JOURNAL OF VIROLOGY, Oct. 1985, p. 135–143
0022-538X/85/100135-09$02.00/0
Copyright © 1985, American Society for Microbiology
Vol. 56, No. 1

Regulation of Cytomegalovirus Gene Expression: α and β Promoters Are *trans* Activated by Viral Functions in Permissive Human Fibroblasts

RICHARD R. SPAETE AND EDWARD S. MOCARSKI*

Department of Medical Microbiology, Stanford University School of Medicine, Stanford, California 94305

Received 5 April 1985/Accepted 13 June 1985

We have fused immediate (α) and delayed (β) early promoter-regulatory sequences taken from the cytomegalovirus (CMV) genome to *Escherichia coli lacZ* (β-galactosidase) as an indicator gene to study regulated expression of these promoters. After transfection of human fibroblast cells with plasmid constructs carrying β-galactosidase fusions, and subsequent infection with CMV, we have demonstrated that viral *trans*-acting functions up-regulate the expression of these genes in a temporally authentic manner. The α promoter is activated even when de novo protein synthesis is blocked and when UV-inactivated virus is used, suggesting that, as for herpes simplex virus type 1 (HSV-1), a virion structural protein is responsible for its up-regulation. We have found that HSV-1, as well as CMV, is capable of *trans* activating the CMV α promoter. The β promoter is activated by CMV but is completely unresponsive to HSV-1 infection. The temporal synthesis of the α and β promoters in the transient expression system conforms with their natural regulation during viral replication. The β-galactosidase fusions we describe provide a most exquisitely sensitive indicator system for the study of *cis*- and *trans*-acting viral regulatory functions.

Human cytomegalovirus (CMV) has a large double-stranded DNA genome of 240 kilobase pairs that promotes coordinately regulated, sequentially ordered gene expression during viral growth in permissive cells (10, 29, 55, 56; reviewed in reference 49). Herpesviruses such as herpes simplex virus (HSV) and CMV regulate their immediate (α) and delayed (β) early gene expression at the level of transcription (8, 10, 12, 14, 19, 20, 24, 40, 49). For HSV-1 early genes, regions upstream of the start site of transcription contain *cis*-acting signals which play a role in transcriptional up-regulation during infection (14, 22, 25–27, 39, 42, 58). This characteristic distinguishes herpesvirus gene regulation from early gene expression in papovaviruses, which utilize transcriptional enhancers (2, 4), and adenoviruses, which appear to regulate early genes by a generalized transcriptional activation initiated by the E1A gene product (5, 21, 34). HSV-1 has defined *cis*-acting regulatory sequences responsible for the coordinate expression of α and β genes. No studies have yet revealed the signals used by CMV to regulate immediate (α) and delayed (β) early gene expression.

Studies originally reported by Post et al. (39) showed that HSV-1 α-gene activation could be detected by fusing α promoters to an indicator gene. Further work by Mackem and Roizman (25–27) showed that a consensus sequence exists upstream of all HSV-1 α genes and is responsible for their coordinate expression. More recent work has established the role of this consensus sequence as well as those of adjacent sequences in the transcriptional activity of α promoters (22). The *trans*-acting component in the induction of HSV-1 α genes appears to be a virion tegument protein (3, 7, 39). The sequences that confer β transcription in HSV-1 are not yet defined because they overlap with sequences essential for RNA polymerase II transcription (14, 42, 58). The *trans*-acting components involved in HSV-1 β-gene regulation include the α4 gene product, possibly working in concert with other α-gene products (13, 15, 38, 40, 57). Although CMV α- and β-gene expression is transcriptionally regulated, neither *cis*- nor *trans*-acting components have been defined. Because in vitro transcription of the CMV major immediate early (MIE) promoter is inhibited by the addition of α-amanitin (52), it appears that this class of CMV genes is transcribed by host cell RNA polymerase II. By analogy with studies on HSV-1 (9), it is likely that the other CMV genes are transcribed by the host polymerase as well. The narrowly restricted host range of CMV (limited to human fibroblast cells in culture) suggests the requirement for additional cellular factor(s) in viral gene expression.

Relying upon the transcription studies from the laboratories of Stinski (48–52, 55, 56) and Spector (29, 30, 47) that identified prototypic α and β genes, we have constructed promoter fusions to an indicator gene and assayed *cis*- and *trans*-acting functions involved in CMV gene regulation. Our studies reveal several important features of CMV gene regulation by using a new, extremely sensitive indicator system. The approach is based on studies reported by Lee and co-workers (17, 23) on mouse mammary tumor virus promoter fusions, in which *lacZ* (β-galactosidase [β-gal]) was used as the indicator gene product. We have separately fused the MIE (α) promoter (48, 52) and a major delayed (β) early promoter (30, 47) to *lacZ* in pBR322-based constructs which carry as well the simian virus 40 (SV40) origin of replication and enhancer (17, 23, 54). We have studied the regulated expression of β-gal after transfection of plasmid constructs into human fibroblast cells and subsequent infection by CMV or HSV-1. Our results support the notion that a virion protein is responsible for some measure of up-regulation of CMV immediate early genes. Unpublished experiments reported by Stinski, Roehr, and Jeang (personal communication) reinforce this conclusion. Our data indicate that HSV-1, as well as CMV, is able to affect this regulation. In contrast to the α promoter, the CMV β promoter is up-regulated by CMV but not by HSV-1 infection. We take these results to mean that the α and β promoters have

* Corresponding author.

