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The 89,000-M_r Murine Cytomegalovirus Immediate-Early Protein Activates Gene Transcription

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To study *trans*-activation of gene expression by murine cytomegalovirus (MCMV) immediate-early (IE) proteins, the IE coding region 1 (ie1), which encodes the 89,000-M_r IE phosphoprotein (pp89), was stably introduced into L cells. A cell line was selected and characterized that efficiently expressed the authentic viral protein. The pp89 that was constitutively expressed in L cells stimulated the expression of transfected recombinant constructs containing the bacterial chloramphenicol acetyltransferase (CAT) gene under the control of viral promoters. The regulatory function of the ie1 product was confirmed by transient expression assays in which MCMV IE genes were cotransfected into L cells together with recombinant constructs of the CAT gene. For CAT activation by the ie1 product, a promoter region was required, but there was no preferential activation of a herpes simplex virus type 1 delayed-early promoter. All plasmid constructs that contained the intact coding sequences for pp89 induced gene expression in *trans*. The MCMV enhancer region was not essential for the expression of a functional IE gene product, and testing of the *cis*-regulatory activity of the MCMV enhancer revealed a low activity in L cells. Another region transcribed at IE times of infection, IE coding region 2, was unable to induce CAT expression and also did not augment the functional activity of ie1 after cotransfection.

Infection of the mouse with the murine cytomegalovirus (MCMV) serves as a model to study a herpesvirus infection in the natural host. The biological features of MCMV infection and human CMV (HCMV) infection are similar (13). Both viruses have a genome of 235 kilobase pairs (kbp), although the organization of the genomes is different (5, 26). Similar to HCMV and other herpesviruses, MCMV genes are expressed in three sequential phases which show coordinated regulation and temporal control (14, 15, 17, 24). The initial class of gene products, the immediate-early (IE) polypeptides, is necessary for the next phase, in which the early polypeptides are produced. Herpesviruses differ with regard to the number of IE genes, and the function(s) of individual IE gene products is unclear. Studies with temperature-sensitive mutants revealed that in herpes simplex virus type 1 (HSV-1) the protein Vmw-175 or ICP4 encoded by IE gene 3 and in pseudorabies virus the single 180,000-M_r (180K) IE gene product are continuously required for early and late gene transcription (28, 31, 39). In HCMV three regions of IE transcription have been defined (36), and a 72K to 75K phosphoprotein may play a dominant role in the regulation of HCMV gene expression (37). In MCMV the major 89K IE phosphoprotein (pp89) is the candidate for a regulatory protein (18).

Transfection assays with recombinant plasmids which contain indicator genes linked to eucaryotic promoter- and transcriptional-control elements provide the means to study the effects of *cis*- and *trans*-activation of gene expression. Imperiale et al. (16) and Green et al. (12) demonstrated that after transfection of cells with plasmids containing the pseudorabies virus IE gene, the pseudorabies virus IE gene product activates in *trans* the expression of genes under control of unrelated promoters. Everett (8) extended these studies to IE genes of HSV-1, HCMV, and varizella-zoster virus. Of the different HSV-1 IE genes the genes encoding the 175K, the 110K, and the 12K proteins appear to play a direct or indirect role in *trans*-activation (8, 28). In addition, Persson et al. (29) demonstrated that cells which constitutively express HSV-1 IE genes can also activate other herpesvirus genes in *trans*.

In this study, we set out to examine whether the gene product of IE coding region 1 (ie1), pp89, can act as an activator of transcription. We transfected mouse L cells with plasmids containing ie1 and selected clones that constitutively express the ie1 gene product. We demonstrate that the ie1 product can stimulate the expression of the chloramphenicol acetyltransferase (CAT) gene under control of eucaryotic promoters, irrespective of whether pp89 is constitutively or transiently expressed. In addition, the functional activity of an IE coding region 2 (ie2) which gives rise to a 1.75-kb RNA was tested. The results suggest that only the product of ie1 stimulates transcription.

MATERIALS AND METHODS

Virus and cell culture. MCMV (mouse salivary gland virus, strain Smith, ATCC VR-194) was used and propagated in BALB/c mouse embryo fibroblasts (MEF) as described previously (5).

Ltk^- clone 1D cells (19) were maintained in Dulbecco modified Eagle medium containing 10% fetal calf serum (Seromed, Munich, Federal Republic of Germany) supplemented with 100 U of penicillin and 100 μg of streptomycin per ml. Cells were grown in plastic tissue culture dishes (Greiner, Nürtingen, Federal Republic of Germany) or microtiter plates (Costar, Cambridge, Mass.). Cultures were maintained at 37°C in 5% CO_2.

Plasmids and cloning procedures. The cloning of MCMV DNA fragments in the vectors pACYC177 and pACYC184 was done by established procedures (23) and has been described before (5, 17). The physical map of the MCMV genome and MCMV DNA fragments contained in plasmids are shown in Fig. 1.

