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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

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ABSTRACT

The expression of Herpes Simplex Virus 1 (HSV-1) glycoprotein C (gC), a well defined herpesvirus late gene, was studied by linking the promoter-regulatory region of this gene to the coding sequences for the bacterial enzyme, ß-galactosidase (ß-gal). A chimeric gene, containing the ß-gal gene under the control of gC sequences from -1350 to +30 relative to the mRNA start site, was inserted by homologous recombination into the thymidine kinase (TK) locus of the HSV-1 genome. Selection of the TK^- recombinant virus by plaque assay was facilitated by addition of a ß-gal indicator to the agarose overlay. Recombinant virus containing the gC promoter-ß-gal chimeric gene faithfully expressed ß-gal as a viral late gene, as shown by the absence of ß-gal expression when viral DNA replication was inhibited with phosphonoacetic acid. In contrast, the inhibition of viral DNA replication had no effect on the expression of ß-gal when the ß-gal gene was under the control of the early HSV-1 TK promoter in a separate recombinant virus. Analysis of recombinant viruses containing 5′ to 3′ deletions in the gC regulatory region revealed no apparent difference in ß-gal expression as deletions extended from -1350 to -109 base-pairs (bp) before the RNA start site, demonstrating that sequences between -109 and +30 are sufficient for regulated gC expression in the viral genome. Analysis of the mRNA made by these recombinant viruses confirmed the results of the ß-gal assays, and demonstrated that the transcriptional start sites of the gC promoter-ß-gal chimeric genes were the same as the start site of the gC gene.

INTRODUCTION

Gene regulation in Herpes Simplex Virus 1 (HSV-1) is a well coordinated temporal process. Viral genes are transcribed in the nucleus of the infected cell by cellular RNA polymerase II and are generally grouped into three classes according to their kinetics of expression and requirements for synthesis; immediate-early (IE), early , and late genes (1). However, viral gene expression is more complex than this simple classification, since not all early genes are expressed at the same time, and the expression of some late genes is detectable at early times even though their maximal expression occurs late in infection (2).

The first genes to be expressed after HSV-1 infection are the IE genes, and

transcription of their mRNAs does not depend upon prior viral protein synthesis (3, 4). There are five known IE genes and the DNA sequences that regulate their expression have been studied in detail (5, 6). An essential promoter element of IE genes is located close to the mRNA start site, probably within 110 base-pairs (bp) (5). An additional regulatory region that responds to a viral trans-acting factor (7, 8) is located far upstream from the start of IE mRNAs (9), and appears to function as an enhancer (10). The expression of the second class of HSV-1 genes, the early genes, is dependent upon the prior synthesis of IE gene products. The DNA sequence elements required for regulated expression of early genes have been identified using the early genes that code for thymidine kinase (TK) and glycoprotein D (gD). Sequences necessary for expression of gD lie within 83 bp upstream of the mRNA start site (11). For the TK gene, upstream sequences to -105 were shown necessary for constitutive expression when the TK gene was microinjected into oocytes (12, 13). In addition, these same sequences were necessary for viral activation of the TK promoter (14, 15).

The third class of HSV-1 genes, the late genes, requires prior viral DNA replication for their full expression. Most of the late proteins identified to date appear to be virion constituents, and late proteins make up approximately half of the known HSV-1 gene products (16). Some viral late genes, such as that coding for the capsid protein VP5 (17), are expressed in the absence of DNA replication but require DNA synthesis for maximal expression (2). Other late genes, such as the one coding for glycoprotein C (gC) (18), stringently require DNA synthesis for their expression.

The mechanisms that regulate late gene expression are not well understood, but studies with temperature-sensitive (ts) mutants indicate that the IE 175K and IE 63K polypeptides are necessary for late gene expression (19, 20). Moreover, the major HSV-1 DNA binding protein may have a role in late gene expression in addition to its role in DNA replication (21). Very little is known about the DNA sequences that are important for late gene expression. An investigation of the VP5 promoter indicated that no more than 125 bp of upstream sequences were necessary for maximal promoter activity (22, 23). A recent compilation of late HSV-1 promoter sequences has revealed very little in common besides the presence of TATA and CAT sequence elements similar to those found in the upstream regions of most eukaryotic genes (24).

