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Expression of the Herpes Simplex Virus Type 1 Glycoprotein C Gene Requires Sequences in the 5′ Noncoding Region of the Gene

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The role of the 5′ noncoding region of the herpes simplex virus type 1 glycoprotein C (gC) gene in viral gene expression was investigated with recombinant herpesviruses that contained the bacterial β-galactosidase gene under the control of the gC promoter-regulatory region. Each of these viruses had the same DNA sequences from the start of gC transcription upstream to −114 but had variable segments of the downstream 140-base-pair sequence that is between the start of gC transcription and translation. Analysis of β-galactosidase expression and mRNA synthesis from these viruses demonstrated the importance of DNA sequences from the start of gC transcription downstream to +38 for optimal expression from the gC promoter.

To define the DNA sequences that are involved in regulation and expression of viral late genes, we have constructed recombinant herpesviruses that express the bacterial enzyme β-galactosidase (β-Gal) from the promoter of the glycoprotein C (gC) gene, a well-characterized herpesvirus late gene. Recombinant viruses are advantageous in the study of late promoter-regulatory regions because herpesvirus late genes that are removed from the viral genome no longer require viral DNA replication for their expression (3, 9, 11, 12). In previous studies using recombinant viruses, we demonstrated that sequences between −1350 and +30 relative to the start of gC transcription regulate β-Gal as a viral late gene and that upstream sequences can be deleted to at least −109 without affecting expression (14). Other studies (5, 6) have shown that upstream gC sequences can be deleted to −34 without affecting expression and that a 15-base-pair (bp) TATA box promoter element is absolutely required for gC expression.

Although gC sequences between −109 and +30 regulate β-Gal expression as a late viral gene in recombinant viruses, it is not clear whether gC sequences downstream from +30 have a role in gC expression. Homa et al. (5) noticed that deletions of DNA segments containing the start of transcription and downstream sequences lower the relative level of gC mRNA, and they suggested that this was due to a shortening in the length of the nontranslated leader that is non-sequence specific. In this report, we describe the construction of a set of recombinant herpesviruses designed to determine whether sequences involved in the regulation of herpes simplex virus type 1 (HSV-1) gC expression are located in the 5′ untranslated region of the gene. Each of these recombinant viruses had the β-Gal gene under the control of the promoter for the late viral gC gene. Each gC promoter contained sequences from the start of transcription upstream to −114 but different segments of the downstream 140-bp nontranslated sequence between the start of transcription and translation.

The DNA sequences of the gC gene containing the 5′ nontranslated leader of the gene were isolated by insertion of a unique *Sal*I restriction enzyme site in the cloned gC gene at position +141 relative to the start of transcription. This changed the ATGGCC that begins the gC coding sequence (4) to GTCGAC but left the 140 bp between the start of gC transcription and translation unaltered. Promoter sequences of the gC gene from −114 to +140 were inserted upstream of the β-Gal gene in the HSV-1 insertion vector pGal8 (J. P. Weir, K. R. Steffy, and M. Sethna, submitted for publication) to generate the plasmid pgCL1. In addition to the gC sequences upstream of the β-Gal gene, gCL1 contained an additional 35 bp from the *Sal*I site to the ATG that begins β-Gal translation (Fig. 1). The construction gCL3 contained the same gC promoter sequences, −114 to +140, upstream of the β-Gal gene as gCL1, but the 35 bp between the gC promoter and the ATG that begins β-Gal translation was eliminated. Thus, the coding sequences for β-Gal began at +141, in the same position in which the coding sequences for gC are normally located in the authentic gC gene. Sequential 3′-to-5′ deletions were made in the gC leader region that left sequences to +71 (gCL5), +38 (gCL6), +16 (gCL6.1), and −3 (gCL7). Two other constructions with deleted sequences between +28 and +99 (gCL3.1) and +2 and +39 (gCL8) were made. Recombinant viruses were made from each plasmid construction as described previously (14).