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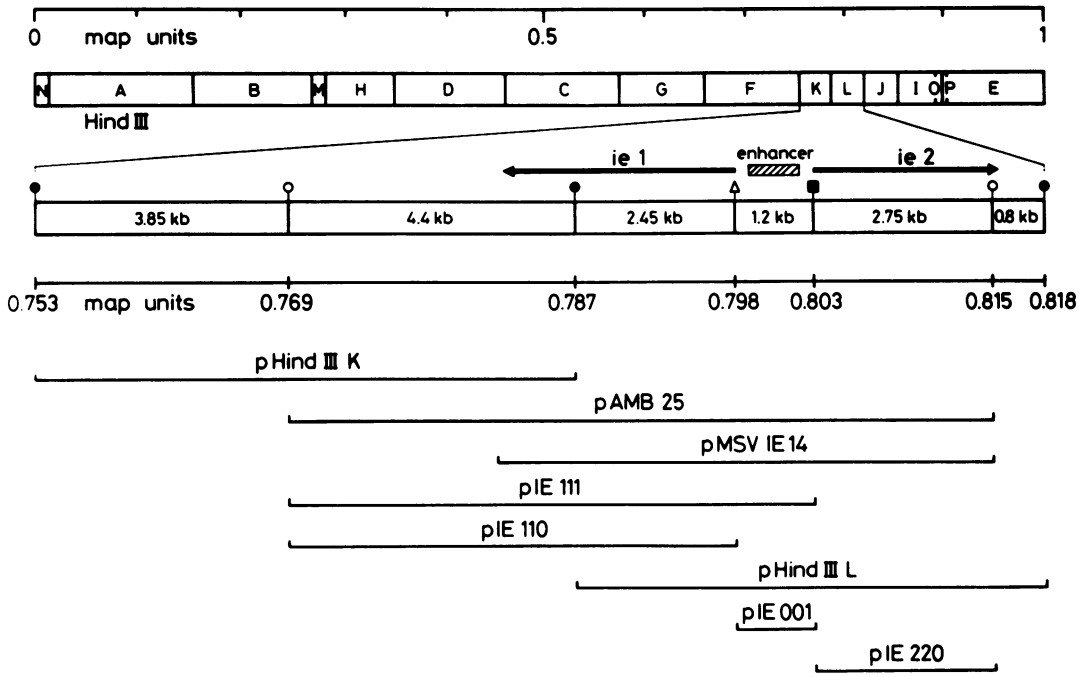

FIG. 1. Physical map of the MCMV genome. The location of the *Hin*dIII cleavage sites is shown. The region encoding the 2.75-kb transcript of ie1 and the 1.75-kb transcript of ie2 separated by the long >700-base-pair enhancer sequence is expanded. The plasmid pMSV-IE14, which contains a 7.8-kb MCMV DNA fragment, was used for the introduction of ie1 into L cells. The 10.8-kb *Bam*HI fragment (plasmid pAMB25) was used as a probe to detect MCMV sequences inserted into cellular DNA and mRNA transcribed in L cells. The other plasmids were used in transient assays to monitor the *trans*-activating function of the IE gene products. The arrows indicate the start and the direction of transcription of the IE genes. Symbols: ●, *Hin*dIII; △, *Dra*I; ■, *Hpa*I; ○, *Bam*HI.

Plasmid pMSV (a gift from J. Doehmer) contains an *Eco*RI fragment with the proviral genome of Moloney mouse sarcoma virus (MSV), 100 base pairs of cellular sequences at the 5′ end of the provirus, and 2.1-kilobase-pair cellular sequences at its 3′ end (38). The plasmid pMSV-IE14 was constructed by insertion of the 10.8-kb MCMV *Bam*HI fragment of plasmid pAMB25 (17) into the unique *Bgl*II site of the MSV vector. The 10.8-kb MCMV *Bam*HI fragment contains the region encoding the major MCMV IE polypeptide (ie1). In the plasmid pMSV-IE14 the gene encoding the major IE polypeptide is transcribed in the direction opposite to that of the retroviral genome. Sequences of 3 kb in size were deleted at the 3′ end of the IE gene during the cloning procedure, probably due to the relaxation of the specificity of the *Bam*HI enzyme (9). After insertion of the MCMV sequences the *Eco*RI fragment was 15.7 kb. The plasmids p*Hin*dIII-K and p*Hin*dIII-L have been described previously (5), and the other constructs (pIE111, pIE110, pIE001, pIE220) were subclones from p*Hin*dIII-K, p*Hin*dIII-L, or pAMB25. The CAT plasmids pA10-CAT2, pSV2-CAT, and pSrM2-CAT (20) were obtained from P. Gruss; pSVO-CAT (10) was from W. Doerfler; and pgD-CAT, which contains the delayed early HSV-1 glycoprotein D promoter (6) in front of the CAT gene, was from R. Everett. The CAT plasmid pMCMV CAT was constructed by insertion of the 1.2-kb *Dra*I-*Hpa*I MCMV DNA fragment cloned in plasmid pIE001 into the *Bgl*II site of pA10-CAT2.

To provide a specific cRNA probe for the CAT gene, the 488-base-pair *Pvu*II fragment of pSV2-CAT was cloned into the *Sma*I site of pSP64 to obtain pSP-CAT.

DNA transfection. For transfection and selection of transfectants, Ltk$^-$ cells (5×10^5 per dish) were seeded into 60-mm-diameter plastic plates 16 h prior to transfection. The calcium phosphate precipitate technique (11) was used, and 1 μg of pAG60 DNA containing the kanamycin-neomycin resistance gene (4) and 10 μg of plasmid DNA were added in 500-μl precipitates per dish. Transfected cell cultures were selected with the aminoglycoside G418 (250 μg/ml; Hoechst, Frankfurt, Federal Republic of Germany). Cultures were refed every 5 to 7 days. Colonies were counted 18 to 24 days after transfection, and single colonies were picked using cloning cylinders and analyzed for IE gene expression by indirect immunofluorescence. Selected colonies were cloned twice by limiting dilution.

