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Involvement of a High-Mobility-Group Protein in the Transcriptional Activity of Herpes Simplex Virus Latency-Active Promoter 2

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Latency-active promoter 2 (LAP 2) is a TATA-less promoter in herpes simplex virus type 1 (HSV-1) that can express genes during viral latency. Four regions of LAP2 are protected from DNase I digestion in vitro by either HeLa cell nuclear extracts or purified Sp1. Transient gene expression assays of LAP2 substitution mutants demonstrate that two of the regions protected by Sp1 and three other regions protected by nuclear extract are important for promoter function. The mutation causing the most significant reduction in expression alters a stretch of 23 thymidine residues (T_{23}) that binds a protein with several properties common to high-mobility-group (HMG) proteins. The T_{23} binding activity is heat stable, can be inhibited by poly(dA-dT) · poly(dA-dT), and is inhibited by minor-groove-binding drugs. Antiserum directed against HMG I(Y) blocked the formation of one of the DNA-protein complexes on the T_{23} oligonucleotide, suggesting that a protein antigenically related to HMG I(Y) binds to LAP2 in vitro. Direct evidence of HMG I(Y) involvement in LAP2 function is provided by the findings that recombinant HMG I(Y) protein facilitates Sp1 binding to LAP2 in mobility shift assays and that antisense HMG I(Y) RNA specifically inhibits LAP2 function in vivo. These results suggest that DNA structure may be an important determinant of the activity of a promoter that is capable of escaping the global shutoff of transcription that occurs during viral latency.

Herpes simplex virus type 1 (HSV-1) is an alphaherpesvirus that establishes latency primarily in the cells of the human peripheral nervous system. During latency, the viral genome becomes a circular episome (41, 44) and is at least partially bound by nucleosomes (13). The normal cascade of lytic gene expression is silenced (49) except for the expression of a set of latency-associated transcripts (LATs) from two identical loci in the inverted-repeat sequences surrounding the unique long (U_L) component of the genome (12, 45, 52, 57). Two separate regions that possess promoter activity have been identified: latency-active promoter 1 (LAP1) (2, 14, 16, 36, 67) and the TATA-less latency-active promoter 2 (LAP2) (8, 22, 65). LAP1 is the predominant promoter during latency, while LAP2 is the predominant promoter driving LAT expression during lytic infection (13, 14). The two regions may be functionally interdependent, since LAP1 in the absence of LAP2 cannot express most foreign genes during latency in an ectopic locus (37) or when the foreign gene is introduced into the LAT locus immediately downstream of the LAP1 TATA box near the 8.3-kb minor LAT transcription start site (40). In contrast, introduction of a *lacZ* reporter gene into the LAT intron downstream of the LAP1-LAP2 complex results in long-term reporter gene expression during latency (7, 23). Long-term expression is also seen when the LAP2 promoter is used to drive the expression of *lacZ* in an ectopic locus such as the glycoprotein C gene (22).

While LAP2 lacks a recognizable TATA box, it does contain sequences found in other promoters. LAP2 from HSV-1 strain 17 contains a stretch of 23 consecutive T residues (T_{23}) and a CT-rich element, the latter of which is found in a variety of TATA-less promoters as well as promoters involved in signal transduction (3, 27, 30, 32, 54, 56). Initial characterization of LAP2 revealed sequences between −388 and −11 to be required in transient expression of a reporter gene following transfection of cells in culture. Both the uninterrupted 23-nucleotide thymidine (T_{23}) sequence (−175 to −151), and a CT-rich region (−92 to −57) were found to be required for full LAP2 activity in mammalian cells (21). Plasmid constructs containing the T_{23} sequence and the CT-rich region were sensitive to S1 nuclease digestion, suggesting that these sequences were capable of forming non-B-DNA structures in vitro and may function to alter chromatin structure, allowing this region of the HSV genome to escape the transcriptional silencing that occurs during latency.

