

The 89,000- M_r Murine Cytomegalovirus Immediate-Early Protein Stimulates *c-fos* Expression and Cellular DNA Synthesis

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Received 4 March 1988/Accepted 26 May 1988

The immediate-early (IE) genes of murine cytomegalovirus (MCMV) are expressed in the absence of prior viral protein synthesis and regulate the transcription of MCMV early genes. The effect of MCMV IE genes on growth induction was studied. Different plasmids containing MCMV IE genes were microinjected into arrested NIH 3T3 mouse fibroblasts. Plasmids containing the *ie1* gene coding for the 89,000- M_r major IE protein pp89 were found to stimulate the expression of the *c-fos* protooncogene. Synthesis of pp89 and its transport to the nucleus appeared to be required for *c-fos* expression. DNA synthesis occurred in cells that were injected with MCMV IE genes and in neighboring cells that were not injected. The results suggest that the phosphoprotein pp89 stimulates cells to enter the cell cycle.

Cytomegaloviruses (CMVs) can establish productive cytolytic as well as nonproductive latent infections in cells, and reactivation from latency to productive infection can occur. Certain observations indicate an interdependence between CMV replication and phase transitions of the cell cycle. Murine CMV (MCMV) replication is dependent on the physiological state of the cell, and the virus requires events associated with the host S phase for initiation of viral DNA synthesis (23). The virus itself, however, can promote cellular conditions favorable for viral replication by stimulating cells to synthesize cellular DNA (31) and to release a growth factor (10). These results suggest that at least one CMV gene should have an effect on the cellular genes that control the cell cycle. Given that the expression of this gene is subject to the control of cellular factors, this gene could play a role in the regulation of productive and nonproductive infection of cells.

Gene expression in MCMV is regulated in a cascade fashion and can be subdivided into three phases, immediate-early (IE), early (E), and late. Because stimulation of cellular DNA synthesis and release of cellular growth factors do not require CMV DNA synthesis (10, 31), viral functions expressed IE or E after infection should be involved. We studied the IE genes of MCMV as candidates. The IE genes of MCMV are clustered in a region of about 10 kilobase pairs (13). Three IE transcription units are contained in this region, *ie1*, *ie2*, and *ie3* (14). The gene *ie1* in transcription unit *ie1* and genes transcribed from *ie3* use the same promoter which is controlled by a very long and complex enhancer located upstream (5, 15). The promoter of the transcription unit *ie2* at a position downstream of the enhancer is also regulated by this sequence. MCMV and the human CMV have similar enhancer sequences (2), and

several cellular factors bind to different sequence motifs and thus regulate the expression of the IE genes (9).

CMV IE gene products play a role in the transcriptional activation of CMV E genes. Among the MCMV IE genes, *ie1* is most abundantly expressed and gives rise to the nonviral phosphoprotein pp89, the major IE protein (16). pp89 activates gene in *trans* without apparent promoter specificity (18). To exert its function in gene regulation, pp89 should directly or indirectly interact with DNA. Direct binding to DNA has not yet been demonstrated, but binding to histones has been reported (24a). As with viral proteins of other viruses that influence cell cycle regulation (11, 27, 29), an association of pp89 with kinase activity has recently been shown (A. Barnekow, J. Schickedanz, and U. H. Koszinowski, manuscript in preparation). These multifunctional properties of pp89 have led us to assume that it plays a role in cell cycle regulation. To investigate this possibility, we looked for stimulation of cellular DNA synthesis by pp89.

To monitor the effect of the *ie1* gene product on cell cycle regulation, we studied the expression of the protooncogene *c-fos*. The characteristics of *c-fos* expression in various cell types have led to the suggestion that it has a role in the control of cellular growth and differentiation. Appropriate expression of *c-fos* appears to be a prerequisite for the reentry of quiescent cells into the cell cycle (25). The expression of *c-fos* is rapidly induced within minutes during the transition from the G_0 to the G_1 phase (3, 34), and both protein and mRNA have very short half-lives (19, 24). The effect of the *ie1* gene product on cell cycle regulation was studied by microinjection of IE genes. The results suggest that the nonstructural regulatory protein pp89 of MCMV stimulates *c-fos* expression and triggers cell cycle activation.