For transient gene expression assays, cells were seeded into 100-mm-diameter plates. The calcium phosphate precipitate technique or the DEAE-dextran method described by Luthman and Magnusson (22) was applied. For transfection of MEF only, the calcium phosphate precipitate technique gave satisfactory results. Although by this technique the highest conversion rates also were obtained with L cells, the more reproducible DEAE-dextran precipitate technique was applied in most of the experiments. This technique required lower amounts of DNA than the calcium phosphate precipitate method (21) for efficient CAT gene expression. The addition of carrier DNA to equalize the amount of DNA used for transfection had no effect on the resulting enzyme activity. The experiments shown were carried out without carrier DNA.

Analysis of DNA and RNA. Cells were harvested by trypsinization and washed twice in Ca- and Mg-free phosphate-buffered saline. For the isolation of DNA, the cells were suspended in 50 mM Tris hydrochloride (pH 7.5)–10 mM EDTA–2% sodium lauroyl sarcosinate (17). The cells were treated with 200 μg of RNase A (Serva, Heidelberg, Federal Republic of Germany) per ml for 60 min at 37°C and

1 mg of proteinase K (Serva) per ml for 60 min at 56°C. The DNA was extracted with phenol, chloroform-isoamyl alcohol (24:1), and ether. After ethanol precipitation the DNA was suspended in 20 mM Tris hydrochloride (pH 8.0).

Purification of whole cell RNA, agarose gel electrophoresis of nucleic acids, and transfer on nitrocellulose was carried out as described previously (5, 17, 18). Plasmids were ^{32}P-labeled by nick translation and hybridized to the immobilized RNA or DNA. Northern and Southern blots were exposed to Kodak X-Omat AR films.

Whole cell DNA from transfected cells was harvested from double plates at various times after transfection. DNA (10 μg) was blotted onto nitrocellulose with a slot blot device (Schleicher & Schuell, Dassel, Federal Republic of Germany). Plasmid DNA was visualized by hybridization with a specific cRNA hybridization probe synthesized from pSP-CAT, a plasmid containing the bacteriophage SP6 promoter (25). For the preparation of cRNA the plasmid was linearized by cleavage with *Pst*I, and cRNA was transcribed in the presence of 50 μCi of [^{32}P]UTP (800 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) as described by Melton et al. (25).

Radioimmunoprecipitation. [^{35}S]methionine labeling of cells, preparation of cell lysates, and immunoprecipitation with monoclonal antibody were carried out as described previously (18).

Assay of CAT activity. Cells were harvested by using a rubber policeman 40 h after transfection, and cell extracts were prepared after sonication by the procedure of Gorman et al. (10). The standard assay mixture contained (in a final volume of 180 μl of 0.25 M Tris hydrochloride [pH 7.5]) 20 μl of cell extract, 20 μl of 4 mM acetyl coenzyme A, and 1.5 μl of [^{14}C]chloramphenicol (0.2 μCi/ml; Amersham). The mixture was incubated at 37°C for 60 to 120 min, and the chloramphenicol was extracted and run on thin-layer chromatography plates. Radioactive chloramphenicol was visualized after overnight exposure to Kodak X-Omat S films. After autoradiography the acetylated chloramphenicol forms were excised and counted in a liquid scintillation counter. Under the conditions described, the assay was linear with respect to time and concentration of standard enzyme until a conversion rate of about 70% acetylated products was obtained. At least two replicate determinations for each transfection protocol were carried out in each individual experiment and were found to differ by not more than 2%. However, some differences were seen between results of individual experiments.

RESULTS

Transient and constitutive expression of the product of ie1. The MCMV region of abundant IE RNA transcription spans from map unit 0.769 to 0.817 and codes for one major IE RNA size class of 2.75 kb and several less-abundant RNA species (17). The 2.75-kb mRNA is translated into pp89 which is cleaved in vivo by host cell proteases into a 76K derivate (pp76). Both proteins are transported to the nucleus (18). In analogy to the major IE gene of HCMV (37), we denote the region encoding pp89, which is contained is plasmids pAMB25, pIE111, pIE110, and pMSV-IE14, as ie1. ie1 and ie2, which is entirely located within *Hin*dIII-L (5), are separated by a long enhancer sequence (K. Dorsch-Häsler, G. Keil, F. Weber, M. Jasin, W. Schaffner, and U. H. Koszinowski, Proc. Natl. Acad. Sci. USA, in press).

For the introduction of ie1 into cellular DNA, Ltk$^-$ cells were cotransfected with pMSV-IE14 and pAG60 (4). Colonies which exhibited antigen-specific fluorescence in the majority of nuclei (Fig. 2a) were cloned. Because dividing cells gave a bright signal while cells grown to confluence gave almost no signal, the expression of the antigen appeared to be cell cycle dependent. For characterization of the antigen, viral polypeptides synthesized in clone 45/1 were compared with products from infected MEF (Fig. 2b). In lysates of infected MEF (Fig. 2b, lane 1) and cell line 45/1