In vitro binding studies revealed that the CT-rich region binds two proteins: Sp1 (5, 29) and NSEP-1 (33). Cotransfection of LAP2 with an Sp1 expression vector in *Drosophila* Schneider SL-2 cells enhanced expression from LAP2 (21). Additionally, the CT-rich region of LAP2 was able to induce expression in *cis* when placed upstream of a minimal promoter. However, deletion of the CT element did not abrogate the ability of Sp1 to induce transcription from LAP2, suggesting that Sp1 can bind elsewhere in LAP2, making the CT element functionally redundant for Sp1 binding. The reduction of LAP2 activity in the CT deletion mutant in mammalian cells suggested that other factors in addition to Sp1 may be interacting with elements within LAP2.

In this report, we identify four sequence elements in LAP2 that are protected from nuclease digestion by either HeLa nuclear extracts and/or purified Sp1. Transient-expression assays with substitution mutants revealed several important *cis*-acting elements within these protected regions. Electrophoretic mobility shift analysis (EMSA) with the prominent positive-acting *cis* element, the poly(T) stretch, identified one of the binding proteins as being related or identical to the high-mobility-group proteins, HMG I(Y) (46, 51, 53, 55). We show that recombinant HMG I(Y) facilitates Sp1 binding to

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LAP2 and that HMG I(Y) antisense RNA specifically inhibits LAP2 promoter function in vivo.

MATERIALS AND METHODS

DNase I footprinting. DNase I footprinting (5) was done with the following modifications. Probes were generated from the HSV-1 KOS LAP2 −388 to +42 subclone pSacIILAP (22), which was cut with *Hin*dIII to make the coding-strand probe and with *Eco*RI to make the noncoding-strand probe, followed by end filling with Klenow polymerase. Probes were incubated with either no protein, 2 footprint units of purified Sp1 (Promega, Madison, Wis.), or 20 μg of HeLa nuclear extract (prepared as previously described [15]) in the presence of 100 ng of poly(dI-dC) · poly(dI-dC) (Pharmacia Biotech, Piscataway, N.J.) and then subjected to DNase I (Sigma Chemical Co., St. Louis, Mo.) digestion. The digestion products were purified by phenol-chloroform extraction followed by ethanol precipitation; they were then resuspended in formamide dye and resolved in a 6% polyacrylamide sequencing gel.

Substitution mutant construction. The wild-type expression plasmid LAP2 substitution mutants were generated from pLCP by overlap extension (26). LCP was generated by ligating the *Pst*I-*Bam*HI fragment of LAP2-CAT (22) into pBSIISK− (Stratagene, La Jolla, Calif.). The coding primer 13-20-GTAAAAC GACGGCCAGT and one of the following LAP2 coding-sequence primers were used: 1A-CGGGGCCTGAGATGAgaagatcttcgcgaCGCCAACGGCCGGCC, 1B-GAACACTCGGGGTTgaagatcttcgcgaGCCCCCGTGGCGGCC, 1C-GGG TTACCGCCAACGgaagatcttcgcgaCGGCCCGGCCCGGGG, 2A-CCCAAGG GGCCCCGGgaagatcttcgcgaACGGCCCGGCGCATGC, 2B-CGGCCCGGGG CCCCAgaagatcttcgcgaATGCGCTGCGTTTTT, 2C-CCACAACGGCCCGGCg aagatcttcgcgaTTTTTTTTTTTTTT, 2D-GGCGCATGCGCTGCGgaagatcttcgc gaTTTTTTTTTCTCGG, 3-CCTTTCCTGTCTCGCgaagatcttcgcgaCCTTCACC CCCAGTACC, 4A-CCCCCCTTCACCCCCgaagatcttcgcgaTCCCTTCCTCCC CCG, 4B-CCCAGTACCCTCCTCgaagatcttcgcgaCCGTTATCCCACTCG, and 4C-CCTCCCTCCCTTCCTCgaagatcttcgcgaTCGTCGAGGGCGCCC. The noncoding mutant strand was generated with the primer 1669-GGTGTAACAAGG GTGAAC and primers complementary to the coding-strand primers. Mutants were sequenced with the Sequenase 7′-deaza-dGTP sequencing kit (United States Biochemical). Large-scale preparations of each mutant were performed with a Plasmid Maxi Kit (Qiagen).