MATERIALS AND METHODS

Cell culture. NIH 3T3 mouse fibroblasts, clone 7 (originally from D. Lowy, National Institutes of Health, Bethesda, Md.), were seeded onto cover slips (10 by 10 mm) with prescratched squares and grown in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum in 7% CO_2 . To arrest the cells in the subconfluent state, medium was changed after 24 h to Dulbecco modified Eagle

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medium containing 2.5% bovine platelet-poor plasma. Cells were injected after an arrest of 3 days.

Plasmid DNA. Cloning of MCMV DNA fragments and plasmids pAMB25, pIE111, and pIE100 have been described before (7, 13, 17, 18). Plasmid DNA was prepared by the method of Holmes and Quigley (12). Restriction enzyme digestion, agarose gel electrophoresis, and ethanol precipitation of DNA were performed by standard methods (21).

Microinjection. Linearized plasmid DNA was suspended in sterile water at concentrations of 50 ng/ml. In selected experiments sterile filtered fluorescein isothiocyanate (FITC)-labeled dextran (FD-150; Sigma Chemical Co., Munich, Federal Republic of Germany) was coinjected at concentrations of 1.5%. The probes were centrifuged for 15 min at $20,000 \times g$. To ensure physiological pH conditions, the cell medium was adjusted with HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) to 1 mM just before injection. Microinjection was performed with an injection system (developed by Ansoorge [1]). Injection capillaries were pulled with an automatic puller (Mechanex, Geneva, Switzerland). In individual squares of the cover slip, the nuclei of about 15 cells were injected with $\sim 10^{-11}$ ml of solution. In each experiment 200 to 300 cells were usually injected during 20 to 30 min. After injection the cells were incubated in 7% CO₂ for different periods of time.

Immunofluorescence. (i) **Detection of MCMV IE proteins.** Cells were fixed with 3% paraformaldehyde in phosphate-buffered saline and treated with 0.2% Triton X-100 in phosphate-buffered saline at 20°C. The cells were then incubated at 37°C with the murine monoclonal antibody 6/56/6 specific for MCMV IE protein pp89 (16). In the second step cells were incubated at 37°C with rhodamine-labeled rabbit anti-mouse immunoglobulin G (Dakopatts, Hamburg, Federal Republic of Germany). For a control, nuclei of cells were stained by incubation for 20 min with 5 µg of bisbenzimidazole (Hoechst 33342; Hoechst-Roussel Pharmaceuticals Inc., Somerville, N.J.) per ml. Cover slips were mounted on slides and fluorescence was visualized on an inverted light microscope (Zeiss) at 495 nm (FITC), 554 nm (rhodamine), or 365 nm (bisbenzimidazole).

(ii) **Detection of *c-fos* antigen.** Cells were fixed and permeabilized as described above. The *c-fos* antigen was detected by polyclonal rabbit anti-*fos* serum, and rhodamine-labeled swine anti-rabbit immunoglobulin served as a second antibody.

DNA synthesis assay. To test the stimulation of cellular DNA synthesis, cells were arrested at the G₀ phase as described above. After plasmid DNA injection, 1 µCi of [³H]thymidine per ml was added, and cells were incubated at 37°C in 7% CO₂ for different periods of time. After fixation of the cells, indirect immunofluorescence, and bisbenzimidazole staining, the mounted cover slips were covered with autoradiographic stripping film (AR 10; Eastman Kodak Co., Rochester, N.Y.). After exposure for 2 to 3 days at room temperature, the films were developed and fixed. [³H]thymidine incorporation was visualized by black silver grains and photographed under a microscope.

RESULTS

Expression of MCMV IE genes in mouse NIH 3T3 fibroblasts after microinjection. For the analysis of regulatory effects of isolated viral genes and their products on cellular functions, microinjection offers some advantages. First, the cells injected with DNA can be identified by coinjection of a fluorescence marker. This allowed us to find again each

individual injected cell. Second, the temporal relation between gene uptake and expression can be determined, as can the number of injected cells that express the gene. Third, the regulatory effects on cellular genes by injected viral genes can be studied, and discrimination between direct effects on injected cells and indirect effects on surrounding cells is possible.