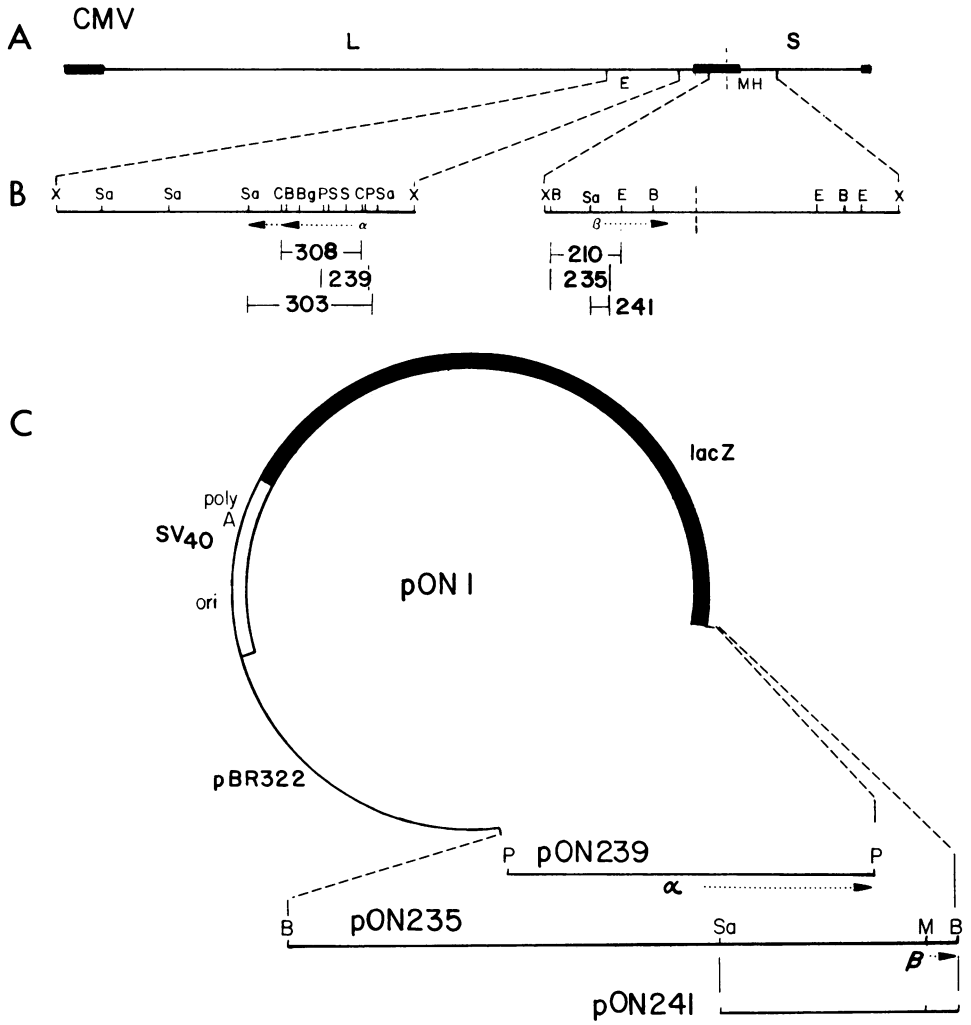

FIG. 1. Plasmid constructs containing CMV α and β promoters fused to β-gal. (A) Schematic representation of standard CMV DNA, displaying the arrangement of the unique and inverted repeat sequences (large boxed areas) of the long (L) and short (S) components (49). The genomic positions of *Xba*I-E and MH are shown. (B) Arrangement of the α-transcriptional region (regions 1 and 2) within the *Xba*I E fragment of CMV (Towne) (48) as well as the β transcript located within the inverted repeat sequences of the L component within *Xba*I MH (30, 47; unpublished data). Restriction enzyme abbreviations: X, *Xba*I; Sa, *Sal*I; C, *Cla*I; B, *Bam*HI; Bg, *Bgl*II; P, *Pst*I; S, *Sac*I; E, *Eco*RI. (C) Representation of β-galactosidase expression vector pON1 and constructs of α (pON239) and β (pON235, pON241) promoter insertions. *lacZ*, β-gal structural gene sequences (17). SV40- and pBR322-derived sequences are as described in Materials and Methods. M, *Mlu*I (other abbreviations are described above).

specific promoter-proximal *cis*-acting sequences which act to discriminate expression of these genes at the appropriate time during viral replication.

MATERIALS AND METHODS

Cells and viruses. Human foreskin fibroblast (HF) cells were isolated in this laboratory and grown in Dulbecco minimum essential medium (DME; GIBCO Laboratories)–10% Nu serum (Collaborative Research, Inc.) in a 5% CO_2 atmosphere. CMV (Towne) and HSV (F^+) were grown in HF cells and Vero cells, respectively (45, 46).

Manipulation of plasmid DNA. The procedures used for preparation of plasmid DNA, restriction enzyme analyses, and transformation of HB101 bacteria have been described previously (43, 46). Plasmids used in transfections were twice banded in cesium chloride gradients. Conditions for BAL 31 nuclease digestion and S1 analysis were as described by Maniatis et al. (28).

Plasmid constructions. The restriction map of the *Xba*I E fragment has been described previously (48, 51) with the exception of the 4.2-kilobase (kb) *Cla*I fragment shown in Fig. 1 which contains the entire transcript (region 1; see reference 48) encoding the MIE gene of CMV (Towne) (unpublished data; M. F. Stinski and R. Stenberg, personal communication). The restriction map of the *Xba*I MH fragment has been described (45, 46) with the exception of the *Sal*I and *Mlu*I sites. pON303 was derived by cloning the 8.1-kb *Sal*I fragment from pXbaE (53) that contains immediate early region 1 and region 2 transcripts (48) into the unique *Sal*I site of the HSV amplicon pP2-103 (43). pON308 was constructed by cloning the 4.2-kb *Cla*I fragment that contains immediate early region 1 into the unique *Cla*I site of the HSV amplicon pP2-204 (44). pON239 was constructed by inserting the 2.1-kb *Pst*I fragment containing 1.1 kb of sequence upstream to the start site of transcription of region 1 (the α gene shown in Fig. 1) into pON1. The α-promoter sequences included in the 2.1-kb *Pst*I fragment were previ-

ously shown by Stenberg et al. (48) to encode a 121-base-pair (bp) leader transcript and an 810-bp intron whose 3′ acceptor site is fortuitously contained within the 3′ proximal *Pst*I site. This *Pst*I fragment was cloned into the unique *Pst*I site in the polylinker of pMT11 (K. Moore and H. Huang, unpublished data) and designated pON233 (data not shown). The splice acceptor sequence was thus changed from a thymine in the first nucleotide of exon 2 (48) to a guanine in pON233. This promoter sequence was cloned into pON1 as a *Sal*I-*Bgl*II fragment, and the fusion was designated pON239 (Fig. 1).

The expression vector pON1 was derived from pJC6S-3 (F. Lee and J. Culpepper, unpublished data), itself a derivative of pCH110 (17). A region from the *Tth*111I site in pBR322 sequences to a *Hin*dIII site 5′ of the β-gal coding sequences was replaced with a 65-bp *Eco*RI-*Hin*dIII polylinker (AATTCCCGGGGATCCGTCGACCT GCAGGCGCAGAACTGGTAGGTATGGAAGATCTCTA GAAGCTT) from pMT11. The parent plasmid pJC6S-3 differs from the published sequence arrangement of pCH110 (17) in that the *Pvu*II-*Hin*dIII fragment containing the SV40 enhancer, ori, and early promoter regions (54) has been placed within an *Hpa*I-*Eco*RI fragment containing the SV40 polyadenylation signals (54) (Fig. 1).

The β-promoter sequences were defined by first generating a restriction map of the region known to encode an abundant β-gene transcript (30, 47; unpublished data). We used the published map position of the 2.7-kb transcript which initiates 600 bp to the left of the *Eco*RI site closest to the L-S junction of the CMV (AD169) genome (30) to compare with CMV (Towne). The Towne and AD169 genomes appeared colinear in this region; thus, the fragment from the *Bam*HI U/S site (0.80 map units) to the *Eco*RI site 0.82 map units) contained the putative promoter-proximal sequences (as well as some sequences extending into the body of the gene). This fragment was subcloned (Fig. 1, pON210) and subjected to BAL 31 nuclease digestion, and *Bam*HI linkers (Collaborative Research) were inserted at the deletion site. From S1 nuclease mapping of RNA prepared from cells at 18 h postinfection (data not shown), a derivative containing 3.2 kb of promoter-proximal sequences and 135 bp of transcribed sequences was identified. This β promoter was cloned in both orientations into pON1; the clones were designated pON234 and pON235 (Fig. 1). The *Sal*I site in the polylinker of pON1 was used to collapse sequences upstream of the β promoter to derive pON241 (Fig. 1). pON241 was derived to remove the 1.2-kb transcript which had been mapped in these sequences (30) and to further define the domain of the regulatory sequences operating in the expression of pON235.

Transfections. The procedure used for transfection of plasmid DNA was as previously described (17). Briefly, 10 to 50 μg of plasmid DNA was mixed with 4 ml of DME–50 mM Tris hydrochloride (pH 7.4) containing 400 μg of DEAE-dextran (Pharmacia Fine Chemicals) per ml and added to 100-mm culture dishes (Falcon Plastics) of HF cells split 1:5 24 h previously. Cells were washed with DME–50 mM Tris hydrochloride (pH 7.4) at 4 h posttransfection and incubated in DME–10% Nu serum at 37°C. Cells were harvested 48 to 72 h posttransfection, depending on the experiment.