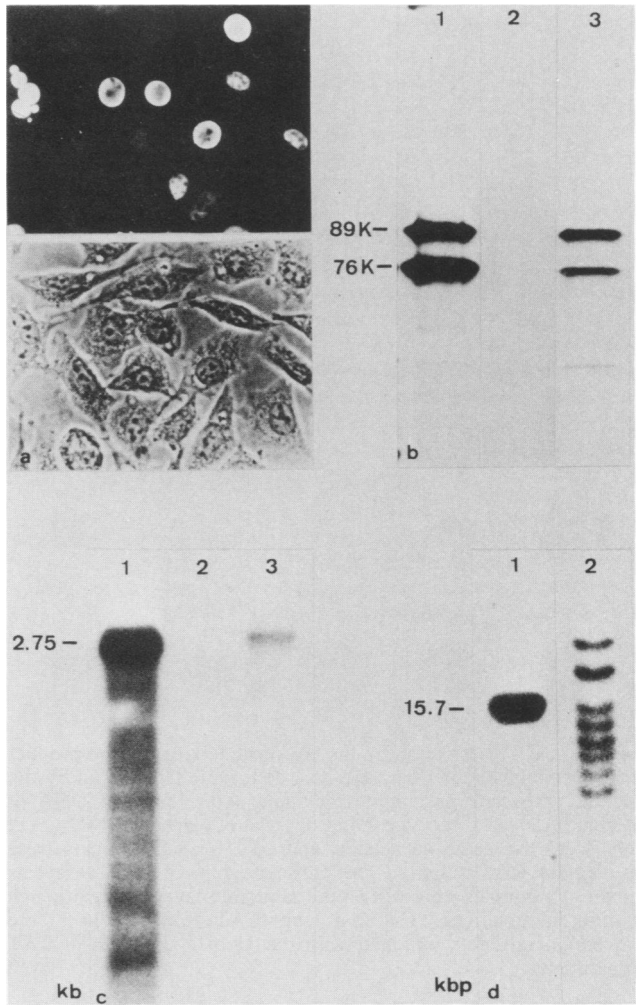

FIG. 2. Characterization of cells expressing pp89. (a, top) Indirect immunofluorescence showing nuclear expression of MCMV IE antigen in the transfected cell line 45/1. (a, bottom) Corresponding phase-contrast microscopic view of cell line 45/1. (b) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% gels) of ^{35}S-labeled proteins immunoprecipitated from cell lysates by monoclonal antibody MCMV to pp89. Cell lysates were from MEF infected (multiplicity of infection, 10) with MCMV in the presence of cycloheximide and labeled after exchange of cycloheximide for actinomycin D 3 h later (lane 1); Ltk$^-$ cells (lane 2); and 45/1 cells (lane 3). (c) Northern blot analysis of RNA from MEF infected with MCMV in the presence of cycloheximide (lane 1); Ltk$^-$ cells (lane 2); and 45/1 cells (lane 3). A 0.5-μg portion of infected MEF RNA and 5-μg portions of RNA from Ltk$^-$ cells and 45/1 cells were fractionated on 1% agarose–formaldehyde gels, transferred to nitrocellulose paper, and hybridized to the ^{32}P-labeled *Bam*HI fragment of plasmid pAMB25. (d) Southern blot analysis of MCMV DNA introduced into L cells. To detect inserted sequences, the *Bam*HI insert of pAMB25 probe was used. Lane 1, 0.5 ng DNA of plasmid pMSV-IE14 added to 10 μg of Ltk$^-$ DNA and cleaved with *Eco*RI; lane 2, 10 μg of DNA of 45/1 cells after digestion with *Eco*RI. Both samples were run on the same gel. kbp, Kilobase pairs.

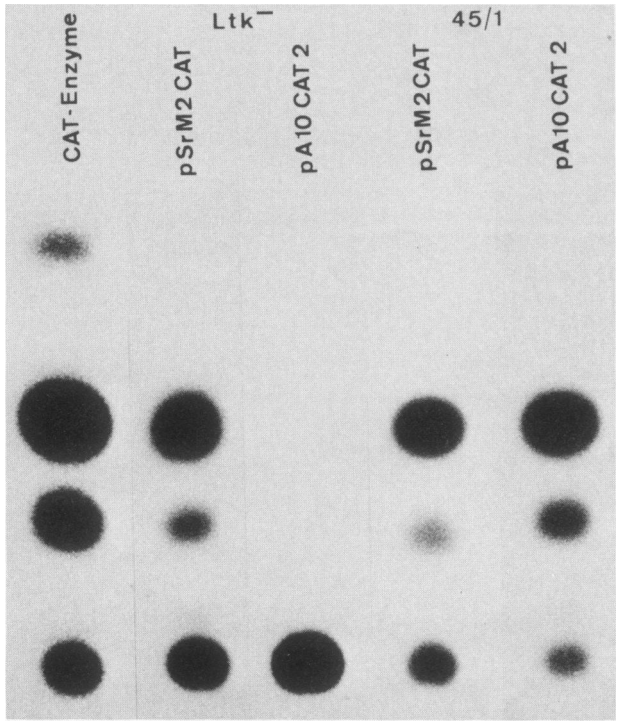

FIG. 3. CAT activation by the constitutively expressed product of ie1. Monolayers of Ltk⁻ cells 45/1 cells were transfected by the calcium phosphate precipitate technique with 25 μg of DNA of plasmid pA10-CAT2 or with 25 μg of DNA of plasmid pSrM2-CAT. Cells were harvested 40 h later, and 20-μl protein extracts were assayed for CAT activity. The chromatogram of CAT assays is shown. All samples were run on the same thin-layer plate, although at different positions. The first lane (CAT-Enzyme) shows the conversion products obtained with 0.6 U of a commercial CAT preparation.