The correct expression of injected MCMV IE genes was tested in growing subconfluent mouse NIH 3T3 fibroblasts by using linearized plasmids containing various regions of the MCMV IE gene region (Fig. 1). FITC-labeled dextran was coinjected to locate the injected cells. Expression of the IE protein pp89 was monitored for 20 h after injection by using a monoclonal antibody specific for pp89 and a rhodamine-labeled second antibody for detection by immunofluorescence. As indicated by the number of FITC-positive cells and the ratio between FITC- and rhodamine-positive cells, about 70% of injected cells survived the injection procedure, and approximately 80% of survivors expressed pp89. Microinjection of all constructs containing the transcription until *ie1* resulted in expression of pp89. Control injections with plasmids linearized by *Hind*III, which cleaves within the fourth exon of the pp89-coding sequence, or injection with the vector pSP62 did not reveal any rhodamine fluorescence.

Subcellular distribution of pp89 after microinjection of the *ie1* gene in arrested mouse NIH 3T3 fibroblasts. Expression of the *c-fos* gene as well as DNA synthesis was low or absent in resting cells. To study the stimulation of the cell cycle following microinjection of MCMV IE genes, NIH 3T3 cells were synchronized in the G₀ phase of the cell cycle by preincubation for 3 days with medium containing 2.5% platelet-poor plasma. Platelet-poor plasma is depleted of platelet-derived growth factor, which is necessary for NIH 3T3 cell proliferation, but possesses all other components that are required (35). This protocol resulted in about 95% of cells being arrested in the G₀ phase, as demonstrated by a lack of [³H]thymidine incorporation into the nucleus during a 24-h incubation period. After the addition of 20% fetal bovine serum for 24 h, incorporation of [³H]thymidine in about 90% of cell nuclei confirmed that the cells could be released from growth arrest.

In contrast to growing cells, only approximately 20% of arrested cells survived after injection, but similar to growing cells, 80% of these expressed pp89 20 h later. It was postulated that prior to any effect on cellular gene functions, the *ie1* gene product had to arrive at the nucleus. During permissive infection with MCMV, the *ie1* gene is the first viral gene that is expressed. The protein is already synthesized in the first 2 h of infection, and the antigen can be detected by immunofluorescence at about the same time in the nucleus but not in the cytoplasm of infected cells. The protein is stable and resides in the nucleus throughout the replication period (13, 16). We examined the kinetics of pp89 antigen expression and the nuclear transport of the antigen following microinjection of the *ie1* gene into quiescent cells. Surprisingly, the kinetics of subcellular distribution of pp89 antigen was different between infection by virus and microinjection of the gene (Fig. 2). Four hours following injection of *ie1* (Fig. 2b) and also 8 h postinjection (data not shown), pp89 antigen was mainly located in the cytoplasm, whereas only as late as 12 h after microinjection the majority of the antigen appeared to be located in the nucleus (Fig. 2d). An exclusive nuclear localization of pp89 antigen was seen at 24 h (Fig. 2f) and 30 h after microinjection (data not shown).