Infection and UV-irradiated virus. Infection of cells was performed in DME–10% Nu serum. Virus was adsorbed for 1 h at 37°C, overlaid with medium, and incubated at 37°C for the required number of hours postinfection (hpi) until the cells were harvested. The protocol for infection in the presence of cycloheximide (CH)-actinomycin D (AcD) was as previously described (39). Briefly, HF cells were transfected with 25 μg of pON239 and incubated for 48 h. CH (50 μg/ml) was added to the HF cells 1 h before infection with CMV. Cells were infected as described above at a multiplicity of infection (MOI) of 10 or 20 PFU per cell in the presence of CH. At 5 hpi, the medium was replaced with maintenance medium containing 50 μg of CH per ml and 5 μg of AcD per ml. After 1 h in the presence of both CH and AcD (6 hpi), the CH was removed with four washes of medium containing 10 μg of AcD per ml. The infection was allowed to proceed 4 h longer (10 hpi) in the presence of 10 μg of AcD per ml, and the cells were harvested.

A 1-ml portion of CMV (Towne) virus stock (1.5×10^8 PFU/ml) was UV irradiated in a 100-mm culture dish as a suspension in 1.5 ml of phosphate-buffered saline containing 1% glucose as described for HSV (3). The virus suspension was exposed to a UV lamp (wavelength, 254 nm; General Electric Co.) such that 3,000 or 6,000 ergs/mm^2 were delivered at a rate of 10 ergs/s. A portion was used to infect cells with 14 PFU per cell as calculated for nonirradiated virus, and a portion was titered to determine residual infectivity by plaque assay.

Enzyme and protein assays. β-gal activity was determined as described by Miller (31). Protein concentration was performed by the procedure of Bradford (6) as described below. Cells were washed with phosphate-buffered saline, scraped off dishes in 1.0 ml of phosphate-buffered saline–2 mM EDTA, and suspended in 5 ml of phosphate-buffered saline. Cells were pelleted (10 min, 200 × *g*), suspended in 50 to 100 μl of freeze-thaw solution (0.25 M sucrose, 10 mM Tris hydrochloride [pH 7.4], 10 mM EDTA) and alternately frozen (−80°C) and thawed (37°C) three times. Extracts were clarified by centrifugation for 10 min at 4°C in a microcentrifuge (Eppendorf). Extract (25 μl) was added to 175 μl of Z buffer (60 mM $Na_2HPO_4 \cdot 7H_2O$, 40 mM $NaHPO_4 \cdot H_2O$, 10 mM KCl, 1 mM $MgSO_4 \cdot 7H_2O$, 50 mM 2-mercaptoethanol; adjusted to pH 7.0) along with 40 μl of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) (4 mg/ml) and allowed to incubate at 30°C in a covered temperature block for 30 min to 4 h. The amount of endogenous β-gal activity from the lysosomal enzyme was minimized by the cell lysis and assay conditions (17, 23, 35). Reactions were stopped by the addition of 100 μl of 1 M Na_2CO_3. The amount of protein in each sample was determined by spectrophotometric analysis (A_{595}) of 2 μl of extract in 1× Bradford reagent (Bio-Rad Laboratories), using a bovine gamma globulin standard curve. Both assays were read on a MicroELISA (MR 580) autoreader (Dynatech Laboratories, Inc.) with an Apple II computer interface. Enzyme and protein assays were read in parallel by using flat-bottomed 96-well microtiter plates (Falcon Plastics) containing 200 μl of the reaction mix. β-gal specific activity was defined as nanomoles of ONPG cleaved (A_{420}/0.0045) per minute per milligram of protein; calculations were done with a locally written computer program coupled directly to the spectrophotometer. Previous studies (17, 23, 35) have shown that there is a dependence of β-gal activity on the amount of input plasmid, the time, and the pH. We have observed similar dependence, most importantly in the amount of input plasmid (unpublished data). All experiments and relevant comparisons were therefore done at the same DNA concentration, usually 25 μg per dish. Day-to-day variability for a given set of conditions was observed in the quantitative but not the qualitative aspects of these assays. Within-experiment variability was negligible. We have made relevant comparisons between samples run on the same day where appropriate.

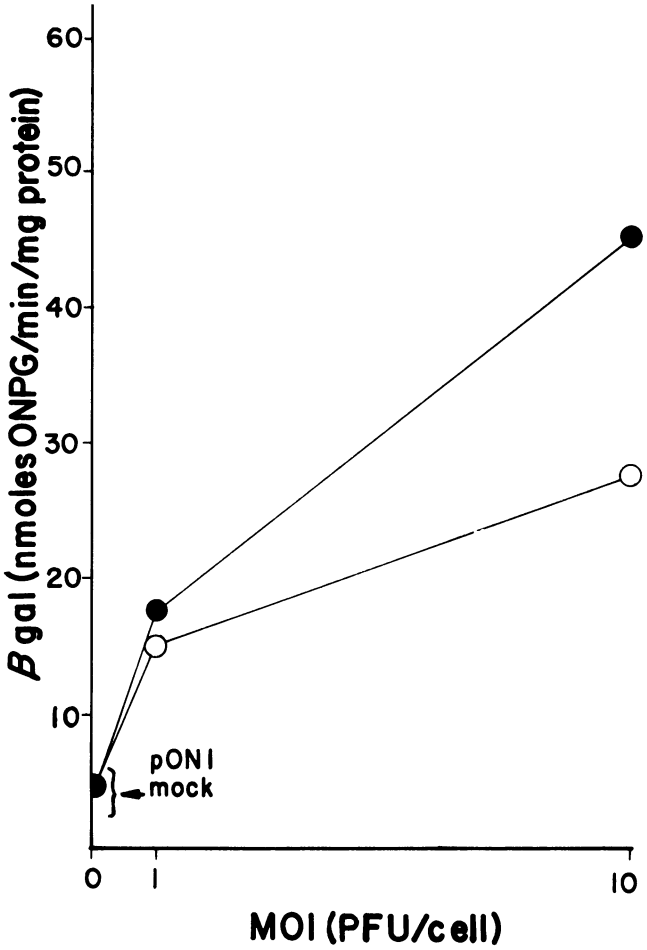

FIG. 2. Induction of the α-promoter–β-gal fusion gene in response to infection by CMV or HSV-1. HF cells were transfected with 25 μg of pON239 as described in Materials and Methods, incubated 48 h, and infected with CMV (Towne) (●) or HSV (F^+) (○) at the MOI indicated. Cells were harvested 24 hpi, and aliquots were assayed for β-gal activity. pON1 and mock, dishes receiving pON1 alone (10 μg) or no plasmid, respectively. The basal level of β-gal activity in cells receiving no plasmid, at 48 h posttransfection with pON239 or pON1, or in cells infected with CMV or HSV was 4 to 6 nmol of ONPG cleaved per min per mg of protein in this experiment.

RESULTS

Induction of the chimeric α-promoter–β-gal fusion gene. The α-promoter fusion gene, p0N239, was transfected into HF cells with DEAE-dextran and 25 μg of DNA per 100-mm dish. After 48 h, cells were either mock infected or infected with 1 or 10 PFU of CMV (Towne) or HSV (F^+) per cell to assay the expression of the indicator gene in response to infection by these viruses. Cells were harvested at 24 hpi, and amounts of β-gal activity were determined for aliquots of each of the dishes. The results of this experiment show an induction in β-gal activity in response to CMV or HSV infection in a multiplicity-dependent manner (Fig. 2). It should be noted that in this and all subsequent assays, dishes which received no plasmid DNA, pON1 with or without virus infection, or pON239 without virus infection produced basal levels of β-gal activity (1 to 6 nmol of ONPG per min per mg of protein) in HF cells. In addition, infection of HF cells with either CMV or HSV (10 PFU per cell) without any plasmid did not induce β-gal activity.

