migrated at a similar position. The relative mobility of this viral protein following synthesis in vivo could not be defined, because antisera obtained either from persistently or latently infected mice or from mice immunized with IE-infected cells did not precipitate this protein. We assume that the amount of protein synthesized during infection is too low to elicit a vigorous antibody response. So far no function in viral gene expression could be assigned to this protein (9).

Transcription of IE RNA from a region downstream of the 3' terminus of the ie1 transcript was found when RNA from cells transfected with the complete ie1 gene was compared with RNA from IE-infected cells. By using oligo(dT)-primed cDNA from poly(A)$^+$ IE RNA, it was found that IE RNA species of low abundance apparently terminate in a region that extends between 0.773 and 0.780 map units. Northern blotting revealed that 5.1-kbp RNA, 2.75-kbp RNA, and 1.0-

to 2.75-kbp RNA smears are transcribed from the strand that codes for the ie1 RNA but terminate in this region. The start site in pAMB33 is shared by the 5.1-kbp RNA. For the other RNA species this start site can only be suggested because first, the transcribed sequences of 50 nucleotides in pAMB33 were too short to detect these minor species with certainty; second, a signal at 2.75-kbp could not be discriminated from the ie1 transcript; and third, hybridization to pAMB33 of an RNA smear without distinguishable size classes could not prove that these RNAs were identical with those hybridizing to sequences downstream of ie1. A 15K polypeptide could be translated from RNA that was selected by DNA sequences which did not select the ie1 transcript. The 15K polypeptide was also seen after selection of IE RNA by several but not all cloned fragments of the ie1 coding region, which indicates splicing. The 15K protein was not translated from the 5.1-kbp transcript, because translation of size-fractionated RNA demonstrated that a 15K polypeptide was only seen in RNA fractions of smaller size. The inability to immunoprecipitate the 15K protein, probably for the reasons discussed above for the ie2 product, precluded definite conclusions with regard to the identity of the 15K proteins seen in different experiments.

A striking homology to IE gene transcription of HCMV prompted us to term the IE region downstream of ie1 as a separate transcription unit (ie3). In HCMV, a second transcription unit (IE2) was found to be located downstream of the major IE gene (IE1) (25), and results of subsequent detailed studies revealed that at IE times a series of IE2 RNAs used the ie1 start site but terminated downstream of IE1. These RNAs differed at the level of RNA splicing and thereby generated mRNAs that were translated to different but related proteins (23). We hypothesize that ie3 of MCMV is homologous to the HCMV IE2. Detailed analysis of ie3 transcripts and sequencing of the ie3 region is required for a more thorough comparison.

Altogether, the organization and expression of IE regions in MCMV and HCMV reveals some common features. (i) A single RNA from ie1 (IE1 of HCMV) represents the most abundant species and is translated into a phosphoprotein which is processed posttranscriptionally in vivo but not in vitro and has a function in gene regulation (6–9, 22, 25, 27) (ii) A second transcription unit is located adjacent to ie1 (IE1) (25), and at IE times a series of RNAs use the IE1 start site but terminate downstream of transcription unit 1 (23). (iii) Upstream of the ie1 (IE1) transcription start site, a complex enhancer is located which regulates ie1 (IE1) gene transcription (1, 3, 24, 26).

The coding region ie2 of MCMV, however, which was separated by the enhancer sequence from the two closely associated and transcriptionally related coding regions ie1 and ie3, is unique for MCMV, whereas an additional coding region 3 in HCMV (25) has a different location and so far no counterpart in MCMV.

LITERATURE CITED

1. **Boshart, M., F. Weber, G. Jahn, K. Dorsch-Häsler, B. Fleckenstein, and W. Schaffner.** 1985. A very strong enhancer is located upstream of an immediate early gene of human

cytomegalovirus. Cell **41**:521–530.

2. **Burns, W. H., and R. Saral.** 1985. Opportunistic viral infection. Br. Med. Bull. **41**:46–49.

3. **Dorsch-Häsler, K., G. M. Keil, F. Weber, M. Jasin, W. Schaffner, and U. H. Koszinowski.** 1985. A long and complex enhancer activates transcription of the gene coding for the highly abundant immediate early mRNA in murine cytomegalovirus. Proc. Natl. Acad. Sci. USA **82**:8325–8329.

4. **Ebeling, A., G. M. Keil, E. Knust, and U. H. Koszinowski.** 1983. Molecular cloning and physical mapping of murine cytomegalovirus DNA. J. Virol. **47**:421–433.

5. **Esche, H., and B. Siegmann.** 1982. Expression of early viral gene products in adenovirus type 12-infected and -transformed cells. J. Gen. Virol. **60**:99–113.

5a. **Hamilton, J. D.** 1982. Cytomegaloviruses and immunity, p. 49. *In* J. L. Melnick (ed.), Monographs in virology. S. Karger, Basel.

6. **Jahn, G., E. Knust, H. Schmolla, T. Sarre, J. A. Nelson, J. K. McDougall, and B. Fleckenstein.** 1984. Predominant immediate-early transcripts of human cytomegalovirus AD 169. J. Virol. **49**:363–370.