CAT assays. Vero cells were transfected in triplicate with lipofectamine (Gibco BRL, Gaithersburg, Md.) with 1.5 μg of LAP2 test plasmid and 0.5 μg of pIEPlacZ (43). Cell lysates were harvested 48 h posttransfection, assayed for CAT activity, and normalized to β-galactosidase activity (47) to control for transfection efficiency and protein concentration. CAT activity was quantified by phosphoimager analysis and expressed as the percentage of the full-length LAP2 promoter activity. For antisense HMG I(Y) experiments, LAP2-CAT or LAP1-CAT fusion constructs (1 μg) were cotransfected with either 0, 1, or 2 μg of the HMG I(Y) antisense expression vector (55) and 2, 1, or 0 μg of the same expression vector (pcDNAI) lacking HMG I(Y) sequences into Vero cells by using lipofectamine. CAT activity was assayed 48 h later, normalized to β-galactosidase activity, and expressed as the percentage of the CAT activity observed in that absence of antisense plasmid.

EMSA. HeLa cell nuclear extracts (2 μg) were incubated for 15 min with or without competitor oligomers in the presence of 3 μg of poly(dG-dC) · poly (dG-dC) (Pharmacia Biotech), 50 mM Tris (pH 8.0), 100 mM NaCl, 10 mM $MgCl_2$, 2 mM EDTA, 2 mM $ZnCl_2$, and 10% glycerol. ^{32}P-labeled specific oligomers were then added, and the mixture was incubated for an additional 30 min. The reaction products were separated on prerun, nondenaturing, 0.25× Tris-borate-EDTA (TBE)–5% polyacrylamide gels. The double-stranded oligomers were made from the following sequences and their complements: T, gatccCTGCGTTTTTTTTTTTTTTTTTTTTTTTCTCGGa; TM, gataccCTGCG GAAGATCTTCGCGATTTTTTTTTCTCGGa; and Sp1, gatccATTCGATC GGGGCGGGGCGAGCa.

Heat-treated extracts were prepared by incubating nuclear extract for 10 min at 65°C. Competition with diminazene aceturate (Berenil) and distamycin (Sigma Chemical Co.) was achieved by preincubating the extract with either of the two drugs at 1 mM, 1 μM, or 1 nM for 10 min and then by adding extract and EMSA as described above.

Antibody-blocking experiments were performed by incubating 2 μg of extract with anti-HMG I(Y) antibody or control preimmune serum (kindly provided by Tom Maniatis, Harvard University) for 1 h at room temperature and then with T probe for 15 min and analyzing the products analysis by EMSA. Supershifting was attempted by incubating extract with the T probe for 15 min, adding antibody, incubating for 1 h, and performing EMSA.

Glutathione-*S*-transferase (GST)-HMG I(Y) and GST proteins (55) were expressed and purified as described previously (50). GST-HMG I(Y) (2 to 128 ng) or GST (8 ng) was incubated with an end-labeled DNA fragment corresponding to bp −189 to −39 of LAP2 for 20 min and then incubated with Sp1 (1 footprint unit; Promega) for 20 min. Complexes were resolved on a 0.25× TBE–4% polyacrylamide gel.