HSV induced β-gal activity at approximately 50% of the levels induced by infection with CMV at 10 PFU per cell. This was unexpected in view of earlier studies by Batterson and Roizman (3) which showed that CMV does not induce expression of HSV α-TK chimeric fusion gene resident in TK-transformed cells. Two potentially important differences between those studies and ours are that we used cells permissive for both viruses and did not stably introduce the β-gal fusion gene into the genome of the cells.

Kinetics of α-gene expression. The temporal response of the α-promoter–β-gal fusion gene to infection by CMV (Towne) was assayed in the following time-course experiment. Dishes of HF cells were transfected with pON239, incubated for 60 h, and infected with 20 PFU of CMV per cell. Starting at 2 hpi, the cells were harvested at 2-h intervals up to 10 hpi. A portion of each extract was assayed for levels of β-gal activity. The results of these assays (Fig. 3) show that there was a steady increase in levels of β-gal activity from 4 to 10 hpi. Figure 2 shows that the accumulation of β-gal continued with time over a 24-h period. These results indicate that α-gene expression was stable and continued during early times after infection, in agreement with the observation that the MIE protein accumulated during the first 24 h of infection (N. Michael and E. S. Mocarski, unpublished data).

Induction of the α promoter in the absence of de novo protein synthesis. To determine whether the CMV α promoter responded to induction by a *trans*-acting virion component, as was first shown for HSV (3, 39), we performed the following experiments. HF cells were transfected with 25 μg of pON239 as described above and incubated for 48 h. Following the protocol outlined in Fig. 4 and described in Materials and Methods, first CH was added to the culture to

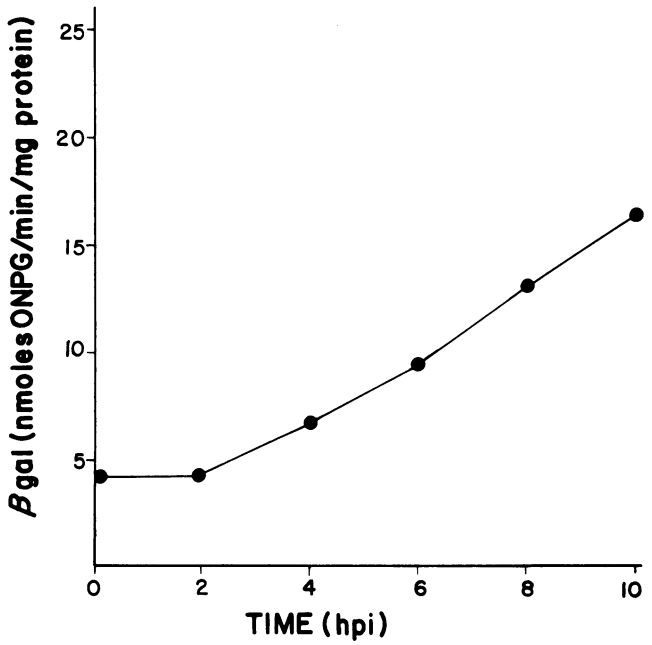

FIG. 3. Kinetics of α-gene expression in response to infection by CMV (Towne). HF cells were transfected with 50 μg of pON239, infected 48 h later with 20 PFU of CMV, and harvested at 2-h intervals up to 10 hpi. A portion of each extract was used to determine β-gal activity.

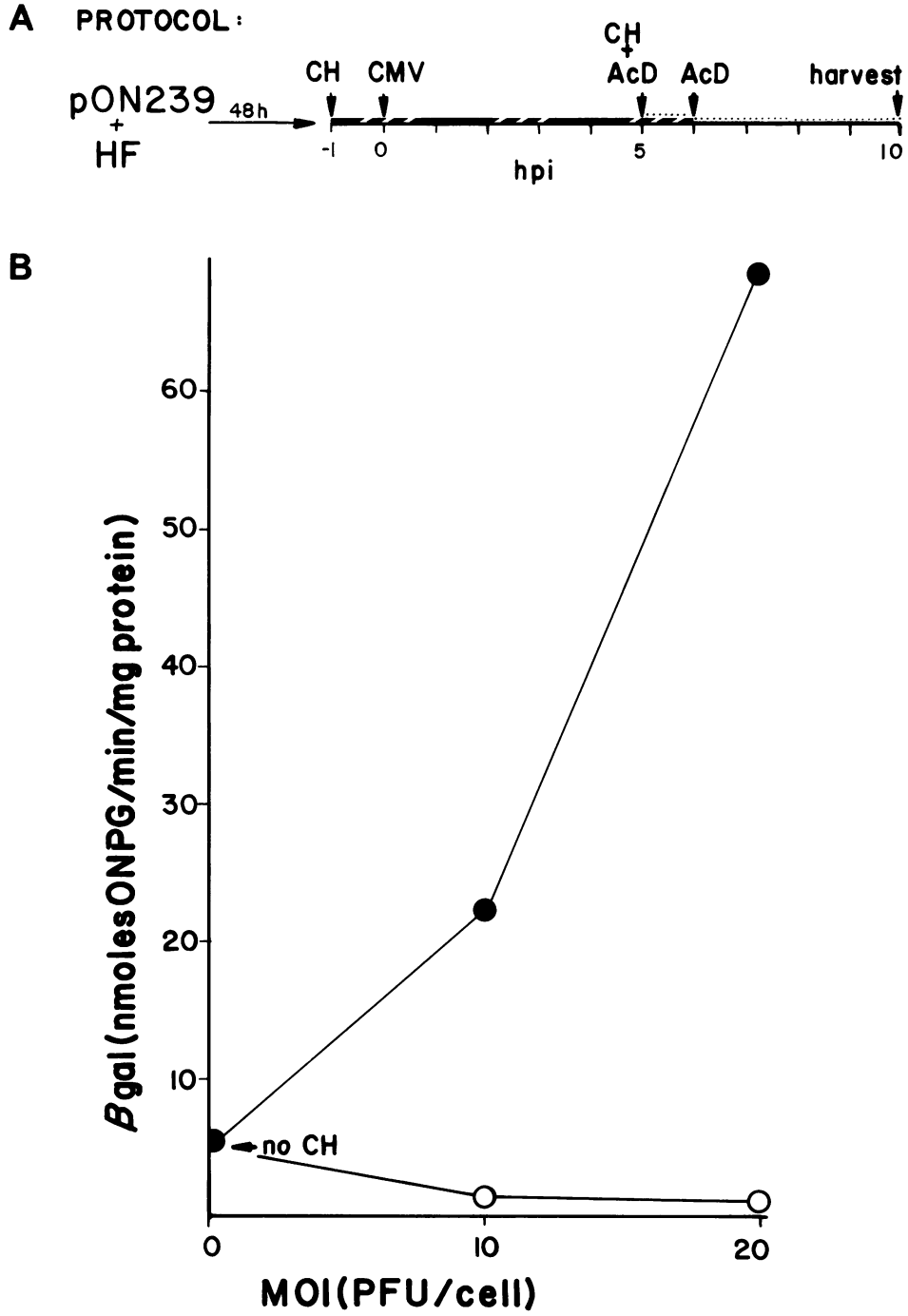

FIG. 4. Induction of the α promoter, but not the β promoter, in the absence of de novo protein synthesis. (A) Protocol of the infection by CMV of HF cells containing pON239 or pON241 in the presence of CH with subsequent reversal in the presence of AcD. (B) Cells containing pON239 or pON241 were infected with CMV (Towne) and harvested at 10 hpi. Aliquots of the extracts were assayed for β-gal activity. Symbols: ●, β-gal activities in cells receiving pON239; ○, β-gal activities in cells receiving pON241. No CH, β-gal activities in cells which received pON239 and drug treatments but which were not infected with CMV (Towne).

block translation, and then AcD was added to block transcription. One hour before infection with CMV, the cells were treated with CH (50 μg/ml). Cells were infected with CMV at an MOI of 10 or 20 PFU per cell in the presence of CH. The infection was allowed to proceed until 10 hpi, at which time the cells were harvested. The results of the assays for β-gal activity (Fig. 4) revealed that high levels of β-gal activity were induced. CH treatment alone did not affect basal levels of β-gal in cells transfected with pON239.

