JOURNAL OF VIROLOGY, Dec. 1991, p. 6454–6460
0022-538X/91/126454-07$02.00/0

Vol. 65, No. 12

Mutational Analysis of Two Herpes Simplex Virus Type 1 Late Promoters

KEVIN R. STEFFY AND JERRY P. WEIR†*

Department of Microbiology, University of Tennessee, Knoxville, Tennessee 37996

Received 1 April 1991/Accepted 19 August 1991

To investigate the *cis*-acting sequence elements that are involved in the regulation of herpes simplex virus type 1 late gene expression, linker-scanning mutations were constructed in the promoters of the glycoprotein C and glycoprotein H genes. Each promoter mutation was inserted upstream of the *Escherichia coli lacZ* gene in a recombinant virus, and the relative activities of β-galactosidase expressed from individual recombinant viruses were compared. This analysis identified three sequence elements in each promoter: a TATA element, an element that overlapped the start of transcription, and an element downstream from the start of transcription. Primer extension analysis confirmed these results and showed that mutations in either the TATA element or the initiation sequence could eliminate normal transcription initiation. Analysis of expression from hybrid promoters revealed that the TATA and the initiation elements were interchangeable, at least when correctly aligned, and that the initiation element plays a pivotal role in determining the actual site of transcription initiation.

Expression of the approximately 70 herpes simplex virus type 1 (HSV-1) genes is regulated primarily at the level of transcription (3, 18) and can be conveniently divided into three phases based upon the temporal order of mRNA synthesis (6). Five immediate-early (IE) genes are expressed soon after infection in the absence of de novo protein synthesis. Functional IE gene products are required for expression of the second set of genes, the early genes, many of whose gene products are involved in viral DNA replication. The expression of the third phase, late genes, requires both viral DNA replication and functional IE proteins.

The *cis*-acting sequences that govern late HSV-1 gene expression have not been clearly defined. A TATA element is the only consensus sequence element identified in the upstream regulatory region of late viral promoters (18), and deletion studies have indicated that little sequence information upstream from this element is required for efficient expression (4, 5). Several studies have indicated the importance of sequences downstream from the TATA element, although no consensus sequence elements have been identified (9, 11, 21). A recent study, for example, showed that a hybrid promoter consisting of the TATA element and upstream sequences from the early thymidine kinase gene, fused to sequences downstream from the TATA element of a late promoter, had properties of both early and late viral promoters (9).

To further define the promoter elements that are in common among HSV-1 late promoters, we have constructed linker-scanning mutations in the promoters of two HSV-1 late genes, those that code for glycoprotein C (gC) and glycoprotein H (gH). Further, by construction of hybrid gC/gH promoters, we have evaluated the ability of the identified sequence elements of these promoters to functionally substitute for one another.

* Corresponding author.

† Present address: Department of Cellular Immunology, Walter Reed Army Institute of Research, 9620 Medical Center Drive, Rockville, MD 20850.

MATERIALS AND METHODS

Cells and viruses. HSV-1(F) was grown and titered in Vero cells. Recombinant herpesviruses were isolated by plaque assay on thymidine kinase-deficient 143B cells as previously described (20, 21). To quantitate β-galactosidase (β-Gal) activity, approximately 3×10^6 Vero cells were infected with virus at a multiplicity of 10 in the presence or absence of 300 μg of phosphonoacetic acid per ml. At 24 h postinfection, the cells were collected by scraping and centrifugation and were assayed for β-Gal as described previously (12, 20). Values reported are the mean of two to five separate experiments.

DNA procedures. Routine procedures were similar to those described by Ausubel et al. (1). Oligonucleotides were made with an Applied Biosystems 380B DNA synthesizer, and plasmid DNA sequencing was done essentially as described by Zagursky et al. (23). Primer extension assays were as previously described (20), using a primer complementary either to +15 to +35 of the *lacZ* coding sequences or to +25 to +49 of the gC coding sequences.

Construction of plasmids. The recombinant viruses vgCL5 and vgHL.1 and their plasmid parents pgCL5 and pgHL.1, respectively, have been described previously (21, 22). To construct the gC linker-scanning mutations, the *Hin*dIII-*Bam*HI fragment from pgCL5, containing gC promoter sequences from −114 to +71, was cloned into the phage vector M13mp18. Oligonucleotide site-directed mutagenesis was performed as described by Kunkel et al. (8) to insert the indicated *Sal*I recognition sites in the promoter. Mutations were verified by sequence analysis and reinserted into pGal8 as *Hin*dIII-*Bam*HI fragments. Plasmid constructions were also verified by sequencing before being used to construct recombinant viruses. To construct the gH linker-scanning mutations, the *Sph*I-*Bam*HI fragment from pgHL.1, containing gH promoter sequences from −83 to +198, was cloned into mp18 and used as the template for oligonucleotide mutagenesis.