In order to identify sequence elements that are important in the regulation of late HSV-1 gene expression, we have linked the promoter-regulatory region of a model late HSV-1 gene, the gC gene, to the coding sequences for the

bacterial enzyme a ß-galactosidase (ß-gal), and have examined the expression of ß-gal from this chimeric gene after insertion into the viral genome. The use of ß-gal as a marker gene facilitates the identification of recombinant viruses and provides a sensitive, quantitative measure of gene expression from the gC promoter-regulatory region. In addition, we show that this approach is useful for the analysis of other viral promoters by construction of a TK promoter-ß-gal recombinant virus. By construction of sequential 5′ to 3′ deletions in the sequences upstream from the gC mRNA start site, we find that sequences between -109 and +30, relative to the start site will regulate ß-gal as a late HSV-1 gene. While this work was in progress, Homa *et al.* (25) reported that the transcriptional control signals of the gC gene lie between -34 and +124 relative to the mRNA 5′ terminus, and that a 15-bp TATA box promoter element is absolutely required for gC expression (26). Taken together, these results seem to indicate that only a very short sequence upstream and downstream of the gC mRNA start site is absolutely essential for gC expression.

(A preliminary report of this work was presented at the 11th Herpesvirus Workshop, Leeds, United Kingdom, July 1986.)

MATERIALS AND METHODS

Cells and viruses

HSV-1 strain F was obtained from the American Type Tissue Culture Collection, Rockville, Maryland, and was grown and titered in Vero cells. Recombinant viruses expressing ß-gal were isolated by plaque assay on TK^- 143B cells using an overlay containing 1% low-melting agarose and 2.5 µg/ml 5-bromodeoxyuridine (BuDR). A second overlay was added approximately 48 hours later containing 0.04% Bluo-gal (Bethesda Research Labs). The recombinant virus vGal was isolated by picking individual TK^- plaques and screening for the presence of ß-gal DNA.

DNA procedures

HSV-1 DNA was isolated from Vero cells infected with HSV-1 at a multiplicity of 0.001 plaque-forming unit (pfu) per cell using the procedure of Denniston *et al.* (27). Plasmid DNAs were isolated by the method of Birnboim and Doly (28) and were further purified by banding in cesium chloride/ethidium bromide gradients. Routine DNA manipulations were similar to those described by Maniatis *et al.* (29). Oligonucleotides were made with an Applied Biosystems 380B DNA synthesizer, and plasmid sequencing was performed as described by Zagursky (30).

Reverse transcriptase was from Seikagaku America, Inc. RNasin was from Promega Biotec.

Construction of recombinant viruses

Recombinant viruses were prepared by transfecting Vero cells with calcium phosphate precipitated DNA (31). Approximately 1 μg of HSV-1 (F) DNA was precipitated with 2-5 μg of plasmid DNA in 1 ml of transfection buffer [0.1% dextrose, 0.14 M NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 20 mM HEPES (pH 7.05)] by the addition of 50 μl of 2.5 M $CaCl_2$, added to 3 x 10^6 cells, and glycerol shocked (32) four hours later. The medium was changed at 48 hours, and the cells and medium collected by scraping at 96 hours. After freeze-thawing three times, the transfected cell extract was plaque-assayed as described above.

Construction of plasmids

Plasmid pTI (obtained from E. Wagner, University of California, Irvine) contains a 3.3 kb DNA fragment of HSV-1 (strain KOS), from a Sal I site (map unit 0.621), approximately 1,350 bp upstream from the start site of transcription, to a Bam HI site (0.643) 380 bp downstream from the end of gC translation (18). The 3.3kb Sal I-Bam HI fragment was inserted into the phage vector M13mp19. The sequence downstream from the reported gC mRNA start site was changed by oligonucleotide mutagenesis of single-stranded phage DNA to generate a new Bam HI site at +30 relative to the mRNA start site (+1 is the first nucleotide of the gC transcript as determined by primer extension analysis). The 1380 bp Sal I-Bam HI fragment containing the mRNA start site, approximately 1.35 kb of upstream sequences, and 30 bp of downstream sequences was isolated and cloned into the plasmid vector pUC18 and designated pgCP. A 3.2 kb fragment of DNA containing the *E. coli* ß-gal gene with translational initiation and termination codons was obtained from the plasmid pPW61 (provided by A. Majundar, National Institutes of Health, Bethesda, Maryland) and cloned downstream of the gC promoter sequences to give pgCP-Gal1. The HSV-1 TK gene (strain F), contained in a 3.6 kb Bam HI clone (33) designated as pRB103 (a gift from B. Roizman, University of Chicago), contains a single Sac I site in its coding region (Fig. 1A). This Sac I site is 443 bp downstream from the start of translation and 684 bp upstream from the end of translation. The DNA fragment containing the gC promoter-ß-gal gene from pgCP-Gal1 was cloned as a blunt fragment into the Sac I site of the TK gene which had been made blunt with T4 polymerase such that the gC-ß-gal chimeric gene ran in the same direction as the TK gene. This plasmid, pgCP-Gal5, was used to make the recombinant virus vgCP-Gal (Fig. 1B). A control plasmid lacking gC promoter sequences was made in the same way and called pGa15. The recombinant virus