In a second approach, HF cells were transfected with pON239 and infected with UV-irradiated CMV 24 h before harvest, as described in Materials and Methods. The induction (Fig. 5) is comparable to that seen at an MOI of 14 with wild-type virus at 24 hpi, even though the UV-irradiated virus MOI was reduced by 10^{-4}-fold. The reported multiplic-

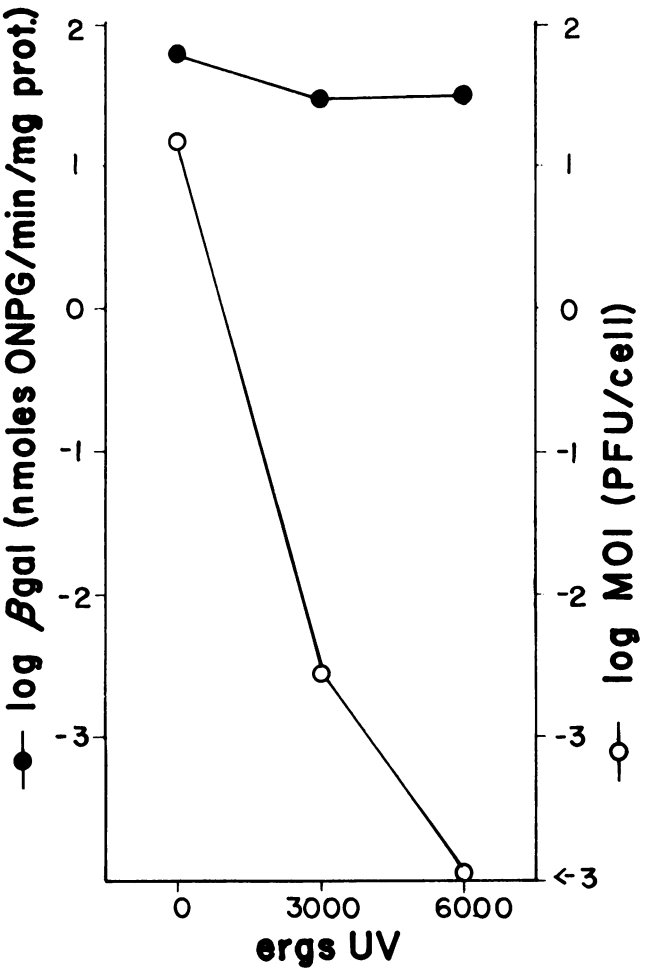

FIG. 5. Induction of the α promoter with UV-inactivated virus. CMV (Towne) was exposed to either 3,000 or 6,000 ergs/mm^2, using a 254-nm UV light source as described in Materials and Methods, or left unirradiated. Portions of virus were both titrated for residual infectivity and used to infect cells (14 PFU per cell) that had been transfected with 25 μg of pON239 48 h previously. At 24 hpi, β-gal activity was measured in the cell extracts. Open circles represent the log of the residual infectivity, and closed circles represent the log β-gal activity. The actual levels of β-gal activity were 60, 27, and 33 nmol of ONPG cleaved per min per mg of protein for the unirradiated sample and the samples irradiated with 3,000 and 6,000 ergs/mm^2, respectively.

ity reactivation of UV-irradiated CMV (1) tempers the interpretation of the UV data alone. Taken together, however, the results of the CH-AcD block experiment and the UV data suggest that a component of the infecting virions was responsible for some level of induction in β-gal activity from the α-promoter construct.

Induction of the β-promoter–β-gal fusion gene. The β-promoter fusion gene, pON235, and the fusion containing the promoter region in reverse orientation, pON234, were transfected into HF cells. After 48 h, dishes receiving pON235 were infected with 1, 10, or 20 PFU of CMV or HSV (F$^+$) or mock infected. Dishes receiving pON234 were infected with CMV at 10 or 20 PFU per cell or mock infected. The cells were harvested at 24 hpi, and portions of the extracts were assayed for β-gal activity. The results of these determinations (Fig. 6) reveal the following: (i) CMV infection of cells containing pON235 resulted in a large multiplicity-dependent induction of levels of β-gal activity; (ii) pON234, with the β promoter in the opposite direction, did not respond to infection by CMV; and (iii) infection by HSV did not induce expression of the CMV β promoter in pON235 (<4 nmol of ONPG cleaved per min per mg of protein). These results indicate that the β promoter is specifically regulated by CMV infection. The lack of induction by HSV suggests that specific *cis*-acting regulatory sequences act in concert with *trans*-acting CMV specific factors.

Kinetics of β-gene expression. pON241 was derived to remove the 1.2-kb transcript which had been mapped in these sequences (30, 49). HF cells were transfected with 25 μg of pON235 or pON241. After 48 h, the cells were infected with an MOI of 20 PFU per cell, and cells were harvested at 0, 4, 8, 12, and 24 hpi. Portions of these cell extracts were assayed for β-gal activity (Fig. 7). A striking induction in β-gal activity began at 12 hpi and continued through 24 hpi. In contrast to the α promoter, no induction was detected at 4 or 8 hpi. Levels of β-gal activity in this experiment were less than those shown in Fig. 6 (for pON235) because of experiment-to-experiment variation as discussed in the Materials and Methods. These results were taken to mean that

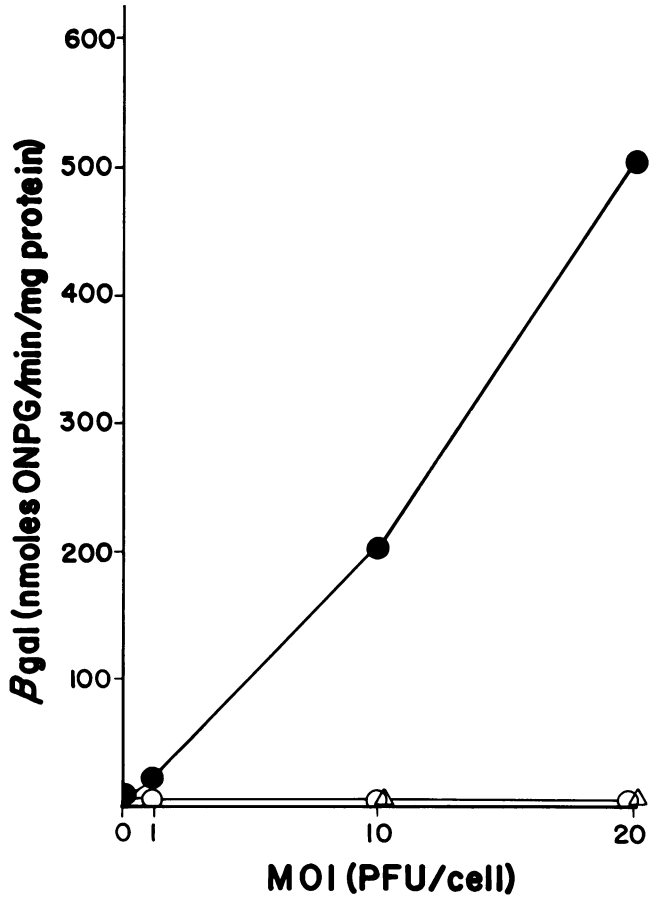

FIG. 6. *Trans* activation of the β-promoter–β-gal fusion in HF cells by infection with CMV but not HSV-1. HF cells were transfected with 25 μg of pON234 or pON235, infected with 1, 10, or 20 PFU per cell of CMV (Towne) or 10 and 20 PFU per cell of HSV (F$^+$), harvested 24 hpi, and assayed for levels of β-gal activity. Symbols: ●, cells transfected with pON235 and infected with CMV; ○, cells transfected with pON234 and infected with CMV; △, cells transfected with pON235 and infected with HSV.