Plasmid pgHC.1 was constructed by removal of the gH promoter sequences from −83 to +13 in pgHL4.4, using the *Hin*dIII site in the vector and the *Sal*I site at +13, and by replacement of the corresponding *Hin*dIII-*Sal*I fragment from pgCP27. Similarly, to generate pgHC.3, the gH pro-

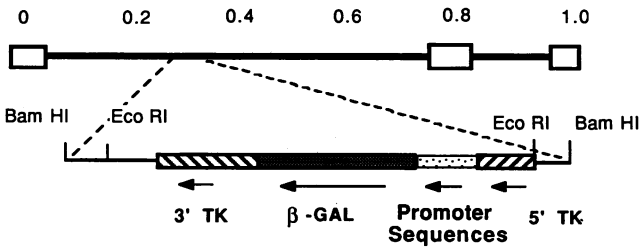

FIG. 1. Structure of recombinant viruses. The top line indicates the HSV-1 genome with approximate map units. The open boxes represent the terminal and internal repeat sequences. The expanded region shows the location and orientation of the HSV-1 thymidine kinase (TK) coding sequences (▧). The coding sequences for β-Gal (▩) and promoter sequences from either the gC or the gH gene (□) are depicted. The promoter sequences and the mutations are described in the text and in Fig. 2 and 4.

moter sequences from −83 to −12 were removed from pgHL4.1 and replaced by gC sequences −114 to −15 derived from pgCP28. Plasmids pgHC.2 and pgHC.4 were constructed by removal of gC promoter sequences (*Hin*dIII-*Sal*I) from pgCP27 and pgCP28, followed by insertion of the corresponding gH fragments from pgHL4.4 and pgHL4.1, respectively.

All recombinant viruses were made by cotransfection of HSV-1 virion DNA and the appropriate plasmid DNA into Vero cells as previously described (20). The general structure of the recombinant viruses is depicted in Fig. 1.

Nucleotide sequence accession numbers. The nucleotide sequence data reported in this paper have been submitted to GenBank and assigned accession numbers M74723 to M74737 for the gC promoter mutations, M74738 to M74741 for the hybrid gH/gC promoters, and M74742 to M74749 for the gH promoter mutations. Nucleotide −45 of the gC promoter in this paper corresponds to nucleotide 96128 of the published U_L sequence (10); nucleotide −37 of the gH promoter corresponds to nucleotide 46618 of that sequence.

RESULTS

Sequence elements of the gC promoter. Previous studies using recombinant viruses have shown that the gC promoter requires a TATA sequence element in the region upstream from the start of transcription (4, 5) as well as sequences downstream from the start of transcription to +38 for optimal expression (21). To identify sequence elements that constitute the HSV-1 gC promoter, a set of linker-scanning mutations was constructed. These mutations changed between two and four bases in separate six-base stretches spanning the gC promoter from −41 to +41 (Fig. 2). Recombinant viruses were made from each linker-scanning mutant as previously described (20). Figure 1 shows the structure of the recombinant viruses with the mutated promoters directing synthesis of the β-Gal gene within the coding sequences of the viral thymidine kinase gene. Expression of *lacZ* was compared with that from the unmutated gC promoter sequences −114 to +71 (vgCL5 [21]). One mutation at the 3′ end of the TATA element lowered β-Gal activity by 41% (vgCP35). Two mutations near the start of transcription, vgCP30 and vgCP24, reduced expression 71 and 74%, respectively. Two other mutations, vgCP20 and vgCP33, that were downstream from the start of transcription lowered expression by 41 and 33%, respectively. No appreciable expression of β-Gal was observed when cells were infected with any of the recombinant viruses under conditions in which viral DNA replication was inhibited with phosphonoacetic acid (data not shown). Thus, this set of linker-scanning mutations implicates three separate sequence elements that are important for maximal expression from the HSV-1 gC promoter.

To further characterize the gC promoter linker-scanning mutations, RNA was isolated from cells infected with the recombinant viruses vgCL5, vgCP35, vgCP30, vgCP24, vgCP20, and vgCP33 and were analyzed by primer extension experiments (Fig. 3). Hybridization and extension of a primer that was complementary to the 5′ end of the β-Gal gene with vgCL5 RNA revealed two major extension products as well as several minor ones. The sizes and patterns of primer extension products were identical to those reported previously for the authentic gC gene as well as for several gC promoter-*lacZ* constructions (20, 21). Similar primer extension results were observed with use of vgCP35, vgCP20, and vgCP33 RNAs, although the intensity of the extension products was reduced relative to the control primer extension (Fig. 3B; Table 1). In contrast, the major primer extension products were absent in reactions using vgCP30 and vgCP24 RNAs. Table 1 shows the relative amounts of β-Gal mRNA, determined by densitometry of the primer extension autoradiographs, compared with the enzymatic levels of β-Gal produced by these mutant viruses. The decrease in intensity of the β-Gal extension products for the promoter mutants correlated with the decrease in enzyme activity observed. These results confirmed the importance of the three identified sequence elements of the gC promoter and indicated that at least one of these elements, the sequence at the initiation of transcription, is necessary for normal transcription from the gC promoter.

Sequence elements of the gH promoter. To determine whether other late HSV-1 promoters contain sequence elements similar to those identified in the gC promoter, a set of linker-scanning mutations was constructed in the gH promoter. Previous results have shown that the gH promoter extends no more than 83 bp upstream from the start of transcription and that expression requires a TATA element. Eight linker-scanning mutations were made between −32 and +40 of the gH promoter (Fig. 4). Recombinant viruses were made, and the expression of *lacZ* was quantitated. Two mutations, one at the gH TATA element (vgHL4.5) and one at the start of transcription (vgHL4.2), resulted in dramatic decreases in β-Gal activity compared with that of the unmutated promoter vgHL1. A third mutation downstream from the start of transcription (vgHL4.8) resulted in a modest decrease in β-Gal activity. None of these recombinant viruses expressed appreciable levels of β-Gal in the presence of phosphonoacetic acid (data not shown).