When Vero cells were infected with each of the recombinant viruses in the presence or absence of phosphonoacetic acid (PAA), an inhibitor of viral DNA replication, all of the viruses that expressed β-Gal did so in the manner of a late viral gene. Expression was first detectable at approximately 6 h after infection and continued to rise for at least 24 h (data not shown). Figure 1 shows the expression of β-Gal at 24 h postinfection in the presence and absence of PAA. Elimination of the 35 bp between the gC leader sequences and the ATG that begins the β-Gal open reading frame (Fig. 1) resulted in a higher level of β-Gal expression in vgCL3- than in vgCL1-infected cells. Since the location of the β-Gal open reading frame has perfectly replaced the gC open reading frame in vgCL3, the β-Gal activity of vgCL3-infected cells was set at 100%. Although the lower level of β-Gal expression in vgCL1-infected cells could be due to the addition of non-gC sequences, we think that a more likely explanation is that the sequence around the ATG in vgCL1 contains a pyrimidine at −3 relative to the A of the ATG. Such a sequence is not predicted to be as favorable for translation as the sequence that exists if a purine is present at this position (7). In each of our other constructions, the sequences

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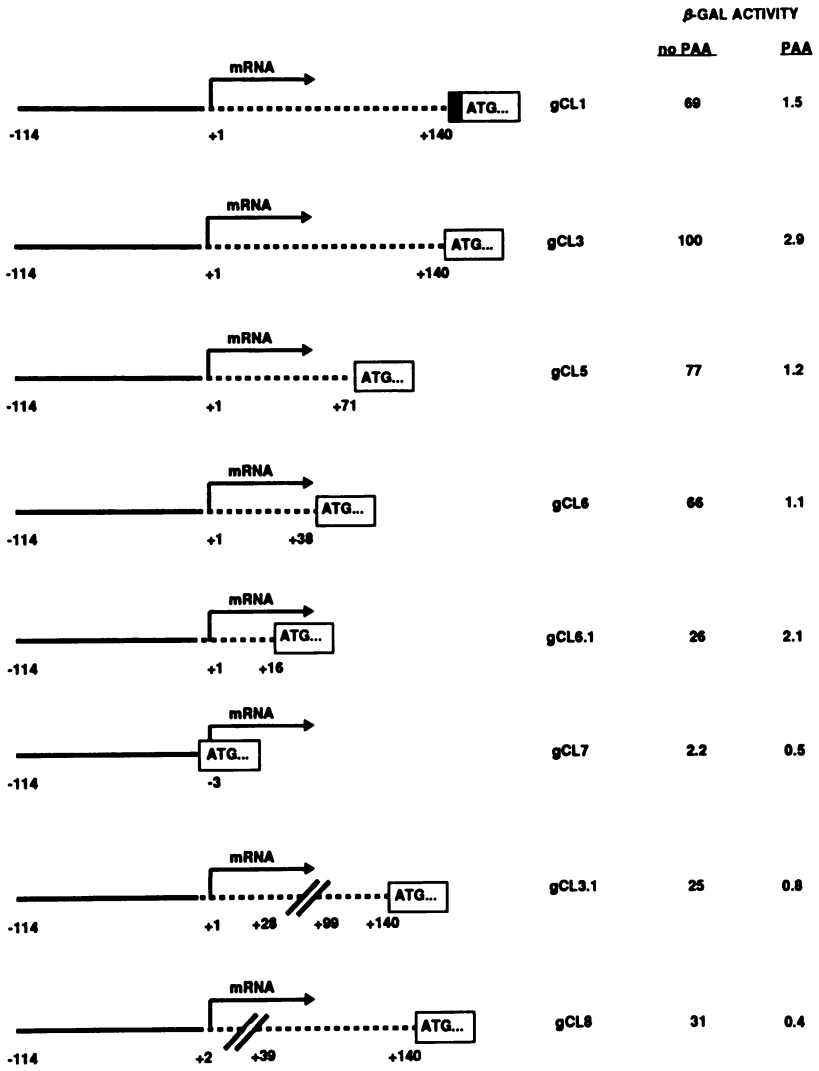