MCMV pp89 stimulates *c-fos* antigen expression. The *c-fos*

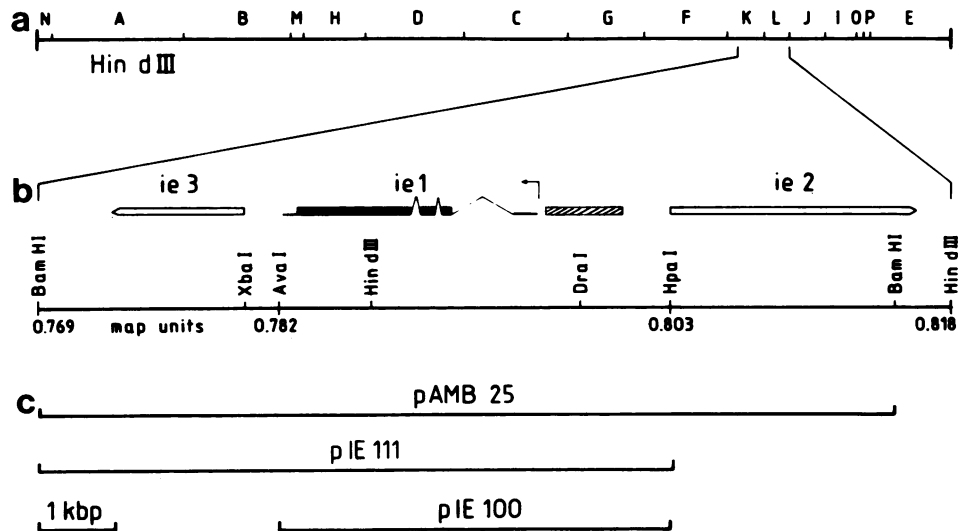


FIG. 1. Physical map of MCMV IE genes. (a) *Hind*III cleavage sites. (b) Organization of the IE region. The locations of the transcription units *ie1*, *ie2*, and *ie3*; the direction of transcription; and the structure of the major RNA transcribed from the *ie1* gene are indicated. The open reading frame is indicated as a segmented solid bar, and the promoter regulatory enhancer region is indicated as a hatched box. The structural organizations of *ie2* and *ie3* are not precisely known; therefore, they are symbolized by open arrows. (c) Plasmid-cloned DNA fragments of the MCMV IE region used for microinjection. kbp, Kilobase pairs.

protooncogene product is present in low amounts in normal cells, and its expression is induced rapidly during cell growth and proliferation (3). Expression of *c-fos* was monitored at different times after injection of the *ie1* gene by immunofluorescence (Fig. 3). We reasoned that different than other stimuli which rapidly turn on *c-fos* expression, any effect of *ie1* gene injection that was associated with the function of pp89 should be delayed. Because pp89 remains in the cytoplasm during the first 4 h postinjection of DNA, *c-fos* expression should be turned on later. No *c-fos* expression was detectable in the nuclei of injected cells after 1 h (Fig. 3b) or 2 and 4 h postinjection (data not shown), whereas at 20 h postinjection, at times when pp89 was found predominantly in the nucleus, a distinct *c-fos* expression was also observed in the nuclei of injected cells (Fig. 3d). On the other hand, *c-fos* expression was already seen after 1 h following stimulation of the cells with 20% fetal bovine serum (Fig. 3e).

Linearization of plasmids containing the *ie1* gene by *Hind*III cleavage with cuts within the fourth exon encoding pp89 (Fig. 1) abolished *trans*-activation (17). Similarly, no effect on *c-fos* expression was seen after injection of plasmids that were linearized with *Hind*III (data not shown). After microinjection with the intact *ie1* gene, expression of *c-fos* was seen in 90% of FITC-positive cells; this was comparable to the number of cells that expressed pp89. No *c-fos* expression was detected in noninjected neighboring cells. After microinjection of the vector plasmid pSP62, *c-fos* expression was not detectable during the 20-h incubation period (data not shown), excluding nonspecific effects of irrelevant DNA on *c-fos* expression. These data suggest that stimulation of *c-fos* expression by the MCMV *ie1* gene requires synthesis of pp89 and its transport to the nucleus.

Stimulation of DNA synthesis in quiescent cells after microinjection of the *ie1* gene. To study whether the growth arrest in quiescent cells is released by pp89, cells were labeled with [³H]thymidine after injection of the *ie1* gene. At different times postinjection, incorporation of the radioactive nucleotide was examined by autoradiography. As described above,

about 80% of cells that survived the injection expressed pp89. After 4 to 12 h no enhanced DNA synthesis was observed (Table 1 and Fig. 4b), whereas 24 h after injection a significant stimulation of DNA synthesis was seen (Table 1 and Fig. 4d). Note that different than *c-fos* expression, there was no stringent correlation between expression of pp89 and DNA synthesis. In some experiments, [³H]thymidine incorporation also occurred in noninjected surrounding cells. This could result in a higher number of cells with labeled nuclei than of cells that express pp89. Results of a typical experiment in which such conditions were observed are represented in Table 1. Conversely, few individual cells were seen that expressed pp89 but that were not active in DNA synthesis (data not shown). Nevertheless, stimulation of DNA synthesis was dependent on the injection and expression of the *ie1* gene, since after injection with plasmid linearized by *Hind*III cleavage or injection with the vector pSP62 DNA for control, there was no induction of DNA synthesis (Table 1), and DNA synthesis in the noninjected control areas remained as low as expected for arrested cells.

Further experiments were carried out to detect a putative additional effect of the other two IE transcription units of MCMV, *ie2* and *ie3*. The data are summarized in Table 2. The *ie1* gene was again sufficient for the induction of DNA synthesis, and there was no evidence that the presence of *ie2* or *ie3* sequences improved the degree of stimulation.