the regulatory domain of the β-promoter fusion gene was contained within 1,000 bp of the start site of transcription. The β promoter on pON241 was further analyzed in a CH-AcD block experiment (Fig. 4) as described for pON239. No induction of the β promoter occurred under these conditions.

DISCUSSION

The coordinately regulated, sequentially ordered expression of herpesvirus early genes is controlled by promoter-proximal sequences which contain *cis*-acting elements (15, 22, 25–27, 38, 39, 41, 42, 58). We have shown that prototypic α and β promoters from the CMV genome contain *cis*-acting signals which interact with viral *trans*-acting functions. The fusion of these promoters to the indicator gene (encoding β-gal) resulted in its expression as either an α or β gene. In transient transfection-infection assays in permissive human fibroblasts, promoter fusion constructs appeared to accurately mimic the regulated expression of these genes from their natural positions in the viral genome. The significant up-regulation we observed for the prototypic α- and β-promoter constructs in the presence of *trans*-acting viral functions showed qualitative and quantitative characteristics of the natural gene regulation; however, until these constructs are inserted into the viral genome, their authenticity can only be implied (41).

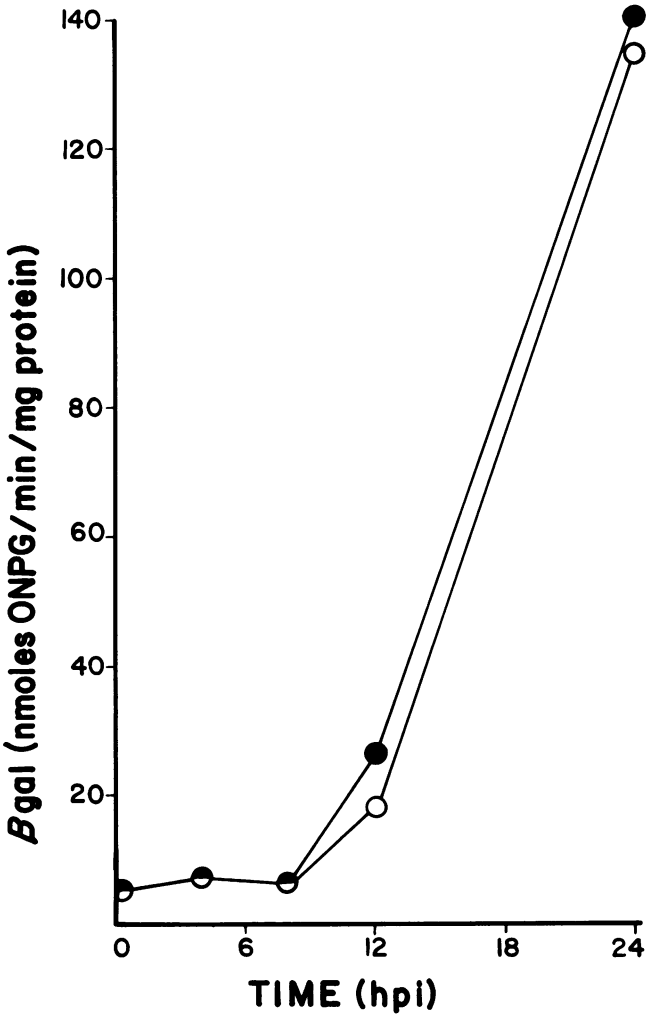

FIG. 7. Kinetics of the induction of the β-promoter–β-gal fusion after infection by CMV (Towne). Cells were transfected with 25 μg of pON241 (●) or pON235 (○), infected with CMV at 48 h posttransfection, and assayed for induction of β-gal activity at 0, 4, 8, 12, and 24 hpi.

The α promoter chosen for fusions has been extensively studied (48, 50, 52). It is active in both virus-infected cells and in vitro, as determined with a HeLa cell transcription system (52). By placing β-gal under the control of this promoter, we have shown that *cis*-acting elements within 1.1 kilobase pair of the start site of transcription act in concert with virion factors to increase the level of expression in a multiplicity-dependent manner. This is consistent with the results obtained with HSV-1 (3, 25–27, 39) and suggest a common mechanism for the activation of herpesvirus α genes. In line with this conclusion are recent experiments (M. F. Stinski and T. J. Roehr, personal communication) reporting activation of the CMV MIE promoter by CMV-specified protein(s). In contrast to previous reports (3) that suggested CMV was unable to activate HSV-1 α promoters in *trans*, our data suggest that the *cis*-acting elements in the promoter region of the CMV α gene can be activated by HSV-1 as well as CMV functions. The most likely reason for this difference resides in the permissiveness of the cells for CMV replication. However, the environment in which the constructs find themselves within the host cell or the assay system itself may contribute to the inconsistency of the two results. A tegument protein has been implicated in the activation of HSV-1 α genes (7) and may be capable of activating CMV α genes as well. This can be tested directly by introducing our CMV promoter construct into the HSV-1 genome by well-established techniques (33) to more stringently assess the *trans* activation of the CMV α promoter by HSV-1 gene products.

The CMV β-promoter fusion was specifically activated in *trans* by CMV gene products, and was totally unresponsive to HSV-1. This is in contrast to results reported for the HSV-1 βγ gene encoding glycoprotein D (16). In those studies, CMV infection of permissive cells into which the HSV-1 gene had been transiently introduced resulted in its transcriptional activation and suggested that early transcriptional signals may be common to both CMV and HSV-1. In contrast to those studies, we measured full gene expression, not solely transcriptional activation. It is probable that CMV gene expression is regulated at many levels (11, 49, 50) and that both transcriptional and posttranscriptional regulation are occurring. The presence of *cis*-acting β-regulatory elements is indicated by our studies as well as by conventional studies on the expression of this gene in permissive cells during viral replication (30, 47, 49, 55, 56). The transient transfection-infection system will enable the dissection of the *cis*- and *trans*-acting functions involved in all levels of β-gene regulation.

The temporal expression of α- and β-promoter fusions was consistent with their accumulated expression from natural templates during viral replication. We have used monoclonal antibody specific for the MIE gene product and detected the accumulation of this product (unpublished data), corroborating the results shown for pON239 (Fig. 2 through 4). The expression of the α-promoter fusion preceded the temporal expression of the β-promoter fusion, which was not detectably induced at 4 or 8 hpi, times when the α promoter was activated. The levels of activation achieved by 24 hpi suggested that the β promoter was quantitatively more highly induced than the α promoter, although the basal levels of

activity from either promoter (in the absence of viral infection) were not perceptibly different. This is consistent with the high level of transcription of the β gene relative to the MIE gene from its natural site within the viral genome at early times during CMV infection (29, 49, 55, 56).