Primer extension experiments were performed by using the RNA from vgHL1-, vgHL4.5-, vgHL4.2-, and vgHL4.8-infected cells and either the β-Gal primer or a mixture of the β-Gal and gC primers (Fig. 5A; Table 1). Extension of the β-Gal primer with vgHL1 RNA produced two closely spaced products at the normal site of gH mRNA transcription (13, 16). A similar result was seen with vgHL4.8 RNA, with no apparent decrease in the β-Gal mRNA extension products (Table 1). These primer extension products were barely detectable when either vgHL4.5 or vgHL4.2 RNA was used with the β-Gal primer. Control primer extension products using the gC primer were similar with all four RNAs (Fig. 5B; Table 1). Taken together, these results indicate that the gC and gH promoters comprise at least two similarly spaced sequence elements, the TATA element and the sequence at

VIRUS	-40 -30 -20 -10 +10 +20 +30 +40	RELATIVE β-GAL ACTIVITY
vgCL5	GATGGGGCCCGGGTATAAATTCCGGAAGGGGACACGGGCTACCCTCACTACCGAGGGCGCTTGGTCGGGAGGCCGCATCGAACGCACACC	100
vgCP34	GATGTCGACCGGGTATAAATTCCGGAAGGGGACACGGGCTACCCTCACTACCGAGGGCGCTTGGTCGGGAGGCCGCATCGAACGCACACC	148±8
vgCP35	GATGGGGCCCGGGTATAAAGTCGACAAGGGGACACGGGCTACCCTCACTACCGAGGGCGCTTGGTCGGGAGGCCGCATCGAACGCACACC	59±4
vgCP25	GATGGGGCCCGGGTATAAATTCCGGAAGTCGACACGGGCTACCCTCACTACCGAGGGCGCTTGGTCGGGAGGCCGCATCGAACGCACACC	81±1
vgCP28	GATGGGGCCCGGGTATAAATTCCGGAAGGGGTCGACGGCTACCCTCACTACCGAGGGCGCTTGGTCGGGAGGCCGCATCGAACGCACACC	93±3
vgCP29	GATGGGGCCCGGGTATAAATTCCGGAAGGGGACACGGGGTCGACTCACTACCGAGGGCGCTTGGTCGGGAGGCCGCATCGAACGCACACC	104±27
vgCP30	GATGGGGCCCGGGTATAAATTCCGGAAGGGGACACGGGCTACGTCGACTACCGAGGGCGCTTGGTCGGGAGGCCGCATCGAACGCACACC	29±12
vgCP24	GATGGGGCCCGGGTATAAATTCCGGAAGGGGACACGGGCTACCCTGTCGACCGAGGGCGCTTGGTCGGGAGGCCGCATCGAACGCACACC	26±6
vgCP31	GATGGGGCCCGGGTATAAATTCCGGAAGGGGACACGGGCTACCCTCACTGTCGACGGCGCTTGGTCGGGAGGCCGCATCGAACGCACACC	98±11
vgCP23	GATGGGGCCCGGGTATAAATTCCGGAAGGGGACACGGGCTACCCTCACTACCGTCGACGCTTGGTCGGGAGGCCGCATCGAACGCACACC	132±25
vgCP27	GATGGGGCCCGGGTATAAATTCCGGAAGGGGACACGGGCTACCCTCACTACCGAGGGCGGTCGACCGGGAGGCCGCATCGAACGCACACC	113±1
vgCP21	GATGGGGCCCGGGTATAAATTCCGGAAGGGGACACGGGCTACCCTCACTACCGAGGGCGCTTGGTCGGGTCGACGCATCGAACGCACACC	92±13
vgCP32	GATGGGGCCCGGGTATAAATTCCGGAAGGGGACACGGGCTACCCTCACTACCGAGGGCGCTTGGTCGGGAGGCGTCGACGAACGCACACC	83±7
vgCP20	GATGGGGCCCGGGTATAAATTCCGGAAGGGGACACGGGCTACCCTCACTACCGAGGGCGCTTGGTCGGGAGGCCGCAGTCGACGCACACC	59±11
vgCP33	GATGGGGCCCGGGTATAAATTCCGGAAGGGGACACGGGCTACCCTCACTACCGAGGGCGCTTGGTCGGGAGGCCGCATCGTCGACACACC	67±4
vgCP26	GATGGGGCCCGGGTATAAATTCCGGAAGGGGACACGGGCTACCCTCACTACCGAGGGCGCTTGGTCGGGAGGCCGCATCGAACGTCGACC	93±7

FIG. 2. Linker-scanning mutations in the gC promoter. Mutations that resulted in the insertion of *Sal*I restriction enzyme recognition sites were generated in the gC promoter as described in Materials and Methods. The altered nucleotides are indicated by underlining. Vero cells were infected with each recombinant virus; β-Gal activity was quantitated and expressed relative to that of vgCL5. Values shown are means and standard deviations of two to five separate experiments.

the start of transcription, which are necessary for promoter activity. A downstream element, in the gC promoter at least, may contribute to maximal promoter activity.