FIG. 1. Construction of HSV-1 recombinant viruses and expression of β-Gal. A 2,000-bp *SalI-EcoRI* fragment of HSV-1 (KOS) DNA containing the first 500 bp of the coding sequence for gC and approximately 1,500 bp upstream from the start of gC translation was cloned into the phagemid vector Bluescribe M13+ (Stratagene). A *SalI* restriction site was generated by oligonucleotide mutagenesis (8) at +141 relative to the start of transcription. A *Hin*dIII site was inserted by mutagenesis at −118 (leaving gC promoter sequences unchanged to −114), and the gC sequences from −114 to +141 were cloned into the HSV-1 insertion vector pGal8. This insertion vector has the coding sequences of the *Escherichia coli* β-Gal gene flanked by sequences from the HSV-1 thymidine kinase gene to direct homologous recombination into the thymidine kinase gene of the viral genome. Recombinant viruses were isolated as described previously (14). The promoter sequences from gCL1 were recloned into the phage vector M13mp18, and the indicated deletions were made by oligonucleotide mutagenesis with oligonucleotides that spanned the desired deletion. After mutagenesis, the promoter sequences were recloned into pGal8 for insertion into the viral genome. The additional 35-bp segment of DNA present in gCL1 between the gC leader and the β-Gal coding sequence (▮) is from the original fragment of DNA that contained the β-Gal gene used to construct pGal8. Vero cells were infected at a multiplicity of infection of 10 in the presence or absence of 300 μg of PAA per ml with each of the recombinant viruses. The β-Gal activity was measured at 24 h postinfection as described previously (14) and expressed relative to that of vgCL3.

brought adjacent to the ATG are predicted to be favorable for translation.

Progressive deletions from the start of translation toward the start of transcription resulted in decreasing levels of β-Gal expression (Fig. 1). Deletion of sequences to +38 (vgCL6) reduced β-Gal expression to 70% of that exhibited by vgCL3. Deletion of another 22 bp to +16 (vgCL6.1) resulted in a more dramatic reduction of expression to 26% of maximal expression, and elimination of the entire 140-bp leader and mRNA start site (vgCL7) abolished detectable β-Gal expression. None of these viruses expressed detectable β-Gal when viral DNA replication was inhibited with PAA, indicating that elimination of gC leader sequences did not affect temporal regulation.

A progressive decrease in observed β-Gal expression could be due to the elimination of the specific DNA sequences that are important for expression or to a progressive reduction in the length of the gC leader that is non-sequence specific. Two deletions, gCL3.1 and gCL8, were constructed to address this question. In vgCL3.1, 70 bp of the gC leader sequence between +28 and +99 was eliminated, with a resulting reduction of β-Gal expression to 25%. Elimination of 36 bp of the gC leader sequence in vgCL8 (sequences between +2 and +39) reduced β-Gal expression in infected