made from this plasmid was vGal (Fig. 1B). A single Bgl II site in pRB103 lies in the 5′ non-translated leader sequence of the TK gene (Fig. 1A), approximately 50 bp downstream from the TK mRNA start site and 58 bp upstream from the start of translation (34). The ß-gal gene from pPW61 was cloned into the TK gene using the Bgl II and Sac I sites, eliminating the 5′ coding sequences of the TK gene, such that the ß-gal gene was under the control of the TK promoter. This plasmid, pTK-Gal, was used to make the recombinant virus vTK-Gal (Fig. 1B).

To generate 5′-3′deletions in the gC promoter sequences, the unidirectional digestion method using Exonuclease III and nuclease S1, as described by Henikoff (35), was employed. Digestion proceeded from the Sal I site at -1350 of pgCP-Gal5 toward the start of gC transcription generating a set of deletions in the upstream gC sequence. Digestion in the opposite direction was prevented by prior digestion with Pst I, which cleaves at a site adjacent to the Sal I site but leaves a 4 base 3′ overhang that cannot be utilized by Exonuclease III. After Exonuclease III and S1 digestion, Sal I linkers were added and the size of the deletions in the resulting plasmids were determined by restriction enzyme analysis. This procedure resulted in plasmids that left approximately 700, 400, 250, and 109 bp of upstream gC sequences.

ß-Galactosidase assays

Vero cells were infected with virus at a multiplicity of 10 pfu/cell and collected by scraping at the indicated times. The cell extracts were freeze-thawed three times and cleared of cell debris by centrifugation. Aliquots of extracts were diluted in phosphate buffered saline and assayed for ß-gal activity as described by Miller (36). Activity was expressed as units of ß-gal per 3 x 10^6 cells. In some experiments, phosphonoacetic acid was added to a final concentration of 300 μg/ml to inhibit viral DNA replication.

Isolation of RNA and primer extension assays

RNA was isolated from approximately 6 x 10^7 Vero cells that had been infected with virus at a multiplicity of 10 pfu/cell. At 24 hours post-infection, cells were scraped, collected by centrifugation, lysed in 5 volumes of guanidinium isothiocyanate solution (29, 37), and the RNA was pelleted through a cushion of 5.7 M CsCl for 16 hours. The RNA pellet was resuspended in water, phenol/chloroform extracted one time, and precipitated with ethanol.

For primer extension experiments, 30 μg of cytoplasmic RNA was hybridized with 2 pmoles of oligonucleotide primer that had been end-labeled with polynucleotide kinase. Hybridization was for 16 hours at 25°C in 80% formamide (29, 38). Following EtOH precipitation, primer-RNA hybrids were taken up in

20 μl of primer extension buffer [50 mM Tris-HCl (pH 8.25), 40 mM KCl, 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.05 mM of each dNTP, 21.5 units reverse transcriptase, and 30 units RNasin] and extended for 2 hours at 42°C before being denatured and run on a 6% sequencing gel.

RESULTS

Construction of recombinant viruses

Homologous recombination has been used previously for the insertion of foreign genes into HSV-1 (39). The technique utilizes the fact that HSV-1 DNA