DISCUSSION

Stimulation of cellular DNA synthesis by CMV (31) caused by the release of a growth factor by permissive and nonpermissive cells infected with CMV (10) demonstrated the effect of CMV infection on cell cycle regulation. Since viral DNA synthesis is not required for induction of host cell DNA synthesis (4), the effect is either due to a viral structural protein transferred by infection or viral gene functions expressed during the IE or E phase of infection. The MCMV *ie1* gene product pp89 appeared to be a likely candidate for a CMV protein that is involved in cell cycle

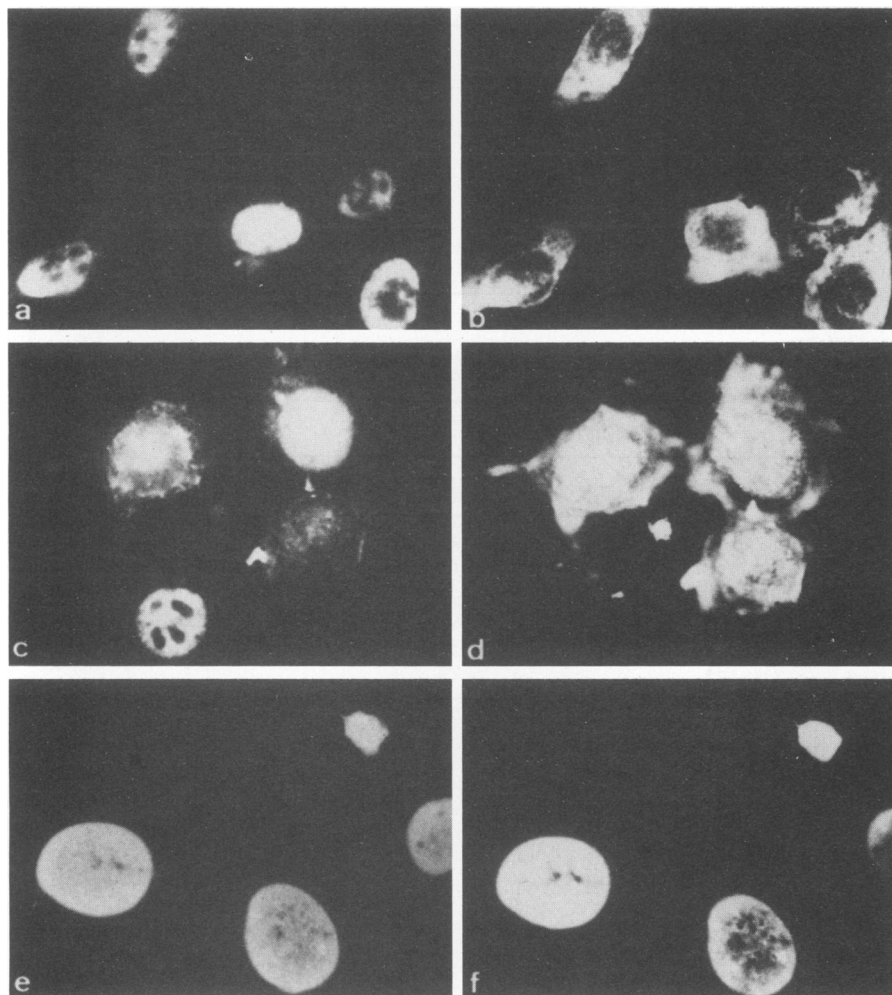


FIG. 2. Immunocytochemical localization of viral pp89 at various times after injection of the *iel* gene. The composite of micrographs shows pairs of identical sections of NIH 3T3 fibroblast cell monolayers. Cells are stained either directly with FITC-labeled dextran to indicate injected cells (a, c, and e) or indirectly with monoclonal antibody to pp89 and rhodamine-labeled second antibody to indicate expression of the injected gene (b, d, and f). Growth-arrested cells were coinjected with FITC and plasmid pIE100 DNA; and indirect immunofluorescence was carried out 4 h (a and b), 12 h (c and d), and 24 h (e and f) later. FITC is located in the nucleus, whereas pp89 is located in the cytoplasm at 4 h, in the cytoplasm and nucleus at 12 h, and selectively in the nucleus 24 h after injection of the *iel* gene. Magnification, $\times 400$.

regulation, because this protein is located in the nucleus of infected cells (16), binds to histones (24a), is associated with a kinase activity (Barnekow et al., in preparation), and acts as a transcriptional regulator (18). We chose to study *c-fos* expression as an indicator gene involved in the early steps of cell cycle regulation. Because the expression of *c-fos* does not necessarily result in the transition of cells into the S phase of the cell cycle (20), cellular DNA synthesis was also tested. The results indicate that the MCMV *iel* gene product pp89 stimulates cells to enter the cell cycle and that *c-fos* induction by pp89 is part of the cell cycle activation pathway.