Hybrid gH/gC promoters. To determine whether the sequence elements of late HSV-1 promoters were functionally similar, experiments were performed that interchanged these elements between the gC and gH promoters. This was made possible since many of the linker-scanning mutations in both promoter regions had a negligible effect on the expression of β-Gal activity. In the first set of experiments, gH promoter elements were substituted into the gC promoter. When gH promoter sequences from −83 to +13, containing both the TATA and initiation elements, were substituted for gC sequences from −114 to +15, expression of β-Gal increased by approximately 50%. However, when gH sequences from −83 to −12, containing only the TATA element, were substituted for gC sequences from −114 to −15, expression of β-Gal decreased by 78% (Fig. 6A). Primer extension experiments using RNA from vgCP27-, vgHC.2-, vgCP28-, and vgHC.4-infected cells showed that the β-Gal mRNA initiated at the normal gC start site in vgCP27- and vgCP28-infected cells and at the normal gH start site in vgHC.2-infected cells. No start site was observed when vgHC.4 RNA was used (Fig. 6B). Thus, although substitution of the gH TATA and initiation sequence together into the gC gene directed efficient transcription, the gH TATA element alone was not sufficient to initiate transcription.

The lack of β-Gal transcription in vgHC.4-infected cells could be due to poor alignment of the gH TATA element with the gC initiation sequence or to the fact that these sequence elements are not interchangeable between the two promoters. To evaluate these two possibilities, sequence elements of the gC promoter were also substituted into the gH promoter. In these experiments, gC sequences from −114 to +15, containing the gC TATA element and initiation sequence, effectively substituted for gH sequences from −83 to +12 (Fig. 7A), and transcription of the β-Gal mRNA in vgHC.1-infected cells initiated from the normal gC start site (Fig. 7B). The measured levels of β-Gal were lower for all of the constructions that contained downstream gH sequences than for gC sequences (Fig. 6A versus Fig. 7A). We suspect that this is due to the presence of a sequence at the start of gH translation that is predicted to be unfavorable for translation (7, 17). Substitution of gC sequences containing only the TATA element (−114 to −15) for gH sequences −83 to −12 also directed β-Gal mRNA transcription (Fig. 7A and B), but mRNA initiation was at the normal gH start of transcription, not at the predicted distance downstream from

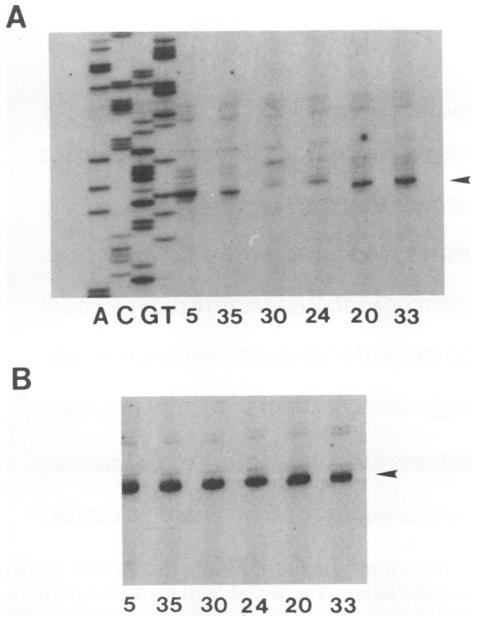

FIG. 3. Primer extension analysis of infected cell mRNA. RNA was isolated from Vero cells infected with vgCL5 RNA (lanes 5), vgCP35 RNA (lanes 35), vgCP30 RNA (lanes 30), vgCP24 RNA (lanes 24), vgCP20 RNA (lanes 20), or vgCP33 RNA (lanes 33), hybridized to both a gC-specific primer and a β-Gal-specific primer in the same reaction, and extended with reverse transcriptase. The extension products (arrowheads) were run alongside a sequencing ladder prepared by using the β-Gal primer and pgCL5 plasmid DNA. (A) Primer extension products of the β-Gal primer and infected cell RNAs; (B) primer extension products of the gC primer and the same RNAs. The gC primer extensions shown in panel B and the β-Gal primer extensions shown in panel A were from different regions of the same gel.

the gC TATA element. These results indicate that the TATA element and initiation element of the gC and gH promoters are interchangeable and that both elements are necessary for late gene expression, and they suggest that the initiation element determines where mRNA transcription begins.

TABLE 1. Comparison of β-Gal activity and mRNA levels from linker-scanning mutants

Mutant	Relative β-Gal activity[a]	Relative mRNA level[b]
gC promoter		
gCP35	59	53
gCP30	29	34
gCP24	26	36
gCP20	59	71
gCP33	67	79
gH promoter		
gHL4.5	16	23
gHL4.2	9	23
gHL4.8	68	116

[a] Values as shown in Fig. 2 and 4.

[b] Calculated from densitometry of primer extension data (Fig. 3 and 5). Values were obtained by multiple scans with a Hoefer GS-300 scanning densitometer. Data were normalized as the ratio of the amount of β-Gal mRNA to the amount of gC mRNA and then expressed relative to the value obtained for vgCL5 (for the gC promoter mutants) or vgHL.1 (for the gH promoter mutants).

DISCUSSION

The HSV-1 gC and gH promoters have been partially defined by previous deletion analysis. For example, both promoters require a TATA element, and the gC promoter requires sequences downstream from the start of transcription for maximal expression (4, 5, 16, 21). However, a complete definition of the composition of late HSV-1 promoters has been lacking. In this study, linker-scanning mutational analysis was performed on both the gC and the gH promoters in order to define and compare the sequence elements that constitute late viral promoters. In this analysis, a small number of nucleotide changes were made in each promoter that resulted in the insertion of a unique *Sal*I restriction site at approximately six- to eight-base intervals. The recognition site for the restriction enzyme *Sal*I was chosen for convenience because of the absence of this recognition sequence in the gC and gH promoters and in the insertion vector pGal8 (22). Promoter constructions that substitute sequences from one promoter to another were facilitated by using the inserted *Sal*I sites.