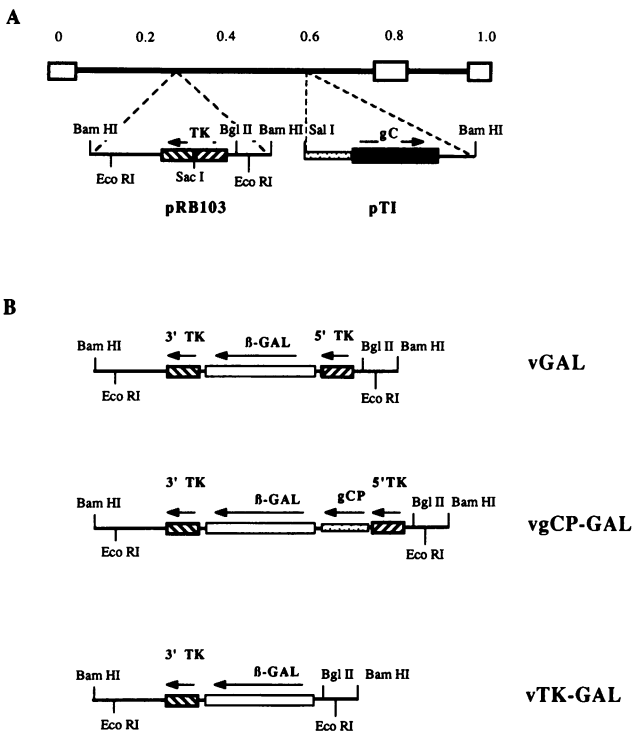

Fig. 1. Construction of recombinant HSV-1 viruses containing the ß-galactosidase gene. A. Sequence arrangement of the prototype HSV-1 genome showing the location and orientation of the thymidine kinase (TK) and glycoprotein C (gC) genes. The coding sequences of these genes are indicated by the large boxes; (▨) for TK and (■) for gC. Restriction sites that were used for cloning and analysis of the construction are indicated. B. Location and arrangements of the ß-gal gene in recombinant viruses vGal, vgCP-Gal, and vTK-Gal. Coding sequences for ß-gal are indicated as a large open box (▭). The promoter-regulatory region of gC is a smaller box (⁘). Promoter sequences for TK are upstream of the TK coding sequences. The Bgl II site that is shown is in the 5′ non-translated leader sequence of the TK mRNA.

is infectious and can be co-transfected into cells as a calcium phosphate precipitate along with another HSV-1 fragment containing a detectable genetic marker; through homologous recombination, progeny virus containing the marker gene will be generated.

In order to study the expression of the late viral gene encoding glycoprotein C, we linked the promoter-regulatory region of the gC gene to the coding sequences for the bacterial enzyme ß-galactosidase (described in Materials and Methods). A detailed analysis of the portion of the HSV-1 genome that contains the glycoprotein C gene has been reported previously (18), including the sequence of the gC coding region, the location of the mRNA start site and 120 bp of sequences upstream from it. The sequences that we used to construct the chimeric gC-ß-gal gene extended from approximately 1,350 bp upstream of the gC mRNA start site to 30 bp downstream. The chimeric gC-ß-gal gene was inserted into the HSV-1 thymidine kinase (TK) gene (Fig. 1A) to direct homologous recombination into the viral genome. To generate the recombinant virus vgCP-Gal (Fig. 1B), the plasmid containing the chimeric gC-ß-gal gene inserted into the TK gene was co-transfected along with HSV-1 wild-type DNA into Vero cells. Infectious virus from the transfection was plaque-assayed under condi-

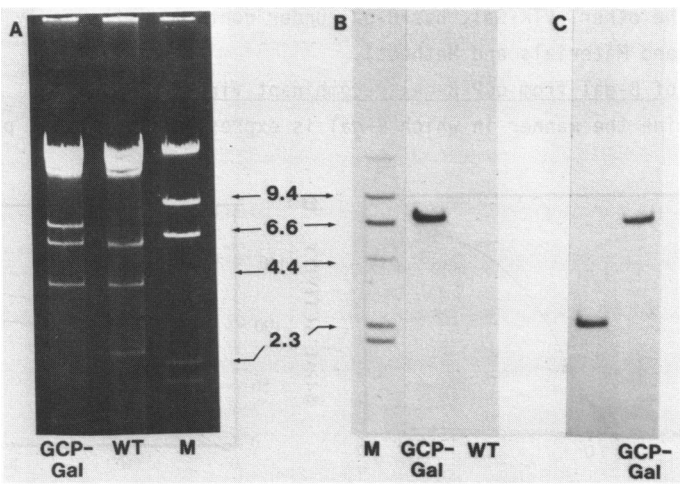

Fig. 2. Structural analysis of vgCP-Gal genomic DNA. DNA was extracted from wild-type (WT) and vgCP-Gal viruses, digested with Eco RI endonuclease and the fragments were separated on a 0.8% agarose gel. A. Staining of the gel with ethidium bromide before transfer to nitrocellulose. B. Nitrocellulose filter hybridized to nick-translated ß-gal DNA fragment. C. A duplicate transfer hybridized to nick-translated TK DNA. Molecular weight size markers, in kilobase pairs, are indicated (M).

tions which select for TK$^-$ virus that expressed ß-galactosidase. Selected plaques were purified at least two more times under the same conditions.