The constructs we have used contained substantial amounts (>1 kbp) of sequence upstream of the transcriptional start site. By using such an extensive upstream region as well as substantial 5′ untranslated regions from both the α and β genes, we had expected to detect maximal activation, thereby creating a powerful tool to dissect *cis*- and *trans*-acting elements in CMV gene expression. The constructs we have described express two of the most strongly activated promoters that have been reported when judged by comparable β-gal assay systems (17, 23, 36) and are active in normal human cells. The MIE promoter strength has been previously studied in the absence of *trans*-acting viral functions and shown to be constitutively strong (52). Our results clearly show that in the presence of *trans*-activating functions, both this and the β promoter are substantially upregulated.

In an elegant series of studies on the mouse mammary tumor virus promoter in which constructs closely related to those used in our studies were used, the role of the SV40 enhancer was shown to be that of increasing the basal level of expression such that *trans*-activation by glucocorticoids became more easily detectable (17, 23). This is in agreement with the results of other studies which suggest that the SV40 enhancer does not qualitatively alter the response of other regulatory elements (18). The precise role of the SV40 enhancer in increasing basal levels of β-gal expression from the CMV promoters studied here has not been established; however, results obtained with controls (pON1 and pON234 compared with pON235, pON239, and pON241) as well as the observed specificity of the *trans* activation by viral infection reaffirm that in our experiments, the enhancer does not confound the detection of physiologically relevant regulatory signals.

There is no difference in whether we deliver the plasmid constructs to HF cells by DEAE-dextran or calcium phosphate precipitation (46), in contrast to the suggestions of others (17, 35). We have tested the *trans* activation of the β-promoter construct pON241 by using both methods in parallel, and neither the basal nor activated levels are significantly different (unpublished data).

The absolute authenticity of the regulatory events observed in these transient transfection-infection assays will require the construction of recombinant CMV which carry these constructs integrated into the viral genome. In the absence of those viral constructs we have used classic parameters of immediate (α) and delayed (β) early gene expression. We expect that this assay might also provide physiologically relevant information on the regulation of CMV γ genes, two of which have been recently mapped (32, 37). The importance of transcriptional controls is implied by the fact that we have studied promoter fusions, although posttranscriptional events may be crucial to activation as well. Because we have used permissive cells and tested a complete set of controls along with the specific promoter constructs, we feel confident in suggesting that the results represent authentic activation comparable to that which occurs when these genes are expressed in the viral genome during infection. The approach we have presented is applicable to the study of any promoter and should enable the isolation of the *trans*-acting regulatory functions affecting the temporal expression of classes of genes during CMV replication.

ACKNOWLEDGMENTS

We are grateful to C. Martens and F. Lee for advice concerning the use of β-gal fusions and for the use of the MR580, to J. Culpepper, K. Moore, M. Stinski, and N. Frenkel for plasmids, and to V. Seewaldt and A. McCormick for excellent technical assistance.

These studies were supported by grants from the American Cancer Society (MV230) and Public Health Service grant 1 ROI AI20211 from the National Institutes of Health. R.R.S. was supported by a fellowship from the Leukemia Research Foundation.

ADDENDUM IN PROOF

Boshart et al. (Cell **41**:521–530, 1985) have shown that a transcriptional enhancer is located within the 2.1-kb *Pst*I fragment used in our studies as the MIE promoter (pON239). Stinski and Roehr (J. Virol. **55**:431–441, 1985) have shown that sequences in proximity to the enhancer element are responsible for some measure of *trans* activation.

LITERATURE CITED

1. **Albrecht, T., S. C. St. Jeor, F. D. Funk, and F. Rapp.** 1974. Multiplicity reactivation of human cytomegalovirus inactivated by ultraviolet light. Int. J. Radiat. & Relat. Stud. Phys. Chem. & Med. **26**:445–454.
2. **Banerji, J., S. Rusconi, and W. Schaffner.** 1981. Expression of a β-globin gene is enhanced by remote SV40 DNA sequences. Cell **27**:299–308.
3. **Batterson, W., and B. Roizman.** 1983. Characterization of the herpes simplex virion-associated factor responsible for the induction of α genes. J. Virol. **46**:371–377.
4. **Benoist, C., and P. Chambon.** 1981. *In vivo* sequence requirements of the SV40 promoter region. Nature (London) **290**: 304–310.
5. **Berk, A. J., F. Lee, T. Harrison, J. Williams, and P. A. Sharp.** 1979. Pre-early adenovirus 5 gene product regulates synthesis of early viral messenger RNAs. Cell **17**:935–944.
6. **Bradford, M. A.** 1976. Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
7. **Campbell, M. E. M., J. W. Palfreyman, and C. M. Preston.** 1984. Identification of herpes simplex virus DNA sequences which encode a *trans*-acting polypeptide responsible for the stimulation of immediate early transcription. J. Mol. Biol. **180**:1–19.
8. **Clements, J. B., R. J. Watson, and N. M. Wilkie.** 1977. Temporal regulation of herpes simplex virus type 1 transcription: location of transcripts on the viral genome. Cell **12**:275–285.
9. **Costanzo, F., G. Campadelli-Fiume, L. Foà-Tomasi, and E. Cassai.** 1977. Evidence that herpes simplex virus DNA is transcribed by cellular RNA polymerase B. J. Virol. **21**: 996–1001.
10. **De Marchi, J. M.** 1981. Human cytomegalovirus DNA: restriction enzyme cleavage maps and map locations for immediate-early, early, and late RNAs. Virology **114**:23–38.
11. **De Marchi, J. M.** 1983. Post-transcriptional control of human cytomegalovirus gene expression. Virology **124**:390–402.
12. **De Marchi, J. M., C. A. Schmidt, and A. S. Kaplan.** 1980. Patterns of transcription of human cytomegalovirus in permissively infected cells. J. Virol. **35**:277–286.
13. **Dixon, R. A. F., and P. A. Schaffer.** 1981. Fine structure mapping and functional analysis of temperature-sensitive mutants in the gene encoding the herpes simplex virus type 1 immediate early protein VP175. J. Virol. **36**:189–203.
14. **ElKareh, A., A. J. M. Murphy, T. Fichter, A. Efstratiadis, and S. Silverstein.** 1985. "Transactivation" control signals in the promoter of the herpesvirus thymidine kinase gene. Proc. Natl. Acad. Sci. USA **82**:1002–1006.
15. **Everett, R. D.** 1984. *Trans* activation of transcription by herpes virus products: requirement for two HSV-1 immediate-early polypeptides for maximum activity. EMBO J. **3**:3135–3141.
16. **Everett, R. D., and M. Dunlop.** 1984. *Trans* activation of plasmid-borne promoters by adenovirus and several herpes group viruses. Nucleic Acids Res. **12**:5969–5978.