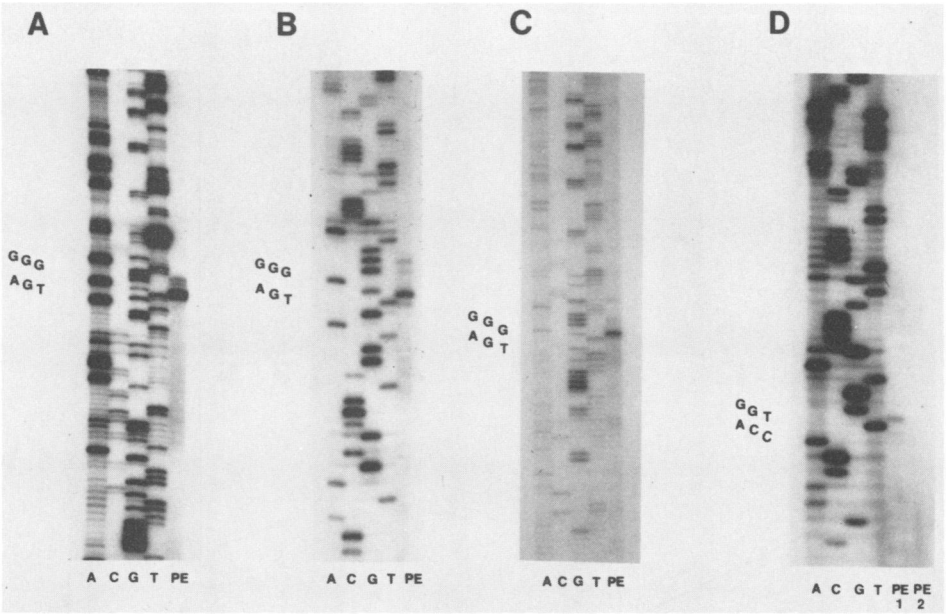

FIG. 2. Analysis of the 5′ ends of β-Gal mRNAs. Vero cells were infected with recombinant viruses at a multiplicity of infection of 10, and total RNA was isolated 24 h later. After hybridization with a 5′-end-labeled oligonucleotide complementary to the 5′ end of the β-Gal gene, primer-RNA hybrids were extended with reverse transcriptase (14). The extended products (lanes PE) were separated on a 6% sequencing gel alongside a sequence ladder prepared with the same primer and the corresponding plasmid DNA. (A) Primer extension analysis of RNA from vgCL3-infected cells; (B) RNA from vgCL6.1-infected cells; (C) RNA from vgCL8-infected cells; (D) RNA from cells infected with vgCL7 in the presence (lane PE2) or absence (lane PE1) of PAA. The short sequence shown is that of the coding strand around the start of transcription.

cells to 31% of maximal expression. The remaining leader sequence in vgCL3.1 and vgCL8 is substantially longer than that remaining in vgCL5 and vgCL6, yet the levels of β-Gal expression are dramatically lower. The results obtained with vgCL3.1 and vgCL8 indicate that the elimination of gC leader sequences upstream of +39 had a much more dramatic effect on expression than the actual length of the gC leader sequence did.

In order to determine whether deletions in the 5′ nontranslated region of gC that resulted in lower levels of β-Gal expression altered the normal start of mRNA transcription, the 5′ ends of RNA from infected cells were analyzed by primer extension. The major extension product observed when RNA from vgCL3-infected Vero cells was used migrated alongside a G residue (coding strand) in the sequence ladder (Fig. 2A) that was previously designated +1 in an analysis of the 5′ end of the gC mRNA (14). As previously observed, minor extension products migrated alongside residues −2, −3, and +2 relative to the major extension product. Extension products obtained when we used RNA from cells infected with each of the other recombinant viruses that expressed β-Gal were observed at the same positions as those from vgCL3. Primer extension analyses in which RNA from vgCL6.1- and vgCL8-infected cells was used are shown in Fig. 2B and C, respectively. Thus, each