7. **Keil, G. M., A. Ebeling-Keil, and U. H. Koszinowski.** 1984. Temporal regulation of murine cytomegalovirus transcription and mapping of viral RNA synthesized at immediate early times after infection. J. Virol. **50**:784–795.

8. **Keil, G. M., M. R. Fibi, and U. H. Koszinowski.** 1985. Characterization of the major immediate-early polypeptides encoded by murine cytomegalovirus. J. Virol. **54**:422–428.

9. **Koszinowski, U. H., G. M. Keil, H. Volkmer, M. R. Fibi, A. Ebeling-Keil, and K. Münch.** 1986. The 89,000-M_r murine cytomegalovirus immediate-early protein activates gene transcription. J. Virol. **58**:59–66.

10. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

11. **Marks, J. R., J. A. Mercer, and D. H. Spector.** 1983. Transcription in mouse embryo cells permissively infected by murine cytomegalovirus. Virology **131**:247–254.

12. **Melton, D. A., P. A. Kreig, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green.** 1984. Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Res. **12**:7035–7056.

13. **Pelham, H. R. B., and R. J. Jackson.** 1976. An efficient mRNA-dependent translation system from reticulocyte lysates. Eur. J. Biochem. **67**:247–256.

14. **Quinnan, G. V., Jr., N. Kirmani, A. H. Rook, J. F. Manischewitz, L. Jackson, G. Moreschi, G. W. Santos, R. Saral, and W. H. Burns.** 1982. Cytotoxic T cells in cytomegalovirus infection: HLA-restricted T-lymphocyte and non-T-lymphocyte cytotoxic responses correlate with recovery from cytomegalovirus infection in bone-marrow-transplant recipients. N. Engl.

J. Med. **307**:7–13.

15. **Quinnan, G. V., J. E. Manischewitz, and F. A. Ennis.** 1978. Cytotoxic T lymphocyte response to murine cytomegalovirus. Nature (London) **273**:541–543.

16. **Reddehase, M. J., H.-J. Bühring, and U. H. Koszinowski.** 1986. Cloned long-term cytolytic T-lymphocyte line with specificity for an immediate-early membrane antigen of murine cytomegalovirus. J. Virol. **57**:408–412.

17. **Reddehase, M. J., M. R. Fibi, G. M. Keil, and U. H. Koszinowski.** Late-phase expression of a cytomegalovirus immediate-early antigen recognized by cytolytic T lymphocytes. J. Virol. **60**:1125–1129.

18. **Reddehase, M. J., G. M. Keil, and U. H. Koszinowski.** 1984. The cytolytic T lymphocyte response to the murine cytomegalovirus. I. Distinct maturation stages of cytolytic T lymphocytes constitute the cellular immune response during acute infection of mice with the murine cytomegalovirus. J. Immunol. **132**:482–489.

19. **Reddehase, M. J., G. M. Keil, and U. H. Koszinowski.** 1984. The cytolytic T lymphocyte response to the murine cytomegalovirus. II. Detection of virus replication stage-specific antigens by separate populations of in vivo active cytolytic T lymphocyte precursors. Eur. J. Immunol. **14**:56–61.

20. **Reddehase, M. J., and U. H. Koszinowski.** 1984. Significance of herpesvirus immediate early gene expression in cellular immunity to cytomegalovirus infection. Nature (London) **312**:369–371.

21. **Reddehase, M. J., F. Weiland, K. Münch, S. Jonjić, A. Lüske, and U. H. Koszinowski.** 1985. Interstitial murine cytomegalovirus pneumonia after irradiation: characterization of cells that limit viral replication during established infection of the lungs. J. Virol. **55**:264–273.

22. **Stenberg, R. M., D. R. Thomsen, and M. F. Stinski.** 1984. Structural analysis of the major immediate early gene of human cytomegalovirus. J. Virol. **49**:190–199.

23. **Stenberg, R. M., P. R. Witte, and M. F. Stinski.** 1985. Multiple spliced and unspliced transcripts from human cytomegalovirus immediate-early region 2 and evidence for a common initiation site within immediate-early region 1. J. Virol. **56**:665–675.

24. **Stinski, M. F., and T. J. Roehr.** 1985. Activation of the major immediate early gene of human cytomegalovirus by *cis*-acting elements in the promoter-regulatory sequence and by virus-specific *trans*-acting components. J. Virol. **55**:431–441.

25. **Stinski, M. F., D. R. Thomsen, R. M. Stenberg, and L. C. Goldstein.** 1983. Organization and expression of the immediate-early genes of human cytomegalovirus. J. Virol. **46**:1–14.

26. **Thomsen, D. R., R. M. Stenberg, W. F. Goins, and M. F. Stinski.** 1984. Promoter-regulatory region of the major immediate early gene of human cytomegalovirus. Proc. Natl. Acad. Sci. USA **81**:659–663.

27. **Wilkinson, G. W. G., A. Akrigg, and P. J. Greenaway.** 1984. Transcription of the immediate early genes of human cytomegalovirus strain AD169. Virus Res. **1**:101–116.