The mutations inserted into the gC and gH promoters identified three relatively short sequence elements that were similarly located in both promoters. The first of these, the TATA element, is a common element found in many eukaryotic and most, if not all, HSV-1 promoters (18). The results presented here confirm that a functional TATA element is necessary for late promoter activity. Mutation of the gH TATA element practically eliminated β-Gal expression (vgHL4.2), and a single base change in the gC TATA element (vgCP35) reduced expression by 41%. Previous work has shown that TATA elements derived from other HSV-1 promoters can effectively substitute for the gH TATA element (16). Substitution of gC sequences from −114 to −15 for gH sequences upstream of −12 also resulted in a functional promoter in vgHC.3. Since transcription initiated at the normal gH start site in this recombinant, 30 bp downstream from the gC TATA element, not 32 bp downstream as in the intact gC promoter, it is unlikely that the TATA element alone determines the site of transcription initiation. The absence of transcription from the hybrid promoter in vgHC.2 confirmed that an intact TATA element is not sufficient for late promoter activity and suggested strongly that this element must be correctly aligned with the downstream initiation element.

A second sequence element was identified in the gC promoter between +31 and +41. Two separate gC mutations in this region decreased β-Gal activity by 33 and 41%. Less dramatic decreases of 21 and 29%, respectively, as determined by primer extension experiments, were observed. What may account for the difference between the effects of these mutations on β-Gal activity and β-Gal mRNA level is unclear at the present time. It is possible that a downstream element both functions as a *cis*-acting DNA element and has a role at the RNA level to increase translation or stability of the mRNA. In a previous report, deletion of gC sequences from +38 to +16 resulted in a more dramatic reduction in expression, from 66% of maximal expression to 26%, than that observed with the linker-scanning mutations (21). We suspect that this is due to the complete removal of this element rather than its alteration.

One mutation in the corresponding region of the gH promoter, +37 to +40, also decreased β-Gal activity, although there was no corresponding reduction in β-Gal mRNA. It may be that this region of the gH promoter has only a minor role in increasing expression of the gH mRNA.

VIRUS	-30 -20 -10 +10 +20 +30 +40	RELATIVE β-GAL ACTIVITY
vgHL1	GACAGAATAAAACGCACGGGTGTTGGGTCGTTTGTTCATAAACGCGGGGTTCGGTCCCAGGGCTGGCACTCTGTCGATACCCCAC	100
vgHL4.5	GACAGTCGACAACGCACGGGTGTTGGGTCGTTTGTTCATAAACGCGGGGTTCGGTCCCAGGGCTGGCACTCTGTCGATACCCCAC	16±3
vgHL4.6	GACAGAATAAAACGTCGACGTGTTGGGTCGTTTGTTCATAAACGCGGGGTTCGGTCCCAGGGCTGGCACTCTGTCGATACCCCAC	103±1
vgHL4.1	GACAGAATAAAACGCACGGGTGTTGGTCGACTTGTTCATAAACGCGGGGTTCGGTCCCAGGGCTGGCACTCTGTCGATACCCCAC	118±8
vgHL4.2	GACAGAATAAAACGCACGGGTGTTGGGTCGTTTGGTCGACAACGCGGGGTTCGGTCCCAGGGCTGGCACTCTGTCGATACCCCAC	9±1
vgHL4.3	GACAGAATAAAACGCACGGGTGTTGGGTCGTTTGTTCATAAACGTCGACTTCGGTCCCAGGGCTGGCACTCTGTCGATACCCCAC	127±5
vgHL4.4	GACAGAATAAAACGCACGGGTGTTGGGTCGTTTGTTCATAAACGCGGGGGTCGACCCCAGGGCTGGCACTCTGTCGATACCCCAC	90±4
vgHL4.7	GACAGAATAAAACGCACGGGTGTTGGGTCGTTTGTTCATAAACGCGGGGTTCGGTCCCAGGGTCGACACTCTGTCGATACCCCAC	106±1
vgHL4.8	GACAGAATAAAACGCACGGGTGTTGGGTCGTTTGTTCATAAACGCGGGGTTCGGTCCCAGGGCTGGCACTCTGTGTCGACCCCAC	68±2

FIG. 4. Linker-scanning mutations in the gH promoter. Mutations that resulted in insertion of unique *Sal*I restriction sites were generated in the gH promoter. The altered nucleotides in each construction are indicated by underlining. The β-Gal activity was determined at 24 h postinfection and expressed relative to that of vgHL1. Values shown are means and standard deviations of two to five separate experiments.

Inspection of this region of the two promoter sequences revealed no obvious similarity other than a four-nucleotide string, TCGA. Interestingly, this four-nucleotide sequence is present in each linker-scanning mutant as part of the *Sal*I recognition sequence and is thus moved only 1 and 3 bp downstream, respectively, in vgCP20 and vgCP33 and 2 bp downstream in vgHL4.8. Since none of the linker-scanning mutations nor the previously reported gC deletion mutation altered the normal start of transcription, we suspect that the downstream sequence elements in the gH and gC promoters probably influence the magnitude of expression but are not involved in determining the actual start site of transcription. However, a more extensive mutational analysis will probably be necessary to clarify both the importance and function of the downstream elements in the gC and gH promoters.