The construction of the recombinant virus was verified by restriction analysis of the DNA followed by Southern blotting as shown in Fig. 2. The TK gene from the F strain of HSV-1 is entirely contained in a 2.4 kb Eco RI fragment (Fig. 1A), and, since there are no Eco RI sites in the gC promoter or the ß-gal gene, this enzyme was used to digest DNA from the wild-type and recombinant viruses. The only apparent difference between the two digests was the disappearance of the 2.4 kb band in the recombinant digest and the appearance of a new 6.8 kb band that would be consistent with the insertion of the 1.4 kb gC promoter and the 3.0 kb ß-gal gene (Fig. 2A). The restriction enzyme digested DNA from the two viruses was transferred onto nitrocellulose and probed with either nick-translated ß-gal DNA or TK DNA. The ß-gal probe hybridized to the 6.8 kb band in the recombinant digest and to no other fragments in either digest (Figure 2B). The TK probe hybridized to the 2.4 kb band in the wild-type digest and to the 6.8 kb band in the recombinant, thus confirming the expected DNA structure (Fig. 2C).

Two other recombinant viruses were constructed in a similar manner for comparison with vgCP-Gal. One, vGal, has no gC sequences upstream from the ß-gal gene, and the other, vTK-Gal, has ß-gal under control of the early TK promoter (Figure 1B and Materials and Methods).

Expression of ß-gal from gCP-ß-gal recombinant virus

To determine the manner in which ß-gal is expressed from the gC promoter in

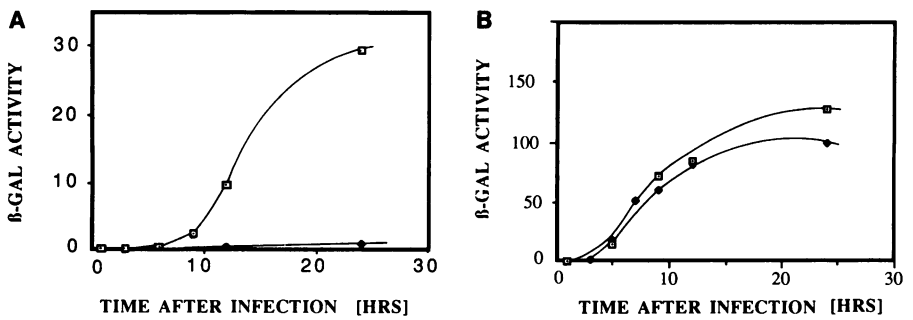

Fig. 3. Time course of ß-gal expression. A. Approximately 3 x 10^6 Vero cells were infected with vgCP-Gal at a multiplicity of 10 pfu per cell in the presence (◆) or absence (⊡) of 300 μg/ml of phosphonoacetic acid. The cells were harvested at the indicated times and assayed for galactosidase activity as described in Materials and Methods. B. Expression of ß-gal from cells infected with vTK-Gal in the presence (◆) or absence (⊡) of phosphonoacetic acid.

vgCP-Gal, we infected Vero cells with the recombinant virus and measured the level of ß-gal activity at different times after infection. As shown in Fig. 3A, ß-gal was expressed by vgCP-Gal under conditions that permit viral DNA replication, becoming detectable at approximately 6 hours after infection and continuing for at least 24 hours. When viral DNA replication was inhibited with phosphonoacetic acid (PAA), the expression of ß-gal was barely detectable even at 24 hours, indicating that this gene was regulated as an authentic HSV-1 late gene. No ß-gal activity was detectable in an infection with wild-type virus (data not shown).

To show that the regulation of ß-gal as a late gene is due to the gC promoter sequences and not an effect of the ß-gal gene itself, Vero cells were infected with vTK-gal and examined for ß-gal activity. Expression of ß-gal from the TK promoter could be observed as early as 4 hours after infection and was not affected when viral DNA replication was inhibited (Figure 3B). In addition, the level of galactosidase activity was much higher from vTK-Gal than vgCP-Gal at all time points examined.

Analysis of the 5′ ends of the gC and gCP-ß-gal mRNAs

Although the gC promoter-ß-gal chimeric gene appeared to be regulated as a viral late gene, we wanted to know if the start site of transcription is the same as that of the authentic gC gene. The start sites of both of these mRNAs were determined by primer extension analysis. For this purpose, an oligonucleotide of 25 bases, complementary to the 5′ region of the gC gene (+25 to +49, relative to the start of translation), was hybridized to total cytoplasmic RNA isolated from vgCP-Gal infected cells and extended with reverse transcriptase. This same oligonucleotide was used as a primer for sequencing plasmid DNA from pTI. Figure 4A shows the primer extension analysis of the corresponding sequence analysis run on the same sequencing gel. A major band representing the start of the gC mRNA migrated alongside a G residue in the sequence ladder, and the complementary C residue was designated as +1 on the gC sequence (Fig. 4C). A similar analysis was done using a primer that is complementary to the 5′ end of the ß-gal gene (+60 to +74, relative to the ATG that initiates translation). When this primer extension product was run alongside a sequence ladder prepared using the same primer and plasmid DNA containing the gCP-ß-gal gene, it was apparent that the mRNA start site for the chimeric gene was identical to that used by the gC gene (Fig. 4B).