(Fig. 2b, lane 3), pp89 and pp76 were detected after immunoprecipitation and were absent in the nontransfected L cells (Fig. 2, lane 2).

The viral RNA synthesized in 45/1 cells was analyzed by Northern blotting and compared with the RNA transcribed at IE times in infected MEF (Fig. 2c). Using the nick-translated *Bam*HI insert of pAMB25 as probe, a 2.75-kb RNA expressed in infected cells (Fig. 2c, lane 1) (17) was also detected in RNA from 45/1 cells (Fig. 2c, lane 3). In RNA from L cells no hybridization was seen (Fig. 2c, lane 2).

To examine the integration of MCMV sequences into cellular DNA, 45/1 DNA was analyzed by restriction enzyme digestion followed by agarose gel electrophoresis and Southern blotting. Figure 2d shows the results obtained after cleavage with *Eco*RI, which cuts pMSV-IE14 on both sides of the MSV vector-plasmid junctions. By using the nick-translated internal *Bam*HI fragment of pAMB25 as probe, strong hybridization to the 15.7-kb *Eco*RI fragment of pMSV-IE14 was seen. The analysis of 45/1 DNA revealed eight discrete bands that hybridized to MCMV DNA. The different size of the hybridizing bands indicated several inserts. Dot blot analysis revealed the presence of about 10 equivalents of the region transferred by pMSV-IE14 (data not shown).

45/1 cells therefore contain at least one active copy of ie1 from which a RNA of the correct size is transcribed. This RNA is translated into a protein that is indistinguishable from the viral pp89.

Induction of CAT gene expression in 45/1 cells. To determine whether the viral IE protein constitutively expressed in L cells could stimulate expression of the CAT gene under control of a eucaryotic promoter, L cells and 45/1 cells were transfected with DNA of pSRM2-CAT, which contains the MSV enhancer upstream of the simian virus 40 (SV40) promoter (22), or with pA10-CAT2, which contains the enhancerless SV40 promoter (20). The enzyme level expressed from the CAT gene in transient assays correlates with the level of mRNA and thus provides a test to estimate promoter activity (10). The expression of pSrM2-CAT was similar in L cells and 45/1 cells (Fig. 3). However, transfection of pA10-CAT2 into 45/1 cells resulted in high levels of CAT activity, whereas with transfection into L cells no enzyme activity could be detected. Results comparable with those obtained with 45/1 cells were obtained also with five other L-cell clones that were stably transfected with ie1 (data not shown).

45/1 cells are a subclone of the L-cell line; still, both lines

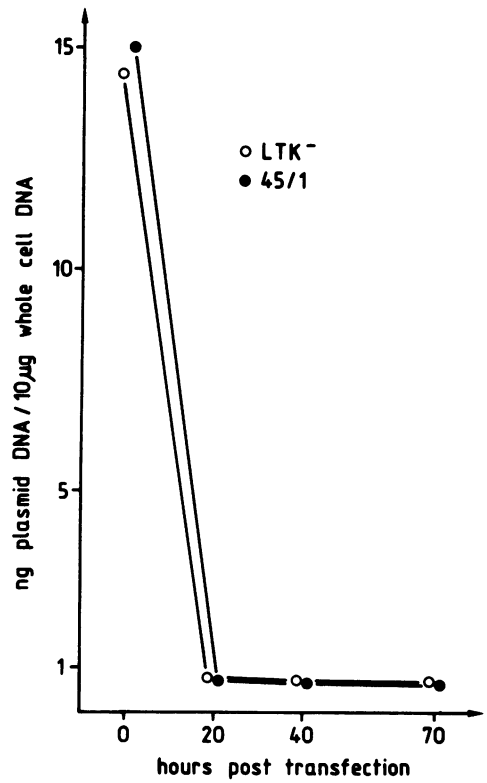

FIG. 4. Stability of transfected plasmid DNA in L cells and 45/1 cells which express pp89 of MCMV. Cells were transfected with 10 μg of pSrM2-CAT, and whole cell DNA was extracted at various times. The time point after removal of the calcium phosphate precipitate was taken as the sample at 0 h. Two replicate plates were extracted. Total DNA (10 μg) was blotted onto nitrocellulose and hybridized to a [^{32}P]cRNA probe specific for the CAT gene. The amount of plasmid DNA in cells was determined by autoradiography using a standard dilution of plasmid DNA. The results were corrected for the number of cells per culture dish determined at 0 h to exclude the effect of different growth rates. Four replicate determinations were carried out at 40 h; two were used for the determination of CAT activity. The chloramphenicol conversion rate by extracts from L cells was 46% and from 45/1 cells was 45%.