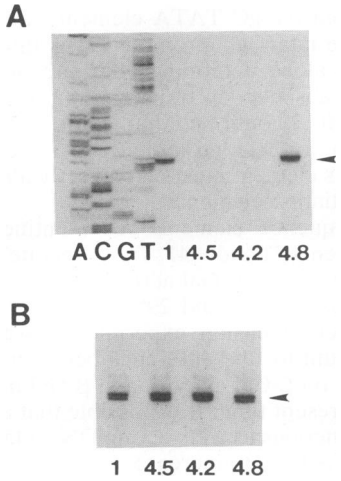

FIG. 5. Primer extension analysis of infected cell mRNA. RNA was isolated from Vero cells infected with vgHL1 (lanes 1), vgHL4.5 (lanes 4.5), vgHL4.2 (lanes 4.2), or vgHL4.8 (lanes 4.8), hybridized to both gC-specific and β-Gal-specific primers in the same reaction, and extended with reverse transcriptase. The extension products (arrowheads) were run alongside a sequencing ladder prepared using the β-Gal primer and pgHL1 plasmid DNA. (A) Primer extension products of the β-Gal primer and infected cell RNAs; (B) primer extension products of the gC primer and the same infected cell RNAs. The gC primer extensions shown in panel B and the β-Gal primer extensions shown in panel A were from different regions of the same gel.

Linker-scanning mutations near the start sites of transcription for both promoters had dramatic effects on promoter activity. These mutations defined a short sequence from −3 to +4 in the gC promoter and one from −6 to +7 in the gH promoter that were required for promoter activity. In this region, the two promoters share 5 of 8 bp (Fig. 8). Mutations in this region did not simply alter the specificity of transcription initiation. Levels of expression were dramatically reduced as measured by both β-Gal activity and β-Gal mRNA levels. In addition, no new mRNA start sites were observed as a result of the inserted mutations, only minor start sites that were utilized in the unmutated promoters. Furthermore, primer extension experiments with the RNA from vgHC.4-infected cells indicated the essential role for the initiation element in determining the start site of transcription. The mechanism by which this initiation element functions is not clear. Its location near the start of transcription resembles that of the recently described initiator elements (Inr) (14, 15). Such elements have been defined as having the ability to independently direct initiation of transcription by RNA polymerase II and the ability to be activated in the absence of a TATA element by an upstream activator element. Although it is not known at this time whether the gC or gH element will function in such a manner in an in vitro transcription assay, evidence to date indicates that in the context of the viral genome, an upstream TATA element is necessary. Figure 8 shows the sequences of two well-characterized Inr elements, that of the murine terminal deoxynucleotidyltransferase (TdT) gene and the adenovirus major late (AdML) promoter. The gC initiation element shares 7 of 8 nucleotides with the TdT Inr and 8 of 10 nucleotides with the AdML Inr; the gH initiation element

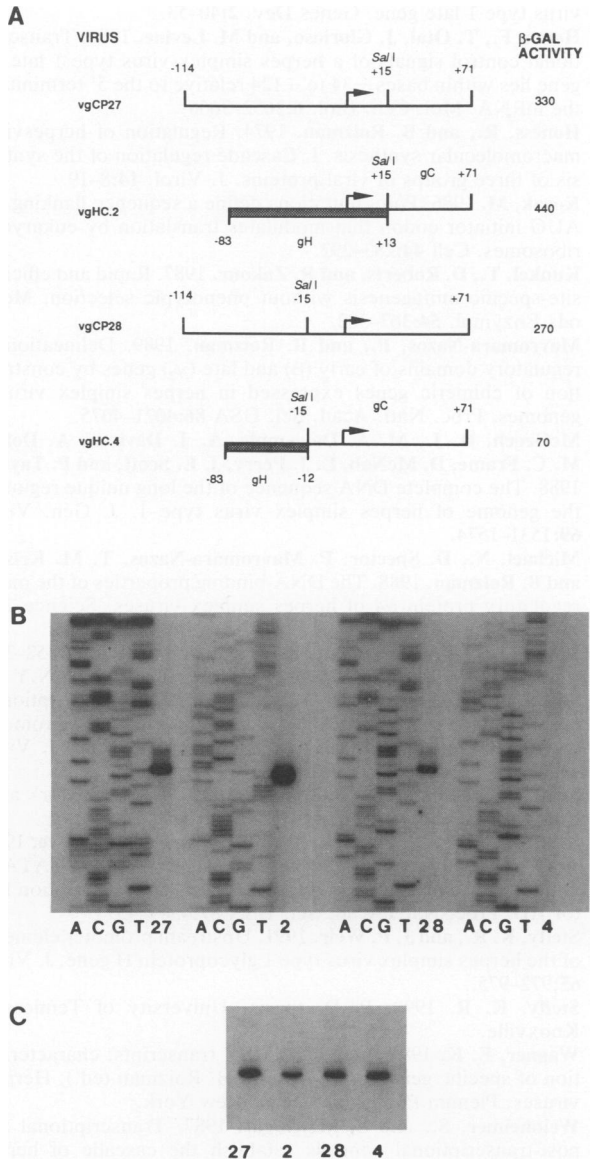

FIG. 6. Substitution of gH promoter sequences into the gC promoter. (A) Sequences from −83 to +13 and from −83 to −12 were inserted into the gC promoter at +15 and −15, respectively, as described in Materials and Methods. The β-Gal activity was measured at 24 h postinfection as previously described (12, 20). (B) Primer extension analysis of RNA from cells infected with vCP27 (lane 27), vgHC.2 (lane 2), vgCP28 (lane 28), and vgHC.4 (lane 4) and a β-Gal-specific primer. Each primer extension was run alongside a sequencing reaction prepared with the β-Gal primer and the corresponding plasmid DNA. (C) Extension of the gC primer and the same RNAs. Each primer extension reaction contained both β-Gal and gC primers. The gC extension products shown here are from the same reaction and the same gel as the β-Gal extension products shown in panel B.