These experiments show that the ß-galactosidase gene is a suitable marker gene for studies of HSV-1 promoter and regulatory sequences. In addition, the region of the gC gene used in these experiments is sufficient for regulated

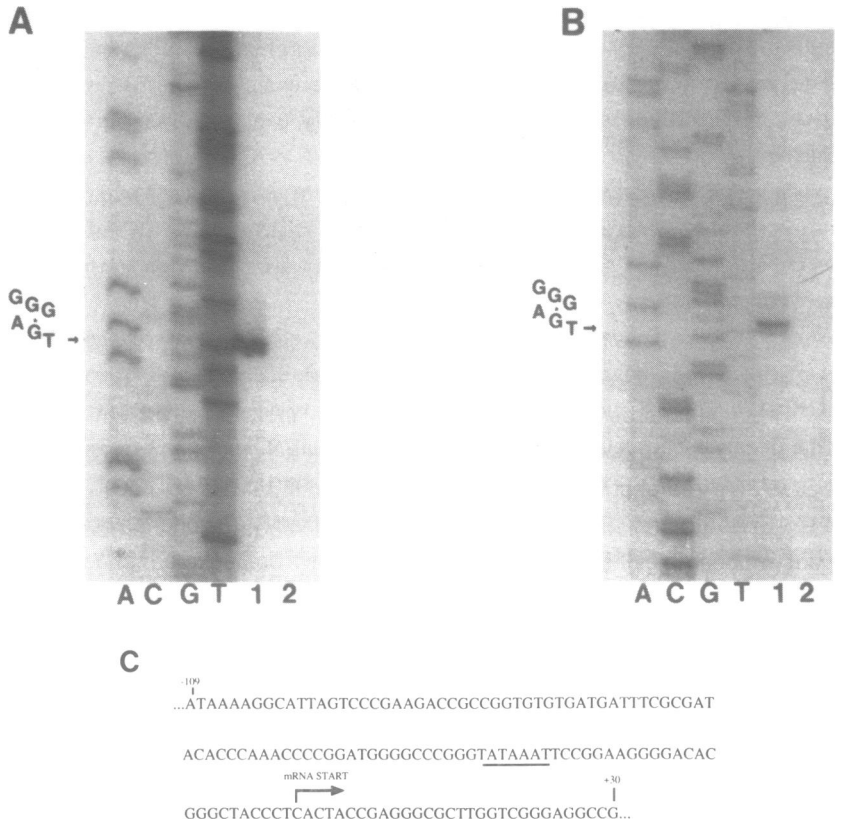

Fig. 4. Analysis of the 5′ ends of gC and ß-gal mRNAs from vgCP-Gal infected cells. A. Primer extension assay using total infected cell RNA (lane 1) or tRNA (lane 2) and the primer that is complementary to the 5′ end of the gC mRNA. Dideoxy sequencing reactions using the same primer and plasmid DNA, pTI, containing the gC gene are shown on the left. B. Primer extension assay using RNA from vgCP-Gal infected cells (lane 1) or tRNA (lane 2) and the primer that is complementary to the 5′ end of the ß-gal mRNA. A sequence ladder prepared using this primer and plasmid DNA containing the gC promoter-ß-gal gene is run alongside for comparison. C. Sequences around the start site of the gC mRNA, including the mapped start site (shown by arrow) and the sequences upstream and downstream of the start site that were used in the viral constructions.

late gene expression and can be used to further define such regulatory signals.

Deletions in the 5′ gC regulatory region

To define DNA sequences in the gC fragment that are important for regulation of ß-gal as a late gene in the recombinant virus, a series of 5′ to 3′ dele-

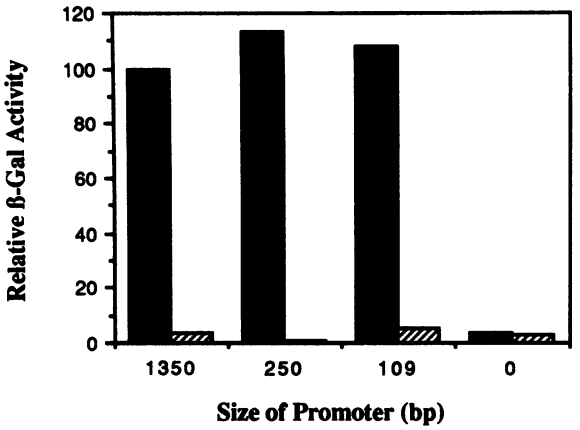

Fig. 5. Expression of ß-galactosidase by recombinant viruses. Vero cells were infected in the presence (▨) or absence (■) of phosphonoacetic acid with recombinant viruses containing deletions in the gC promoter region. The galactosidase activity was measured at 24 hrs post-infection and expressed relative to that of vgCP-Gal. The ß-gal activity expressed by vGal is shown as 0 size promoter.