Results of our experiments demonstrate that the *c-fos* protein is accumulated after injection of the MCMV *iel* gene DNA into quiescent NIH 3T3 cells. For two reasons, the increased *c-fos* expression is not due to mechanical irritation of cells during injection or to nonspecific effects of plasmid DNA. First, *c-fos* protein and mRNA have very short half-lives (19, 24); for example, *c-fos* protein accumulation following wounding of NIH 3T3 cells has been demonstrated to occur within 1 h, to remain high for another 1 h, and then

gradually to decrease within 3 h to barely detectable levels (34). This kinetics of *c-fos* expression differs from that following injection of the MCMV *iel* gene. Second, injection of the *iel* gene linearized within the coding region or of vector DNA did not lead to the accumulation of *c-fos*.

The induction of *c-fos* was delayed after microinjection of the *iel* gene, and *c-fos* was detected at 20 h but not during the first 4 h. We suggest that the delayed *c-fos* accumulation is caused by the delayed appearance of pp89 in the nucleus. Although pp89 is synthesized after gene injection, it remains located exclusively in the cytoplasm during the first 4 h, and it probably cannot act as a *trans*-activator during this period. As yet there is no explanation for the difference between nuclear transport after virus infection and injection of the gene. Additional MCMV proteins probably do not play a role in the nuclear transport of pp89, because in cells that stably express the transfected *iel* gene (17), pp89 is detected selectively in the nucleus.

Delayed induction of *c-fos* by a viral regulatory protein is, however, not unique for pp89. Polyomavirus can also cause a delayed and sustained accumulation of *c-fos* mRNA be-