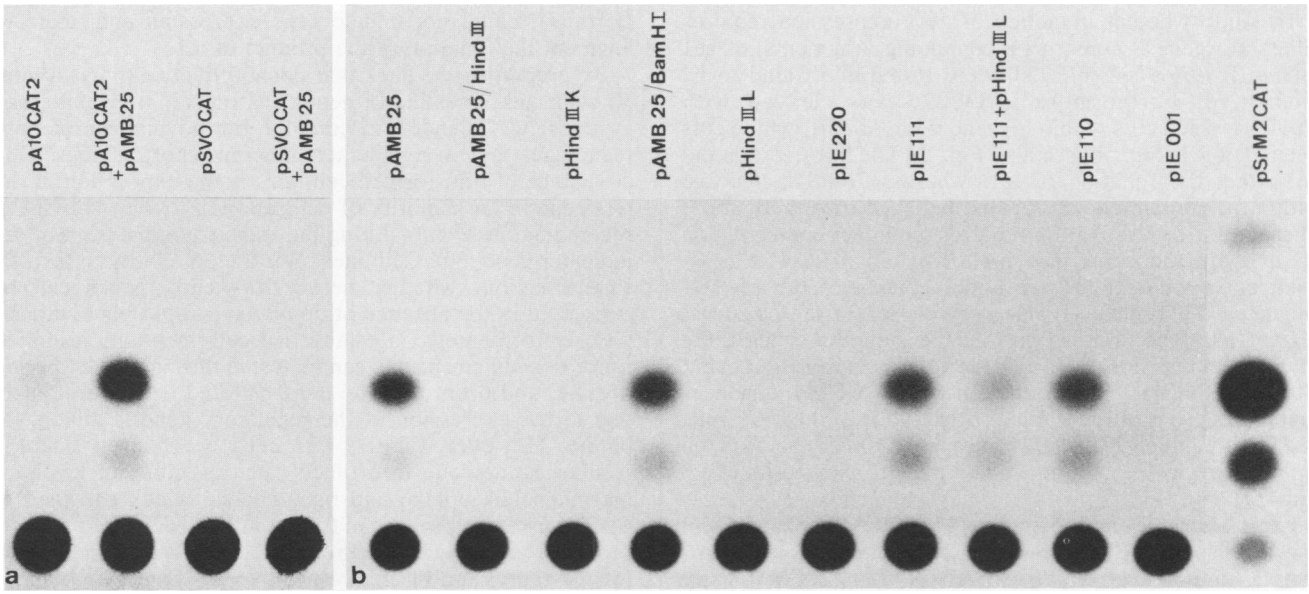

FIG. 5. CAT expression after cotransfection of MCMV sequences. (a) Requirement of promoter sequences in the CAT construct. DNA (10 μg) of plasmids containing the CAT gene constructs with (pA10-CAT2) or without (pSVO-CAT) the SV40 promoter region were transfected by the DEAE-dextran precipitate technique into L cells in the presence or absence of 2 μg of DNA of plasmid pAMB25 containing ie1. Extracts were prepared and assayed for CAT activity 40 h later. (b) MCMV genes and sequences required for CAT induction. DNA (10 μg) of plasmid pA10-CAT2 was transfected together with 2 μg of DNA of plasmids containing sequences of the MCMV IE region (Fig. 1). pAMB25 contained the complete ie1 sequences and the ie2 lacking the 3′ end of the gene. *Hin*dIII cleaved inside and *Bam*HI cleaved outside the ie1 protein coding sequences. p*Hin*dIII-K lacked the 5′ end of the gene in ie1. p*Hin*dIII-L contained the complete ie2 sequences, and pIE220 contained ie2 without the enhancer sequences and the 3′ end of the ie2 gene. pIE111 and pIE110 contained the complete ie1 protein coding sequences, but pIE110 lacked the enhancer sequences. pIE001 contained the enhancer sequences but not the transcription start sites in ie1 and ie2. A control for transfection efficiency (pSrM2-CAT) was included. Cell extracts were prepared 40 h after DEAE-dextran precipitate transfection and assayed for CAT activity.

could be differentially susceptible to transfection procedures. The percentage of transfected cells can vary in different cell lines, and the amount of plasmid DNA maintained by a transfected cell can also be variable and lead to different levels of gene expression. A control therefore was needed to compare the transfection conditions of L cells and the 45/1 derivative. The amount of transfected plasmid DNA present in cells at various times after transfection was determined and used as a standardization parameter (1). The stability of pSrM2-CAT DNA during the 70 h following transfection was analyzed (Fig. 4). Within the first 20 h about 94% of the transfected DNA was lost. At 40 h after transfection the level of CAT expression was tested. There was no difference between the two cell lines with regard to uptake of plasmid DNA, plasmid DNA stability, and CAT expression. Thus, the presence of pp89 in L cells stimulated the expression of a transfected gene in *trans*.

Activation of CAT after transient expression of ie1. The conclusion that pp89 activates transcription was confirmed and extended by transient expression assays. After cotransfection of L cells with ie1, which is contained in pAMB25, and pA10-CAT2, followed by incubation for 40 h, the amount of synthesized CAT activity was assayed. The results (Fig. 5) reveal the *trans*-activating function of the ie1 product also under these conditions. To test whether CAT gene expression requires the presence of transcriptional control elements for *trans*-activation, the enzymatic activity after cotransfection of ie1 and pSVO-CAT (10), which lacks the promoter-regulator region, was tested. The absence of detectable CAT activity with pSVO-CAT indicated that promoter elements are essential for gene expression stimulated by the MCMV IE gene product (Fig. 5a).

In a study on transient gene activation by HSV-1 IE genes, O'Hare and Hayward (28) observed a requirement for homologous promoters. While the SV40 promoter in pA10-CAT2 could only weakly be activated by HSV-1 IE gene products and had to be replaced by HSV-1 delayed-early promoters (28), the presence of pp89 resulted in up to a 50-fold induction of CAT activity with plasmid pA10-CAT2 (see above). The specificity for homologous promoters could not be tested in our study because no early MCMV promoter has been characterized so far. To test the induction of CAT activity with the CAT gene under control of at least a herpesvirus promoter, we tested plasmid pgD-CAT, which contains the delayed early HSV-1 gD promoter (6; data not shown). Different to the results obtained with pA10-CAT2, the transfection of pgD-CAT alone resulted in an increased basal level of enzyme activity. The finding that the stimulation due to ie1 cotransfection was usually about threefold led us to conclude that induction of transcription by the ie1 product does not preferentially act on a herpesvirus promoter.