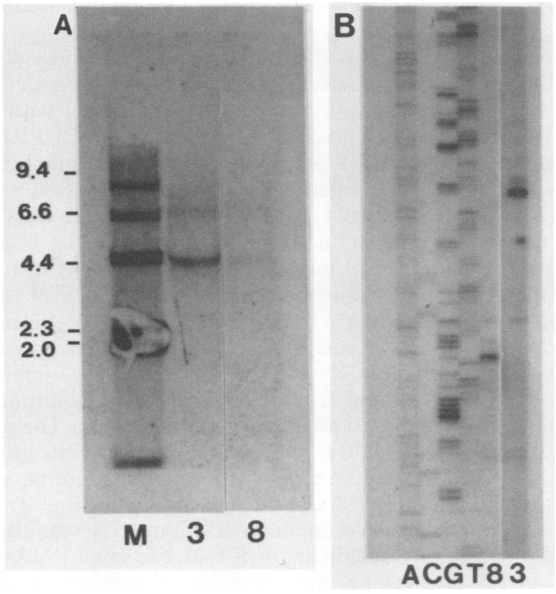

FIG. 3. Accumulation of β-Gal mRNA in cells infected with vgCL3 (lanes 3) and vgCL8 (lanes 8). (A) RNAs (40 μg) from vgCL3- and vgCL8-infected cells were analyzed for the presence of β-Gal mRNA by Northern blotting. Nitrocellulose membranes were hybridized to a ^{32}P-labeled β-Gal-specific DNA probe, washed, and subjected to autoradiography. The relative amounts of β-Gal mRNA were determined by densitometry of the autoradiographs. Labeled DNA markers (lane M) were denatured in the same way as the RNA and were run on the gel for comparison. Sizes shown are in kilobase pairs. The relative β-Gal mRNA ratio (vgCL8/vgCL3) was 0.30. (B) Primer extension analysis of β-Gal mRNAs from vgCL3- and vgCL8-infected cells was as described in the legend to Fig. 2. The sequence ladder was generated from the β-Gal primer and pgCL8 DNA. The relative amounts of β-Gal primer extension products were determined by densitometry of the autoradiograph. The amount of gC mRNA was quantitated in each sample to serve as an internal control for the amount of RNA used in each assay. The relative β-Gal mRNA ratio (vgCL8/vgCL3) was 0.38.

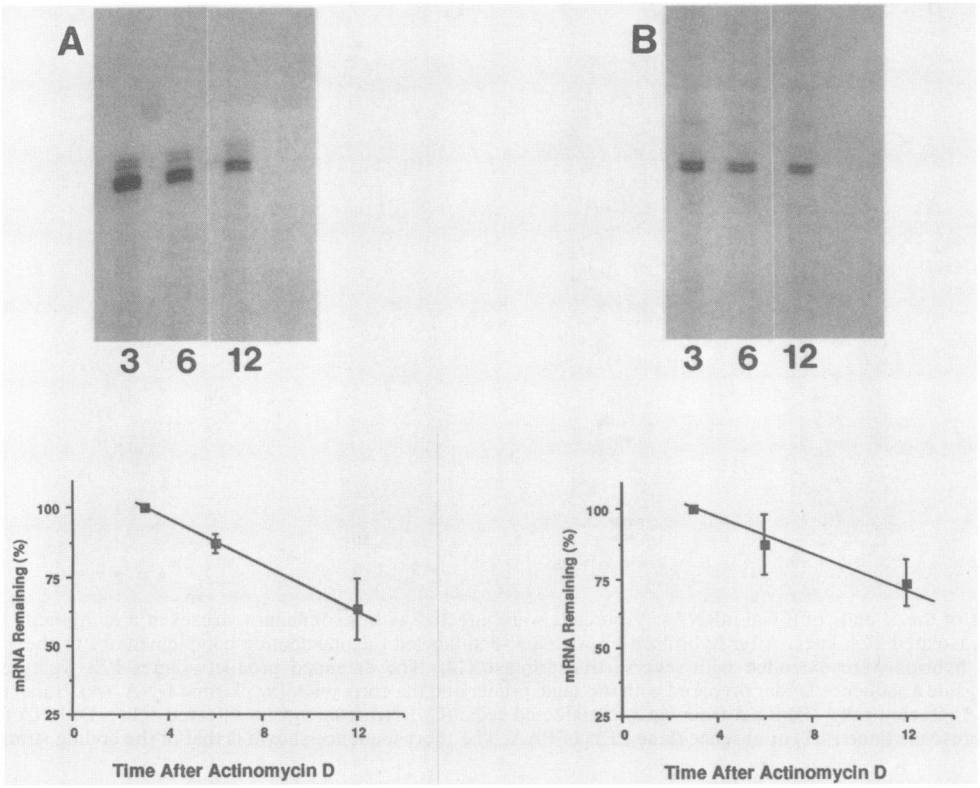

FIG. 4. Stability of β-Gal mRNA in cells infected with vgCL3 (A) and vgCL8 (B). Vero cells (1×10^7) were infected with recombinant virus at a multiplicity of infection of 10. At 12 h postinfection, dactinomycin (actinomycin D) was added to a final concentration of 10 μg/ml. RNA was isolated 3, 6, and 12 h later and used for primer extension analysis (lanes 3, 6, and 12, respectively). The relative amounts of β-Gal primer extension products were determined by densitometry of autoradiographs.

recombinant virus that expressed β-Gal retained the original mRNA start site, regardless of the level of β-Gal expression.