tions was made from the Sal I site at -1350 of the gC promoter-regulatory region of the plasmid pgCP-Gal5 (Materials and Methods). Deletions were obtained that left approximately 700, 400, 250, and 109 bp of gC sequences upstream from the mRNA start site as determined by restriction enzyme analysis. The deletion to -109 was determined by sequence analysis.

Recombinant viruses were made as described above that contained deletions in the gC 5′ regulatory region to -250 and -109 from the mRNA start site and were designated vgCPΔ250-Gal and vgCPΔ109-Gal, respectively. Vero cells were infected with each of these viruses in the presence and absence of PAA, and the expression of ß-gal was determined at 24 hours post-infection (Fig. 5). In the absence of PAA, there was no diminution of ß-gal expression when the deletions extended from -1350 to -109 bp, but deletion of all gC sequences reduced the level of ß-gal expression to background levels. Determination of ß-gal activity in cells infected in the presence of PAA indicated that each recombinant virus that expressed ß-gal required viral DNA replication. Time course experiments indicated that deletions to -109 had no effect on the temporal expression of ß-gal (data not shown), and there was no expression of ß-gal from vGal at any time after infection.

Analysis of the 5′ ends of mRNA

In order to determine whether the deletion of upstream gC sequences resulted

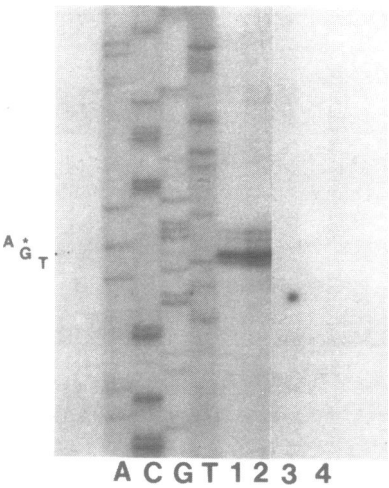

Fig. 6. Analysis of the 5′ ends of ß-gal mRNAs made in cells infected with promoter deletion viruses. Primer extension assays were done as described in Fig. 4 using the ß-gal primer and total RNA from cells infected with vgCP-Gal (lane 1), vgCPΔ109-Gal (lane 2), vGal (lane 3), or tRNA (lane 4). Dideoxy sequencing reactions using the same primer and plasmid DNA containing the gC promoter-ß-gal gene is run alongside for comparison.

in any alterations at the start of mRNA transcription, the 5′ ends of RNA synthesized in cells infected with recombinant viruses were analyzed by primer extension experiments (Fig. 6). RNA was isolated from cells infected with either vgCP-Gal, vgCPΔ109-Gal, or vGal, hybridized to the primer that is complementary to the 5′ end of the ß-gal gene, and extended with reverse transcriptase. Extension products were observed using the RNA from vgCPΔ109-Gal at the same position as that from vgCP-Gal. Extension products were not detected using RNA isolated from vGal.

DISCUSSION

The present study was undertaken to determine the DNA sequences in the 5′ flanking region of a herpesvirus late gene, gC, that are important for regulated expression. A region of DNA containing the reported start site of the gC mRNA, the 30 bp immediately downstream, and approximately 1350 bp upstream from the start site, was isolated and linked to a marker gene, the coding sequences for the bacterial enzyme ß-galactosidase.

In preliminary experiments, we examined the expression of ß-gal from this chimeric gene in transient expression assays. When Vero cells were trans-

fected with plasmids containing the chimeric gene, expression of ß-gal was directed by gC sequences, and expression was dependent upon concomitant HSV-1 infection. However, expression was not dependent upon viral DNA replication. Further, expression was also observed if, instead of virus infection, the 110K, 175K, and 63K immediate early-genes were co-transfected with the chimeric gene (J.P.W., unpublished results). Thus, the regulation of ß-gal by the gC promoter seemed to be like that of an early viral gene. The observation that herpesvirus late genes not resident in the viral genome no longer require viral DNA replication for their expression has been made previously (40, 41, 42, 43), and, for this reason, further analysis of sequences involved in late regulation was performed using recombinant viruses.