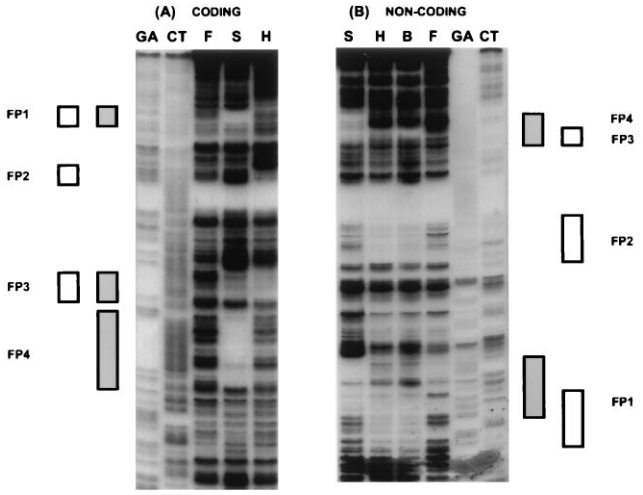

FIG. 1. DNase I protection of LAP2. DNase I footprinting was performed on both coding and noncoding strands of LAP2 with either purified Sp1 or HeLa nuclear extracts. GA and CT indicate chemical sequencing standards from the same probe. F represents free probe (no extract); S represents purified Sp1; H represents HeLa nuclear extract; B represents B103 nuclear extract. Protected regions are identified as FP1 through FP4. Shaded bars represent regions protected by Sp1; open bars indicate regions protected by HeLa nuclear extract.

RESULTS

Four regions of LAP2 are protected in footprinting assays. Four regions of LAP2 exhibited protection from DNase I with either nuclear extract (HeLa) or purified Sp1 on both the coding (Fig. 1A) and noncoding (Fig. 1B) strands. We have designated these FP1 (−290 to −250), FP2 (−200 to −175), FP3 (−110 to −96), and FP4 (−88 to −45), numbered in order from 5′ to 3′ on the coding strand of the promoter. Footprinting with nuclear extracts from the neuroblastoma cell line B103 revealed similar protection to that observed with the HeLa extracts (Fig. 1B); therefore, no cell-type-specific protections were observed. Purified Sp1 protein protected FP1, FP3, and FP4, whereas nuclear extracts protected FP1, FP2, and FP3, indicating that FP2 was unique to the nuclear extracts and FP4 was unique to Sp1. Additionally, the patterns of protection within FP1 were different between Sp1 and the nuclear extracts. Sp1 bound at a sequence further downstream within LAP2, while the extracts bound further upstream (Fig. 1B). Sp1 titration in the footprinting assay demonstrated that Sp1 binds to the sequences protected in FP4 at lower concentrations than it binds to the sequences protected by FP3 (data not shown), suggesting that Sp1 has a higher affinity for sequences in FP4. The DNase I protection results and a comparison of HSV-1 LAP2 sequences from HSV-1 KOS and HSV-1 17 are presented in Fig. 2.

Substitution mutations in the footprinted regions of LAP2 have significant effects on reporter gene activity in Vero cells. To test the relative importance of each of the protected regions to promoter activity, 11 individual 14-bp substitution mutants were generated and tested in transient-transfection assays for reporter gene expression (Fig. 3) in Vero cells. Vero cells (an African green monkey kidney cell line) were used for transfections since they are commonly used to study HSV-1 lytic infections. However, abundant protease activity in Vero cell extracts limited their utility for footprinting and gel shift experiments. Transfection of LAP2 promoter constructs showed that mutants 1A and 1C displayed reduced promoter activity. Mutant 1A corresponds to the region within FP1 pro-