No *trans*-activation by ie2. In HSV-1 more than one IE gene product can act as activator of transcription, and the combination of certain IE genes provides a higher level of activation than a single IE gene (5, 16). Therefore, plasmid p*Hin*dIII-L, which contains ie2, was tested either alone or in combination with ie1. The results show clearly (Fig. 5b) that the transfection of ie2 sequences alone does not stimulate gene expression. After cotransfection of ie1 (pIE111) and ie2 (p*Hin*dIII-L), there was no augmented activity of pp89. In fact, in the majority of experiments the effect of the ie1 product was even lower after cotransfection of both ie1 and ie2.

To study whether induction of gene expression requires intact IE genes, constructs containing fragments of ie1 (p*Hin*dIII-K) or ie2 (pIE220) were tested and found to be ineffective. Furthermore, pAMB25 was cleaved with *Hin*dIII, which cuts within ie1, and with *Bam*HI, which cuts outside of ie1 (Fig. 5b; see also Fig. 1). The linearization did not affect the function of ie1, whereas *Hin*dIII cleavage within the protein-coding region abolished *trans*-activation. Therefore, *trans*-activation requires the intact coding region of pp89. In the constructs pAMB25 and pIE111, the sequences encoding pp89 are under control of the MCMV enhancer. The enhancer also controls ie2 in the construct p*Hin*dIII-L. The proposition that the induction seen in the transient expression assays is due to a recombination event (30), resulting in the introduction of the MCMV enhancer sequences in a position leading to activation of the CAT gene in *cis*, is unlikely, because cotransfection of the isolated enhancer region cloned in plasmid pIE001 showed no effect (Fig. 5).

Expression of ie1 in the absence of the MCMV enhancer and low activity of MCMV enhancer sequences in L cells. Attempts to demonstrate *trans*-activation of MCMV early genes after superinfection of 45/1 cells failed, and the analysis of MCMV transcriptional activity in L cells revealed that pp89 is not expressed after infection (data not shown). We reasoned that in L cells the MCMV enhancer has a low activity. To test the enhancer requirement for ie1 gene function, the enhancer element was deleted from plasmid pIE111 to give plasmid pIE110. Results (Fig. 5b) show that the enhancer is not essential for ie1 function.

Because these results suggest that there is a strong ie1 promoter but do not prove the low activity of the MCMV enhancer in L cells, the enhancer sequences were inserted upstream of the SV40 promoter in pA10-CAT2 to give pMCMV-CAT. The *cis*-acting activity of the MCMV enhancer sequences on CAT expression was studied in L cells and MEF, in which MCMV can replicate. For comparison the cells were transfected also with pSrM2-CAT and pA10-CAT2. The results (Fig. 6) show that the activity of the MCMV enhancer in L cells is lower than that of the MSV enhancer. However, when tested in MEF the activation by the MCMV enhancer was higher. This observation was independent of the transfection protocol used (data not shown).

Although activation of CAT gene expression in L cells by pMCMV-CAT was low, the IE gene was expressed in L cells after transfection of pIE110, and the inability to demonstrate IE gene expression after infection remained unexplained. MEF and L cells were therefore infected with 5 to 500 PFU of MCMV in the presence of actinomycin D to prevent transcription (33). After infection with 5 PFU, about 80% of nuclei of infected MEF were positive by in situ hybridization, while the nuclei of L cells showed grain numbers in the range of the uninfected control cells, even after infection with 500 PFU of MCMV, which indicates the resistance of this cell line to infection (K. Münch, unpublished data).

DISCUSSION

In this report we have described experiments designed to study the effect of MCMV IE genes on the expression of unrelated genes to identify viral IE genes that are active in transcriptional regulation. We demonstrated that the product of ie1, pp89, can act as an activator of gene expression, independent of whether the gene is constitutively or transiently expressed after transfection. Another IE gene, which is located in ie2 but which is less abundantly transcribed at IE times, could not induce gene expression and failed to augment the function of the product of ie1.

By preparing cell lines that constitutively express a viral IE gene and by testing IE gene constructs in transient assay systems, we intended to combine the advantages of both techniques and to avoid their inherent shortcomings. One advantage of transformed cell lines is the expression of the test gene in the majority of cells and the low probability of recombination events during the transient expression of the indicator gene (30). Cell lines offer the possibility to test, by superinfection, whether herpesvirus early genes can be expressed in the absence of de novo viral protein synthesis (29). Unfortunately, in established cell lines only a limited range of gene constructs can be tested during a given period of time, and proof that the demonstrated effects are solely due to the expression of the regulatory gene is difficult to obtain. However, for the 45/1 cells we showed that the results are not due to artifacts such as different levels of plasmid DNA uptake and maintenance which can lead to CAT expression because of the cumulative effect of the basal promoter activity (1). Furthermore, there was no indication for the repression of enhancer-induced stimulation of transcription by pp89, as has been shown for 293 cells which express the adenovirus E1a and E1b products (2).