Because the start of β-Gal translation in vgCL7 was positioned upstream from the normal start of gC transcription, the lack of β-Gal expression did not necessarily reflect the lack of mRNA expression from the −114 to −3 gC promoter sequences. The lack of β-Gal expression might be due to the inability of an expressed mRNA to translate a functional β-Gal protein. To address this possibility, RNA was also isolated from vgCL7-infected cells in the presence or absence of PAA and was analyzed by primer extension (Fig. 2D). A faint primer extension product was observed to migrate alongside the G residue (coding strand) at −3 (lane PE1). However, neither the major extension product nor the other minor extension products were observed. Furthermore, in the presence of PAA, no primer extension products were seen (lane PE2). Therefore, deletion of the entire gC leader region and normal mRNA start site eliminated mRNA synthesis from the major gC start site.

The reduced β-Gal expression that resulted from deletions in the gC noncoding leader could be the result of a decrease in the level of β-Gal mRNA or a decrease in translation of β-Gal mRNA. To evaluate these two possibilities, RNA was isolated from cells infected with either vgCL3 or vgCL8 and analyzed by Northern (RNA) blot hybridization. RNA from infected cells was probed initially for gC mRNA with a ^{32}P-labeled DNA fragment specific for gC. The size of the major RNA species detected by the gC probe was approximately 2.5 kilobases (data not shown), which corresponded to the size previously reported for the gC mRNA (4). The amount of gC mRNA was quantitated by densitometry of the autoradiographs to serve as an internal control for the amount of RNA loaded onto each well of the gel. The gC probe was then removed, and the membrane was probed with a second ^{32}P-labeled fragment that was specific for β-Gal. The size of the major RNA species detected by the β-Gal probe was approximately 4.3 kilobases (Fig. 3A), which corresponded to the size expected for the β-Gal transcript. The relative amounts of β-Gal mRNA in vgCL8- and vgCL3-infected cells were quantitated by densitometry of the autoradiographs.

The accumulation of β-Gal mRNA in vgCL8- and vgCL3-infected cells was also determined by primer extension (Fig. 3B). After autoradiography of the sequencing gel with the β-Gal primer extension products of vgCL8 and vgCL3 RNA, the relative amounts of β-Gal mRNA were again quantitated by densitometry. The relative steady-state levels of β-Gal mRNA produced in vgCL3- and vgCL8-infected cells reflected the relative β-Gal activity observed (Fig. 1 and 3).

The decrease in β-Gal mRNA accumulation that resulted from the deletion of sequences between +2 and +39 could be due to either a decrease in the rate of mRNA synthesis or a decrease in mRNA stability. To determine the relative stability of the β-Gal mRNA in vgCL3- and vgCL8-infected cells, RNA was isolated 3, 6, and 12 h after the addition of dactinomycin and quantitated by primer extension. The level of β-Gal mRNA in vgCL3- and vgCL8-infected cells gradually diminished in the 12 h after dactinomycin addition, with an estimated half-life of β-Gal mRNA of >10 h (Fig. 4). For comparison, the relative stability of gC mRNA was determined by primer extension analysis with the same RNAs. In both vgCL3- and vgCL8-infected cells, gC mRNA decayed

at similar rates with an estimated half-life similar to that of β-Gal mRNA (data not shown). Thus, the decrease in relative β-Gal mRNA levels in vgCL8- and vgCL3-infected cells cannot be accounted for by a decrease in mRNA stability. Although these results suggest that sequences between +2 and +39 are important for mRNA synthesis, this has not been shown directly. Unfortunately, results from direct measurement of HSV-1 initiation at late times during infection are difficult to interpret (13).