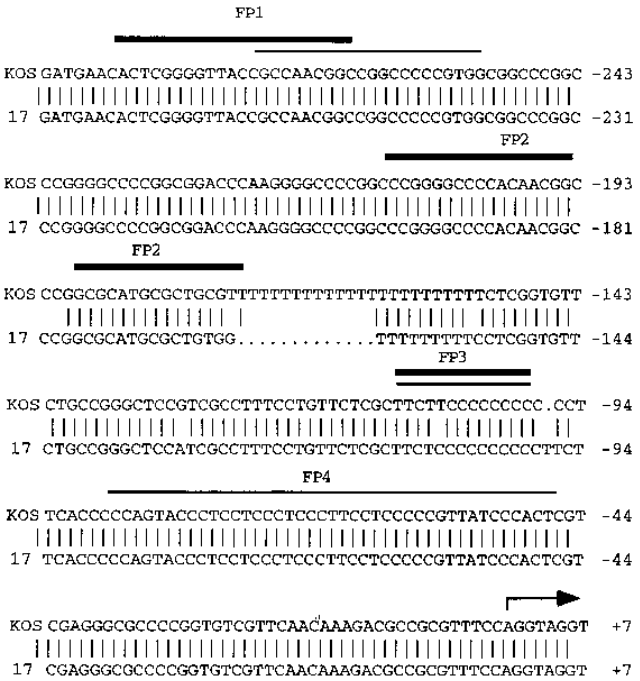

FIG. 2. Summary of DNase I protection assays. Sequences of the HSV-1 LAP2 regions (−293 to +7 relative to the 5′ end of the 2-kb LAT) are shown for HSV-1 KOS and HSV-1 17 strains. Footprinting was performed with DNA from the KOS strain. Thick bars indicate regions protected by HeLa nuclear extract; thin bars indicate regions protected by purified Sp1. The arrow denotes the 5′ end of the 2-kb LAT. The DNA sequence for HSV-1 17 can be retrieved from GenBank as accession number X14112. The DNA sequence for the KOS strain was reported earlier (59).

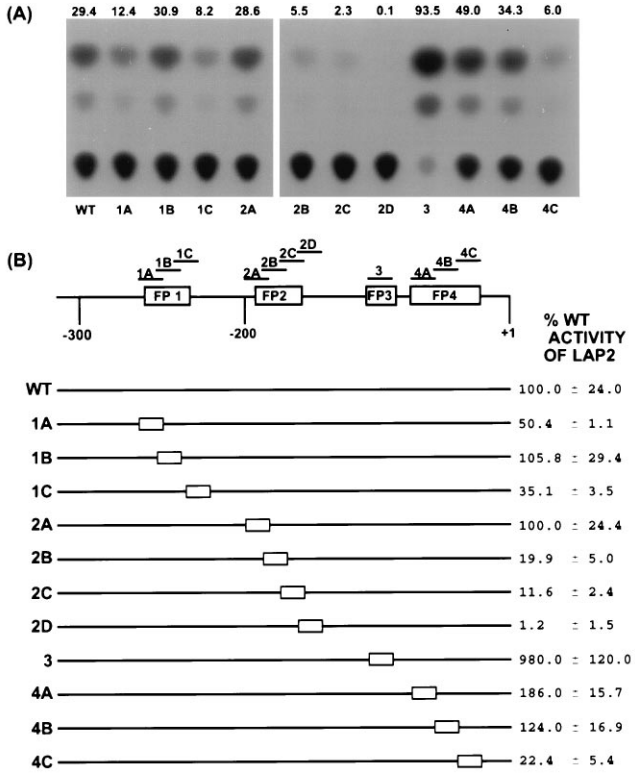

FIG. 3. CAT assays performed on Vero cells transiently transfected with substitution mutants of LAP2. All assays were performed in triplicate. (A) Typical results obtained in the assay. Percent acetylation is indicated above the assay results. (B) Summary of the CAT assays results performed with LAP2 substitution mutants. Reporter gene activity is listed with its standard deviation on the right side of each construct as a percentage of the acetylation obtained from the wild-type (WT) construct. The extracts used to determine the activity of construct 3 had to be diluted to bring the sample to within the linear range of the CAT assay.

tected by nuclear extract, while 1C corresponds to the region protected by Sp1 in FP1.