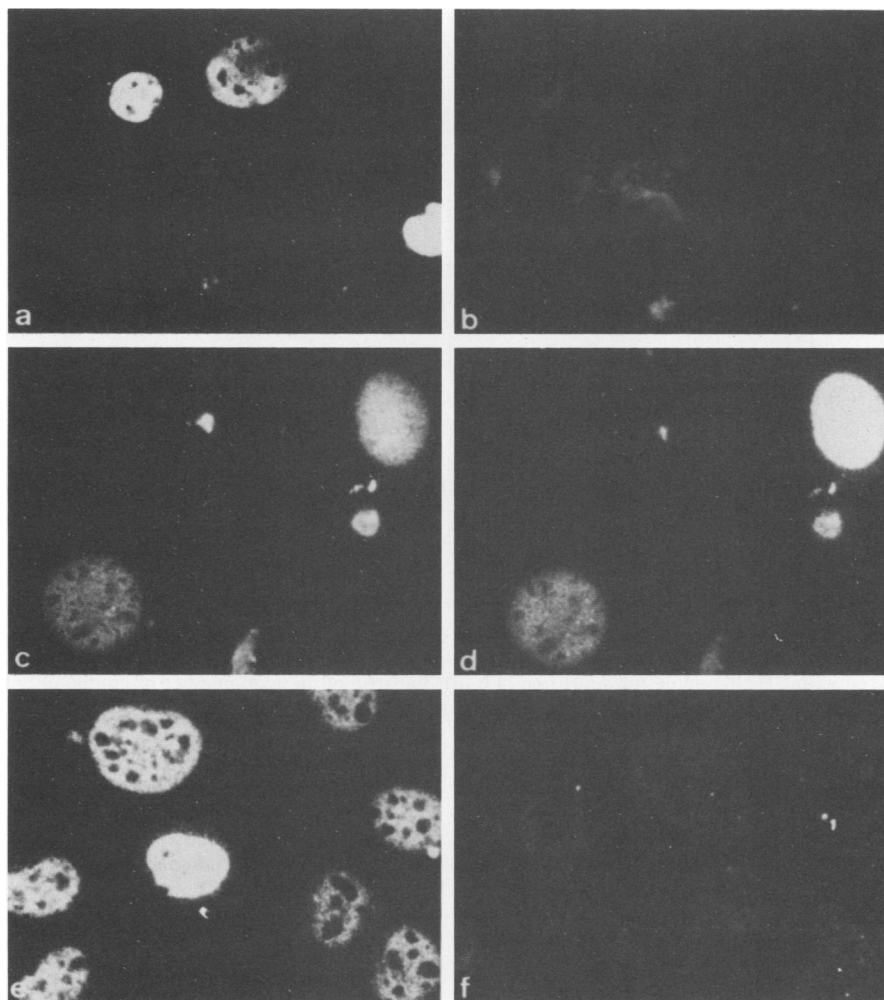


FIG. 3. Induction of the *c-fos* protein by viral pp89 at various times after *iel* gene injection. The composite of micrographs shows *c-fos* antigen expression in NIH 3T3 fibroblast cell monolayers. Growth-arrested cells were coinjected with FITC and plasmid pIE100 DNA, and indirect immunofluorescence with antiserum to *c-fos* and rhodamine-labeled second antibody was carried out 1 h (a and b) and 20 h (c and d) later. FITC fluorescence is seen as an injection marker in panels a and c, and immunofluorescence to detect *c-fos* antigen expression is shown in panels b and d. The micrographs in panels e and f show *c-fos* antigen 1 h after stimulation with 20% fetal bovine serum (e) and no *c-fos* in cells without stimulation (f). Magnification, $\times 400$.

TABLE 1. Relationship between pp89 expression and DNA synthesis in arrested NIH 3T3 fibroblasts after microinjection of DNA

Probe injected ^a	Labeling time (h)	No. of injected cells	No. of FITC-positive cells	No. of pp89-positive cells	No. of cells with DNA synthesis ^b
pIE100	4	342	80	61	0
	8	310	61	54	0
	12	304	123	74	0
	24	321	40	35	50
pSP62	24	350	70	0	5
pIE100- <i>HindIII</i> ^c	24	157	ND ^d	0	3

^a Results are of one representative experiment.

^b The total number of cells with silver grains was determined in areas containing injected cells. From this value, the basal DNA synthesis activity was subtracted and evaluated by counting the number of positive cells in a noninjected area with a comparable number of cells.

^c The plasmid was linearized by *HindIII* cleavage to interrupt the coding region of the *iel* gene.

^d ND, No coinjection by FITC.

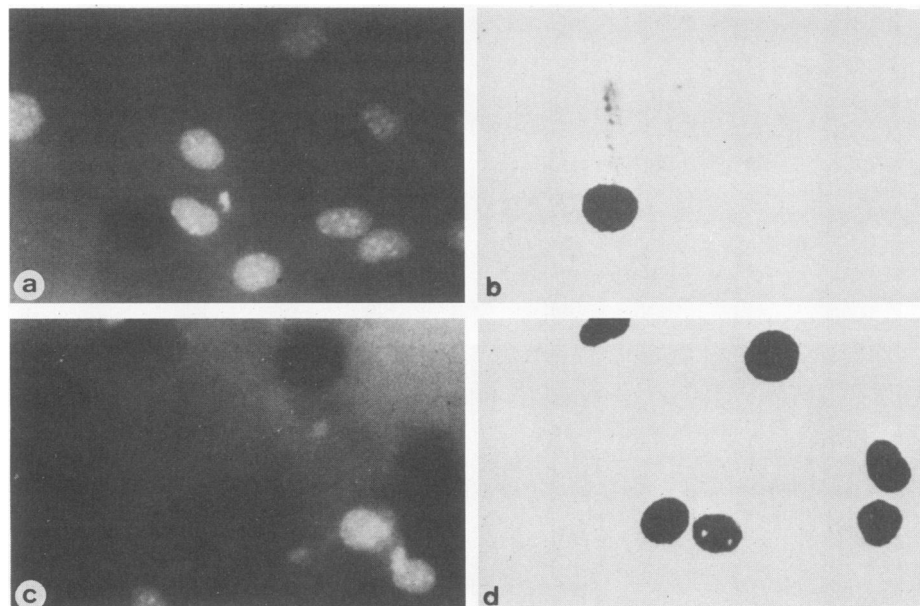


FIG. 4. DNA replication in cells injected with plasmid pIE100 DNA. The composite of micrographs shows either identical sections of cells stained with bisbenzamide (a and c) or autoradiography of [³H]thymidine incorporated into NIH 3T3 cells (b and d). After DNA injection cell monolayers were incubated with [³H]thymidine for 4 h (b) or 24 h (d). Because of the high number of grains, only those nuclei that did not synthesize DNA were detectable by bisbenzamide staining. Magnification, $\times 250$.