17. **Hall, C. V., P. E. Jacob, G. M. Ringold, and F. Lee.** 1983. Expression and regulation of *Escherichia coli lacZ* gene fusions in mammalian cells. J. Mol. Appl. Genet. **2**:101–109.
18. **Hamer, D. H., and M. Walling.** 1982. Regulation *in vivo* of a cloned mammalian gene: cadmium induces the transcription of a mouse metallothionein gene in SV40 vectors. J. Mol. Appl. Genet. **1**:273–288.
19. **Honess, R. W., and B. Roizman.** 1974. Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. J. Virol. **14**:8–19.
20. **Honess, R. W., and B. Roizman.** 1975. Regulation of herpesvirus macromolecular synthesis: sequential transition of polypeptide synthesis requires functional viral polypeptides. Proc. Natl. Acad. Sci. USA **72**:1276–1280.
21. **Jones, N., and T. Shenk.** 1979. An adenovirus 5 early gene product function regulates expression of other early viral genes. Proc. Natl. Acad. Sci. USA **76**:3665–3669.
22. **Kristie, T. M., and B. Roizman.** 1984. Separation of sequences defining basal expression from those conferring α gene recognition within the regulatory domains of herpes simplex virus 1 α genes. Proc. Natl. Acad. Sci. USA **81**:4065–4069.
23. **Lee, F., C. V. Hall, G. M. Ringold, D. E. Dobson, J. Luh, and P. E. Jacob.** 1984. Functional analysis of the steroid hormone control region of mouse mammary tumor virus. Nucleic Acids Res. **12**:4191–4206.
24. **Mackem, S., and B. Roizman.** 1980. Regulation of herpesvirus macromolecular synthesis: transcription-initiation sites and domains of α genes. Proc. Natl. Acad. Sci. USA **77**:7122–7126.
25. **Mackem, S., and B. Roizman.** 1982. Differentiation between α promoter and regulator regions of herpes simplex virus 1: the functional domains and sequence of a movable α regulator. Proc. Natl. Acad. Sci. USA **79**:4917–4921.
26. **Mackem, S., and B. Roizman.** 1982. Regulation of α genes of herpes simplex virus: the α 27 gene promoter-thymidine kinase chimera is positively regulated in converted L cells. J. Virol. **43**:1015–1023.
27. **Mackem, S., and B. Roizman.** 1982. Structural features of the herpes simplex virus α gene 4, 0, and 27 promoter-regulatory sequences which confer α regulation on chimeric thymidine kinase genes. J. Virol. **44**:939–949.
28. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
29. **McDonough, S. H., and D. H. Spector.** 1983. Transcription in human fibroblasts permissively infected by human cytomegalovirus strain AD169. Virology **125**:31–46.
30. **McDonough, S. H., S. I. Staprans, and D. H. Spector.** 1985. Analysis of the major transcripts encoded by the long repeat of human cytomegalovirus strain AD169. J. Virol. **53**:711–718.
31. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
32. **Mocarski, E. S., L. Pereira, and N. Michael.** 1985. Precise localization of genes on large animal virus genomes: use of λgt11 and monoclonal antibodies to map the gene for a cytomegalovirus protein family. Proc. Natl. Acad. Sci. USA **82**:1266–1270.
33. **Mocarski, E. S., L. E. Post, and B. Roizman.** 1980. Molecular engineering of the herpes simplex virus genome: insertion of a second L-S junction into the genome causes additional genome inversions. Cell **22**:243–255.
34. **Nevins, J. R.** 1981. Mechanism of activation of early viral transcription by the adenovirus E1A gene product. Cell **26**:213–220.
35. **Nielson, D. A., J. Chou, A. J. MacKrell, M. J. Casadaban, and D. F. Steiner.** 1983. Expression of a preproinsulin-β galactosidase gene fusion in mammalian cells. Proc. Natl. Acad. Sci. USA **80**:5198–5202.
36. **Norton, P. A., and J. M. Coffin.** 1985. Bacterial β-galactosidase as a marker of Rous sarcoma virus gene expression and replication. Mol. Cell. Biol. **5**:281–290.
37. **Nowak, B., A. Gmeiner, A. Sarnow, A. J. Levine, and B. Fleckenstein.** 1984. Physical mapping of human cytomegalovirus genes: identification of DNA sequences coding for a virion phosphoprotein of 71 kDa and a viral 65 kDa polypeptide. Virology **134**:91–102.
38. **O'Hare, P., and G. S. Hayward.** 1985. Evidence for a direct role for both the 175,000- and 110,000-molecular-weight immediate-early proteins of herpes simplex virus in the transactivation of delayed-early promoters. J. Virol. **53**:751–760.
39. **Post, L. E., S. Mackem, and B. Roizman.** 1981. Regulation of α genes of herpes simplex virus: expression of chimeric genes produced by fusion of thymidine kinase with α gene promoters. Cell **24**:555–565.
40. **Preston, C. M.** 1979. Control of herpes simplex virus type 1 mRNA synthesis in cells infected with wild-type virus or the temperature-sensitive mutant *tsK*. J. Virol. **29**:275–284.
41. **Silver, S., and B. Roizman.** 1985. γ_2-Thymidine kinase chimeras are identically transcribed but regulated as γ_2 genes in herpes simplex virus genomes and as β genes in cell genomes. Mol. Cell. Biol. **5**:518–528.
42. **Smiley, J. R., H. Swan, M. M. Pater, A. Pater, and M. E. Halpern.** 1983. Positive control of the herpes simplex virus thymidine kinase gene requires upstream DNA sequences. J. Virol. **47**:301–310.
43. **Spaete, R. R., and N. Frenkel.** 1982. The herpes simplex virus amplicon: a new eucaryotic defective-virus cloning vector. Cell **30**:295–304.
44. **Spaete, R. R., and N. Frenkel.** 1985. The herpes simplex virus amplicon: analyses of *cis*-acting replication functions. Proc. Natl. Acad. Sci. USA **82**:694–698.
45. **Spaete, R. R., and E. S. Mocarski.** 1984. *Trans*-acting functions encoded by herpes simplex virus-1 recognize *cis* cleavage/packaging signals present on cytomegalovirus DNA. UCLA Symp. Mol. Cell. Biol. New Ser. **21**:359.
46. **Spaete, R. R., and E. S. Mocarski.** 1985. The *a* sequence of the cytomegalovirus genome functions as a cleavage/packaging signal for herpes simplex virus defective genomes. J. Virol. **54**:817–824.
47. **Spector, D. H., S. H. McDonough, S. I. Staprans, J. C. Tamashiro, T. Friedmann, and D. Filpula.** 1984. Organization and expression of the repeats at the termini and L-S junction of human cytomegalovirus strain AD169. UCLA Symp. Mol. Cell. Biol. New Ser. **21**:441.
48. **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.
49. **Stinski, M. F.** 1983. The molecular biology of cytomegaloviruses, p. 67–113. *In* B. Roizman (ed.), The herpesviruses, vol. 2. Plenum Publishing Corp., New York.
50. **Stinski, M. F., R. M. Stenberg, and W. F. Goins.** 1984. Structure and function of the human cytomegalovirus immediate early genes. UCLA Symp. Mol. Cell. Biol. New Ser. **21**:399.
51. **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.
52. **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.
53. **Thomsen, D. R., and M. F. Stinski.** 1981. Cloning of the human cytomegalovirus genome as endonuclease *Xba*I fragments. Gene **16**:207–216.
54. **Tooze, J. (ed.).** 1981. DNA tumor viruses, Molecular biology of tumor viruses, 2nd ed., p. 799–829. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
55. **Wathen, M. W., and M. F. Stinski.** 1982. Temporal patterns of human cytomegalovirus transcription: mapping the viral RNAs synthesized at immediate early, early, and late times after infection. J. Virol. **41**:462–477.
56. **Wathen, M. W., D. R. Thomsen, and M. F. Stinski.** 1981. Temporal regulation of human cytomegalovirus: transcription at immediate early and early times after infection. J. Virol. **38**:446–459.
57. **Watson, R. J., and J. B. Clements.** 1980. A herpes simplex virus type 1 function continuously required for early and late virus RNA synthesis. Nature (London) **285**:329–330.
58. **Zipser, D., L. Lipsich, and J. Kwoh.** 1981. Mapping functional domains in the promoter region of the herpes thymidine kinase gene. Proc. Natl. Acad. Sci. USA **78**:6276–6280.