To construct a recombinant HSV-1 virus containing the a ß-gal gene under control of the gC promoter-regulatory region, we inserted the chimeric gene into the viral genome by means of homologous recombination. The choice of ß-gal as a marker gene was partially due to its ease of assay and quantitation, as demonstration previously in transient expression studies of cytomegalovirus gene expression (44). More importantly, it also facilitated the isolation of recombinant virus plaques using Bluo-gal as a color indicator. This approach has been used previously in the construction of recombinant vaccinia viruses (45) and should also be useful for isolating recombinant viral genomes with insertions into any non-essential region of the virus, not only the TK locus. Such an approach was recently used to show that HSV-1 ribonucleotide reductase is not essential for viral growth in cultured cells (46).

The recombinant virus, vgCP-Gal, expressed ß-gal as a viral late gene under the control of the gC promoter-regulatory region. When viral DNA replication was inhibited, ß-gal expression was reduced to background levels. Furthermore, if gC regulatory sequences were not present, there was no expression of ß-gal. This recombinant virus, vGal, has the complete ß-gal gene inserted into the coding sequence of the TK gene at the same position as in vgCP-Gal, 445 bp downstream from the start of TK translation.

Primer extension analysis of RNA from vgCP-Gal infected cells showed an mRNA start site for the chimeric gene that was identical in position to that identified for the authentic gC gene. Analogous start sites were not seen using RNA from vGal infected cells. Although the TK promoter is upstream of the ß-gal gene in both virus constructs, and presumably still directs synthesis of an mRNA, ß-gal would not be produced from such an mRNA unless the ß-gal coding sequences were in-frame with the 5′ TK coding sequences. This is not possible in the case of vgCP-Gal because of the gC sequences between the

TK and ß-gal coding sequences. In the case of vGal, sequence analysis showed that the ß-gal gene is not in-frame with the 5′ TK coding sequences (data not shown). Alternatively, the upstream TK promoter could affect ß-gal expression if initiation, or reinitiation, of translation occurred at the ATG that begins the ß-gal coding sequence on the mRNA produced from the upstream TK promoter. The lack of expression of ß-gal from vGal demonstrates that this does not occur, and therefore, is highly unlikely to occur with vgCP-Gal.

Another recombinant virus, vTK-Gal, served as a useful comparison to vgCP-Gal. Expression of ß-gal by this virus was independent of viral DNA replication. Although this would be expected from the early TK promoter, it shows that the ß-gal gene, when inserted into the HSV-1 genome, is not restricted to late expression and that this approach should be useful for investigating other HSV-1 promoters. Interestingly, the level of ß-gal expression from the TK promoter construct is 3 to 4-fold higher than that from the gC promoter construct. If the amount of gC made in an HSV-1 infected cell is actually greater than that of TK, there could be several possible explanations. First, even though the information necessary for regulated expression of gC resides between -109 and +30, other sequence elements, either further upstream or downstream, might quantitatively affect the level of gC expression. Alternatively, the stability of the gC-ß-gal mRNA may be different from that of the authentic gC mRNA. A third possible explanation is that removal of TK sequences in the 5′ untranslated region of the gene from +51 to +107 leads to increased expression in vTK-Gal. Experiments are currently underway to test these possibilities.

Taken together, expression and mRNA analysis demonstrate that the recombinant virus, vgCP-Gal, is an appropriate construct to study late gene regulation in HSV-1. This construction was the starting point for making a series of 5′ to 3′ deletions in the gC regulatory region. There was no effect on either the magnitude of expression or the requirement for viral DNA replication as deletions were extended to within 109 bp of the mRNA start site. Elimination of the entire gC promoter reduced ß-gal expression to background levels. These results were corroborated by primer extension analysis using RNA isolated from cells infected with the recombinant viruses. All of the viruses that expressed appreciable levels of ß-gal synthesized a detectable mRNA with the same start site as the authentic gC mRNA.

There have been few other reports to date that have investigated late gene regulatory sequences in HSV-1. One report showed that no more than 125 bp of upstream sequence were necessary for maximal activity of the viral gene VP5

(22). Although this gene is expressed late during HSV-1 infection, expression is detected at early times after infection and is not stringently dependent upon viral DNA replication. Recently, Homa *et al.* (25), using a different methodology, reported that in recombinant viruses, sequences between -34 and +124 were sufficient for accurate expression of gC as a late viral gene. Further, they have shown that a specific 15-bp TATA box promoter element is absolutely required for gC expression (26). These results, taken together with the ones presented in this report, indicate that sequences required for late gene regulation in HSV-1 may reside in short regions both upstream and downstream of the mRNA start site. Thorough mutational analysis of this region should lead to a better understanding of the DNA sequence elements involved in herpesvirus gene expression, and the factors that interact with them.

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