tween 12 and 36 h after infection which was identified as a tumor antigen function (36). pp89 neither immortalizes nor transforms cells, which is different than the functions of the tumor antigens of polyomavirus or simian virus 40 and the products of the E1A region of adenovirus that all stimulate the cell cycle and that are involved in cell transformation (6, 8, 22, 26, 32, 33, 36). Whether this is a qualitative or merely a quantitative difference between pp89 and the early proteins of DNA tumor viruses is not yet clear. Transient *c-fos* expression appears not to be sufficient to induce cell transformation, and only when *c-fos* mRNA and protein are

expressed constitutively in fibroblasts do the cells become morphologically transformed (24). Although cells that constitutively express the *ieI* gene after transfection (17) do not form colonies in agarose medium (unpublished data), this does not necessarily indicate a qualitative difference, because the expression of the transformed phenotype after transfection of the simian virus 40 early gene is also dependent on the amount of large tumor antigen in cells (28).

Similar to the results of microinjection of DNA tumor virus genes (30) and gene products (33), replicative cellular DNA synthesis could be induced by the *ieI* gene. Mere DNA repair synthesis caused by the damage inflicted on cells by the injection procedure was considered unlikely, because DNA synthesis (i) required the injection of a functional *ieI* gene, (ii) was not seen before 24 h, and (iii) was not restricted to injected cells. There is clear evidence that early proteins of DNA tumor viruses cause cells to release growth factors (26). The evidence for induction of growth factor production following *ieI* gene expression is only indirect and was concluded from the observation that in some experiments a variable percentage of cells surrounding the microinjected cell incorporated [³H]thymidine. The amount of growth factor production was certainly very low, because, although cells that expressed pp89 accumulated *c-fos*, some cells were detectable that expressed pp89 but that were not active in cellular DNA synthesis. Attempts to demonstrate growth factor activity in conditioned media of cells that express the transfected *ieI* gene have failed so far.

Induction of cell proliferation probably plays a role in the productive lytic pathway of MCMV replication. Muller and Hudson (23) have described that, in contrast to asynchronous cells, which produce viral progeny within the first 12 h postinfection, after infection of cells arrested early in the G₁ phase, replication of viral DNA begins first after 16 to 20 h postinfection and reaches a maximum between 20 and 24 h postinfection. The *ieI* gene is the first viral gene that is expressed after infection. The ability of the *ieI* gene product

TABLE 2. Induction of DNA synthesis in arrested NIH 3T3 fibroblasts after injection of MCMV IE genes

Probe injected	Labeling time (h)	No. of experiments	% Cells with DNA synthesis ^a	Stimulation factor ^b
pIE100	4	2	1	0.1
	12	5	13	1.4
	18	5	6	0.7
	24	9	63	7.0 ^c
	30	2	44	4.9 ^c
pAMB25	24	2	62	6.9 ^c
pIE111	24	3	77	8.6 ^c
pSP62	24	7	9	1.0
pIE100- <i>Hind</i> III	24	3	12	1.5
FITC	24	5	7	0.8

^a The percentage of cells with DNA replication is represented by the ratio between the number of cells with nuclei containing silver grains and the number of microinjected cells (represented by FITC-positive nuclei) $\times 100$. Values are the averages of independent experiments.

^b Number of fold compared with DNA synthesis after injection of pSP62 DNA.

^c The number of nuclei with silver grains 24 h following *ieI* gene injection was significantly higher than the number of nuclei with silver grains after injection of pSP62 DNA ($P < \alpha = 0.1$), as calculated by the exact rank sum test (Wilcoxon-Mann-Whitney test).

to stimulate cells to proliferate supports viral replication. The release of growth factors would stimulate surrounding cells to become target cells for secondary infection. Whether the success or the failure to induce the cell cycle could also play a role in the maintenance of viral latency and the reactivation to productive infection is a matter of speculation.

ACKNOWLEDGMENTS

This study was supported by grant Ko 571/8 from the Deutsche Forschungsgemeinschaft. J.S. was supported by a short-term fellowship from the European Molecular Biology Organization.

We thank R. Müller, EMBL, Heidelberg, Federal Republic of Germany, for *c-fos* antisera and A. Habenicht, University of Heidelberg, for platelet-poor plasma. S. Grau provided skillful typing of the manuscript.

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