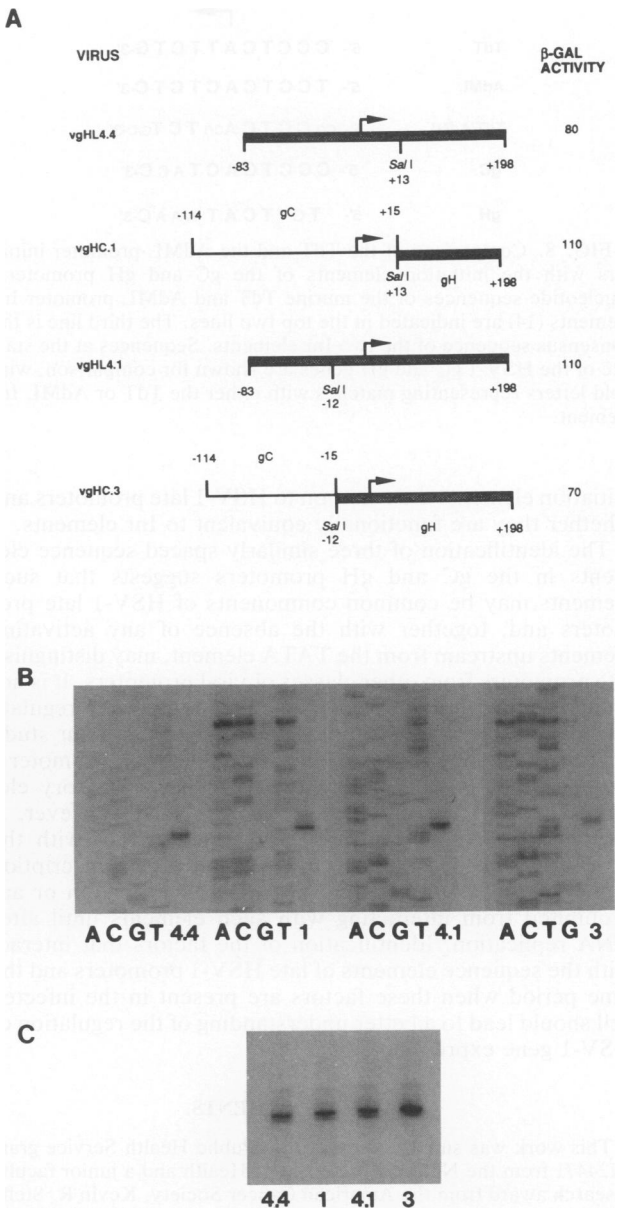

FIG. 7. Substitution of gC promoter sequences into the gH promoter. (A) Sequences from −114 to +15 and −114 and −15 of the gC promoter were inserted into the gH promoter at +13 and −12, respectively. The β-Gal activity was measured at 24 h postinfection. (B) RNA was isolated from cells infected with vgHL4.4 (lane 4.4), vgHC.1 (lane 1), vgHL4.1 (lane 4.1), and vgHC.3 (lane 3) and used for primer extension with both the β-Gal and gC primers in the same reaction. The β-Gal extension products are shown alongside sequence ladders prepared by using the β-Gal primer and the corresponding plasmid DNA. In the sequence ladder alongside the vgHC.3 RNA primer extension, the T and G lanes are reversed. (C) The extension products of the gC primer from the same reaction and same gel shown in panel B.

shares 4 of 4 and 5 of 8 nucleotides with the TdT and AdML Inr, respectively. Figure 8 shows the nucleotides of the gC and gH initiation elements (in bold) that match a corresponding nucleotide in either the TdT or AdML Inr element. Preliminary inspection of sequences from selected HSV-1 genes (10) did not reveal obvious sequence similarities at the start site of the IE 110K, 175K, or 63K gene or the early thymidine kinase, ribonucleotide reductase, or DNA polymerase gene. Similarities could be found in the gE and U_L48 (VP16) genes but not in other reported late genes such as U_S11 or U_L42. It remains to be determined whether the

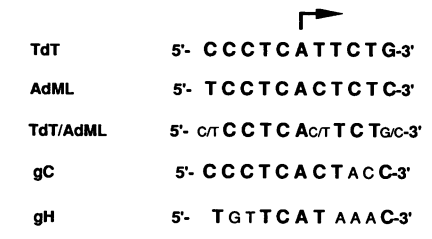

FIG. 8. Comparison of the TdT and the AdML promoter initiators with the initiation elements of the gC and gH promoters. Nucleotide sequences of the murine TdT and AdML promoter Inr elements (14) are indicated in the top two lines. The third line is the consensus sequence of the two Inr elements. Sequences at the start site of the HSV-1 gC and gH genes are shown for comparison, with bold letters representing matches with either the TdT or AdML Inr element.

initiation elements are common to HSV-1 late promoters and whether they are functionally equivalent to Inr elements.