Although the results from recombinant virus construction show the importance of gC leader sequences upstream from +39, the actual role of these sequences in gene expression remains to be resolved. A recent report showed that the immediate-early 175-kilodalton protein (α4) of HSV-1 binds to DNA fragments derived from the 5′ noncoding leader region of two viral late genes (10). It remains to be determined whether the gC leader sequences determined here to be important for gC expression will also bind α4. Inspection of the leader sequences of the three genes did not reveal any obvious similarities.

Other reports have indicated a role for the 5′ leader regions in the expression of HSV-1 genes other than gC. Using transient assays, Blair et al. (1) showed that sequences from −3 to +77 in the leader region of the viral gene VP16 are necessary for virus-induced transcription. Coen et al. (2) observed that a mutation in the +5-to-+15 region of the thymidine kinase gene, when introduced into the viral genome, significantly lowered thymidine kinase mRNA accumulation and transcription rate. Further studies, including mutational analyses, of the 5′ nontranslated region of immediate-early, early, and late HSV-1 genes should lead to a better understanding of the role of this part of the gene in viral gene expression and regulation.

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LITERATURE CITED

1. **Blair, E. D., C. C. Blair, and E. K. Wagner.** 1987. Herpes simplex virus virion stimulatory protein mRNA leader contains sequence elements which increase both virus-induced transcription and mRNA stability. J. Virol. **61**:2499–2508.
2. **Coen, D. M., S. P. Weinheimer, and S. K. McKnight.** 1986. A genetic approach to promoter recognition during trans induction of viral gene expression. Science **234**:53–59.
3. **Dennis, D., and J. R. Smiley.** 1984. Transactivation of a late herpes simplex virus promoter. Mol. Cell. Biol. **4**:544–551.
4. **Frink, R. J., R. Eisenberg, G. Cohen, and E. K. Wagner.** 1983. Detailed analysis of the portion of the herpes simplex virus type 1 genome encoding glycoprotein C. J. Virol. **45**:634–647.
5. **Homa, F. L., J. C. Glorioso, and M. Levine.** 1988. A specific 15-bp TATA box promoter element is required for expression of a herpes simplex virus type 1 late gene. Genes Dev. **2**:40–53.
6. **Homa, F. L., T. M. Otal, J. C. Glorioso, and M. Levine.** 1986. Transcriptional control signals of a herpes simplex virus type 1 late (γ_2) gene lie within bases −34 to +124 relative to the 5′ terminus of the mRNA. Mol. Cell. Biol. **6**:3652–3666.
7. **Kozak, M.** 1986. Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell **44**:283–292.
8. **Kunkel, T. A., J. D. Roberts, and R. A. Zakour.** 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol. **154**:367–382.
9. **Mavromara-Nazos, P., S. Silver, J. Hubenthal-Voss, J. L. C. McKnight, and B. Roizman.** 1966. Regulation of herpes simplex virus 1 genes: gene sequence requirements for transient induction of indicator genes regulated by β or late (γ_2) promoters. Virology **149**:152–164.
10. **Michael, N., D. Spector, P. Mavromara-Nazos, T. M. Kristie, and B. Roizman.** 1988. The DNA-binding properties of the major regulatory protein α4 of herpes simplex virus. Science **239**: 1531–1534.
11. **Shapira, M., F. L. Homa, J. C. Glorioso, and M. Levine.** 1987. Regulation of the herpes simplex virus type 1 late (γ_2) glycoprotein C gene: sequences between base pairs −34 and +29 control transient expression and responsiveness to transactivation by the products of the immediate early (α) 4 and 0 genes. Nucleic Acids Res. **15**:3097–3111.
12. **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.
13. **Weinheimer, S. P., and S. L. McKnight.** 1987. Transcriptional and posttranscriptional controls establish the cascade of herpes simplex virus protein synthesis. J. Mol. Biol. **195**:819–833.
14. **Weir, J. P., and P. R. Narayanan.** 1988. The use of β-galactosidase as a marker gene to define the regulatory sequences of the herpes simplex virus type 1 glycoprotein C gene in recombinant herpesviruses. Nucleic Acids Res. **16**:10267–10282.