The main advantage of transient assays is the quick information about certain functional aspects of complete viral genes or viral control elements. The identical results obtained by using both techniques revealed that pp89 acts as transcriptional activator, and putative effects of *cis*-acting enhancer sequences due to recombination events (30) can be excluded.

Although certain transfected herpesvirus IE genes can *trans*-activate the expression of genes under control of

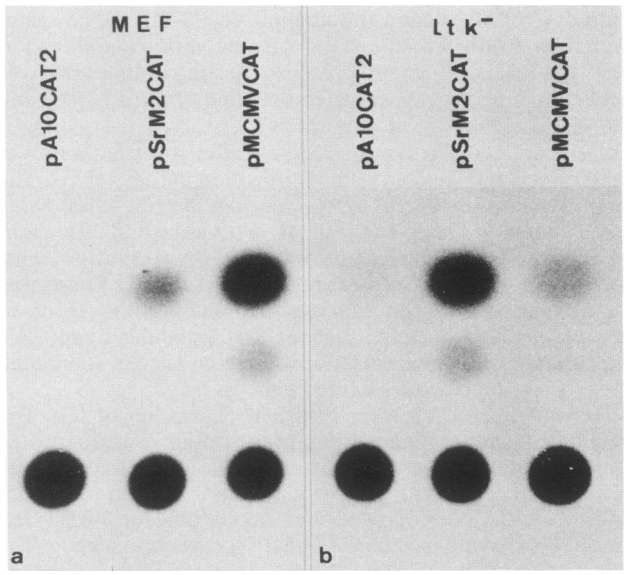

FIG. 6. Cell-specific activity of the MCMV enhancer. DNA of plasmids containing the CAT gene construct and the SV40 promoter alone (pA10-CAT2) or, in addition, the MSV long terminal repeat enhancer (pSrM2-CAT) or the MCMV enhancer sequences (pMCMV-CAT) were transfected into MEF (a) or L cells (b). For transfection of MEF, 20 μg of calcium phosphate-precipitated DNA was used, and Ltk⁻ cells were transfected with 5 μg of DNA by the DEAE-dextran precipitate technique. Cell extracts were prepared 40 h later and assayed for CAT activity.

unrelated promoters (7, 8, 12, 16), there may be differences with regard to the degree to activation. O'Hare and Hayward (27, 28) have reported that homologous HSV-1 promoters are required for efficient CAT gene expression after transfection of HSV-1 IE genes. Everett (7) observed low transcriptional activation of the unrelated HSV-1 gD promoter after cotransfection with the HCMV IE gene. We have shown here that pp89 can activate both the SV40 and the HSV-1 gD promoter and that there is no preferential activation of the herpesvirus promoter. The identification of a homologous MCMV promoter to be tested in these assays is under way.

Single IE gene products of pseudorabies virus and varizella-zoster virus are efficient *trans*-activators (8, 12, 16). A different situation is found in HSV-1. In HeLa cells Vmw-175 (ICP4) alone was found to act as an activator of transcription, and the combination of Vmw-175 and Vmw-110 (ICP0) led to an increase of indicator gene expression (8). In Vero cells, Vmw-110 alone had also *trans*-activating activity, and the 12K IE protein augmented the stimulatory activity of Vmw-175 and Vmw-110 (28). It thus appears that the cell lines used for transient expression assays may influence *trans*-activator function and promoter specificity. Although we demonstrated transcriptional regulation by pp89 after constitutive and transient expression in L cells, the L cells and 45/1 cells were refractory to MCMV gene expression and replication after infection. This is probably due mainly to the failure of virion uptake or DNA transport to the nucleus or both.

There was no indication that the product of ie2 can stimulate transcription or support the activity of ie1. Because we could not demonstrate activation of MCMV early genes in 45/1 cells by superinfection, we currently cannot exclude the possibility of a role of ie2 in the activation of homologous early promoters. The testing of cells that are permissive for MCMV infection and susceptible to efficient transfection experiments should help to clarify the role of ie2. We are now in the process of screening various murine cell lines.

From their study on the activation of gene expression by adenovirus and herpesvirus regulatory genes, Imperiale et al. (16) predicted that there are enhancer elements in the region of herpesvirus IE genes. A strong enhancer has been detected in the IE region of HCMV (3). Similar to HCMV, a very long enhancer sequence is present also in MCMV which separates ie1 and ie2. The MCMV enhancer is, apart from the HCMV enhancer, the strongest one found to date, and in addition the MCMV enhancer has a wide host cell range (K. Dorsch-Häsler et al., in press). The first event of CMV replication, the constitutive transcription of IE genes, is suggested to be under the control of the IE enhancer (3) which directs gene expression in *cis*. It was therefore surprising that in L cells the enhancer had a low activity, whereas the expression of the IE protein did not require the presence of the enhancer sequence. This indicates a tissue-specific function of the MCMV enhancer and suggests that the IE gene encoding pp89 contains an effective promoter.

Cell lines that constitutively express IE gene products may serve to help study other aspects of CMV infection. MCMV IE antigens are recognized by cytolytic T-lymphocyte populations (33, 34) and by IE antigen-specific cytolytic T-lymphocyte clones (32). Because cytolytic T lymphocytes are involved in the control of the spread of MCMV infection, IE antigen expression can be implicated in immune surveillance (35). IE gene products expressed in transfectants in association with the appropriate class I gene products of the major histocompatibility complex will help to define the molecular properties of the antigen(s) recognized by effector T cells.

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