The identification of three similarly spaced sequence elements in the gC and gH promoters suggests that such elements may be common components of HSV-1 late promoters and, together with the absence of any activating elements upstream from the TATA element, may distinguish late promoters from other classes of viral promoters. It is not known at this time how such elements temporally regulate late gene transcription. None of the mutations in our study altered the temporal expression from the two promoters, although it is possible that there are other regulatory elements that were undetected in our analysis. However, it seems most likely that the factors that interact with the *cis*-acting sequence elements to direct late gene transcription either are not available until after DNA replication or are prohibited from interacting with such elements until after DNA replication. Identification of the factors that interact with the sequence elements of late HSV-1 promoters and the time period when these factors are present in the infected cell should lead to a better understanding of the regulation of HSV-1 gene expression.

ACKNOWLEDGMENTS

This work was supported in part by Public Health Service grant AI24471 from the National Institutes of Health and a junior faculty research award from the American Cancer Society. Kevin R. Steffy was supported by a training grant from the National Institutes of Health.

REFERENCES

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. A. Smith, J. G. Seidman, and K. Struhl (ed.).** 1987. Current protocols in molecular biology. Greene Publishing Associates, Brooklyn, N.Y.
2. **Cole, C., and T. Stacy.** 1985. Identification of sequences in the herpes simplex virus thymidine kinase gene required for efficient processing and polyadenylation. Mol. Cell. Biol. **5**:2104–2113.
3. **Godowski, P., and D. Knipe.** 1986. Transcriptional control of herpesvirus gene expression: gene functions required for positive and negative regulation. Proc. Natl. Acad. Sci. USA **83**:256–260.
4. **Homa, F., J. Glorioso, and M. Levine.** 1988. A specific TATA box promoter element is required for expression of a herpes virus type 1 late gene. Genes Dev. **2**:40–53.
5. **Homa, F., T. Otal, J. Glorioso, and M. Levine.** 1986. Transcriptional control signals of a herpes simplex virus type 1 late (γ) gene lies within bases −34 to +124 relative to the 5′ terminus of the mRNA. Mol. Cell. Biol. **6**:3652–3666.
6. **Honess, R., 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.
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., D. Roberts, and R. Zakour.** 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol. **54**:367–382.
9. **Mavromara-Nazos, P., and B. Roizman.** 1989. Delineation of regulatory domains of early (β) and late (γ_2) genes by construction of chimeric genes expressed in herpes simplex virus 1 genomes. Proc. Natl. Acad. Sci. USA **86**:4071–4075.
10. **McGeoch, D. J., M. A. Dalrymple, A. J. Davison, A. Dolan, M. C. Frame, D. McNab, L. J. Perry, J. E. Scott, and P. Taylor.** 1988. The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. J. Gen. Virol. **69**:1531–1574.
11. **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 viruses. Science **239**: 1531–1534.
12. **Miller, J.** 1972. Experiments in molecular genetics, p. 352–355. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
13. **Sharp, J., M. Wagner, and W. Summers.** 1973. Transcription of herpes simplex virus genes in vivo: overlap of a late promoter with the 3′ end of the early thymidine kinase gene. J. Virol. **45**:10–17.
14. **Smale, S. T., and D. Baltimore.** 1989. The 'initiator' as a transcription control element. Cell **57**:103–113.
15. **Smale, S. T., M. C. Schmidt, A. J. Berk, and D. Baltimore.** 1990. Transcriptional activation by Sp1 as directed through TATA or initiator: specific requirement for mammalian transcription factor IID. Proc. Natl. Acad. Sci. USA **87**:4509–4513.
16. **Steffy, K. R., and J. P. Weir.** 1991. Upstream promoter elements of the herpes simplex virus type 1 glycoprotein H gene. J. Virol. **65**:972–975.
17. **Steffy, K. R.** 1990. Ph.D. thesis. University of Tennessee, Knoxville.
18. **Wagner, E. K.** 1985. Individual HSV transcripts: characterization of specific genes, p. 45–104. *In* B. Roizman (ed.), Herpesviruses. Plenum Publishing Corp., New York.
19. **Weinheimer, S., and S. McKnight.** 1987. Transcriptional and post-transcriptional controls establish the cascade of herpes simplex virus protein synthesis. J. Mol. Biol. **195**:819–833.
20. **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.
21. **Weir, J. P., and P. R. Narayanan.** 1990. Expression of the herpes simplex virus type 1 glycoprotein C gene requires sequences in the 5′ noncoding region of the gene. J. Virol. **64**:445–449.
22. **Weir, J. P., K. R. Steffy, and M. Sethna.** 1990. An insertion vector for the analysis of gene expression during herpes simplex virus infection. Gene **89**:271–274.
23. **Zagursky, R., N. Baumeister, N. Lomax, and M. Berman.** 1985. Rapid and easy sequencing of large linear double-stranded DNA and supercoiled plasmid DNA. Gene Anal. Tech. **2**:89–94.
24. **Zhang, F., R. Denome, and C. Cole.** 1986. Fine-structure anaysis of the processing and polyadenylation region of the herpes simplex virus type 1 thymidine kinase gene by using linker scanning, internal deletion, and insertion mutations. Mol. Cell. Biol. **6**:4611–4623.