Since DNase I did not cleave naked LAP2 DNA in the poly(T) stretch (Fig. 1), making it impossible to detect protein binding in this region, mutations within the T stretch were generated to further evaluate the role of this element in promoter function. Mutants 2B, which corresponded to the majority of FP2 upstream of the T stretch, reduced expression fivefold. Mutant 2C, which disrupted part of FP2 as well part of T_{23}, reduced expression almost ninefold. The 14-base alteration of T_{23} (mutant 2D) reduced expression to negligible levels and thus has the most pronounced deleterious effect on promoter function. FP2 was still detected upon footprint analysis of mutant 2D, suggesting that a separate factor binds upstream of T_{23} (data not shown).

In contrast to the other mutants, mutation of FP3 (mutant 3) caused a surprising 10-fold increase in expression from LAP2. Mutant 4A almost doubled reporter gene activity, whereas mutant 4C reduced LAP2 activity nearly fivefold.

Transient-expression assays were also performed in HeLa cells to eliminate the possibility that different transcription factors in Vero cells were interacting with LAP2 compared with those available in HeLa cells. Expression from wild-type LAP2CAT, as well as mutants 2D and 3, was similar to expression from these constructs in Vero cells, indicating that *cis* elements in LAP2 behave similarly in both cell types (data not shown).

The T_{23} region is bound by a HeLa cell protein. Since mutant 2D exhibited the most severe reduction in gene expression, we performed EMSA on probes of either wild-type (T) or mutant 2D (TM) (eliminating 14 bases of the T stretch) poly(T) region. EMSA performed in the presence one of three nonspecific competitors, poly(dA-dT) · poly(dA-dT), poly(dG-dC) · poly(dG-dC), or poly(dI-dC) · poly(dI-dC), revealed strong binding only in the presence of poly(dG-dC) · poly(dG-dC) (data not shown). The mobility shift observed when this probe was bound by HeLa extract was small relative to that observed with Sp1, suggesting that a binding protein with a molecular size smaller than that of Sp1 was complexing with this region of LAP2. EMSA with the poly(T) region revealed three or more complexes (Fig. 4). Complex 1 showed a weaker interaction than did complexes 2 and 3, which ran as an intense

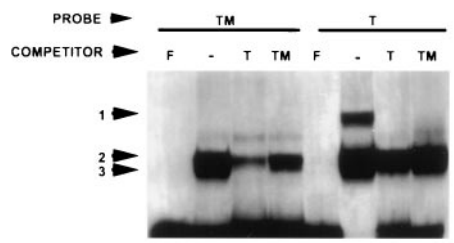

FIG. 4. EMS of the T_{23} element. EMSA was performed on the wild-type thymidine (T) stretch probe and the 2D mutant (TM). EMSA was performed with either T or TM with 100× cold competing T or TM. F represents free probe. T represents wild-type T stretch oligomer. TM represents mutant 2D oligomer of the T stretch. Complexes 1 through 3 are indicated.

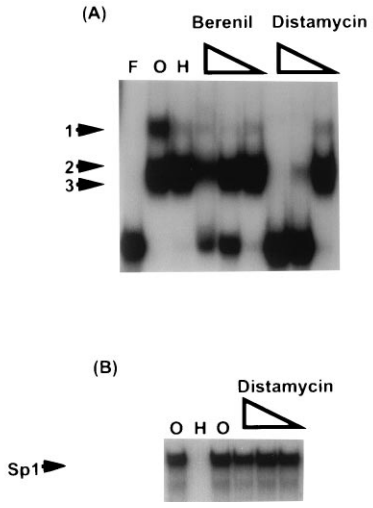

FIG. 5. T_{23}-binding activity has properties of HMG proteins. Minor-groove-binding drug EMSA competition low-molecular-weight nuclear proteins for the T stretch probe (A) the control consensus Sp1 probe (B) are shown. EMSA was performed with the LAP2 T stretch probe by preincubating the probe with either 0, 1 mM, 1 μM, or 1 nM diminazene aceturate (Berenil) or distamycin followed by binding with either heat-treated or non-heat-treated nuclear extracts and subsequent electrophoresis. All drug competitions were done with heat-treated extract. F, free probe; O, non-heat-treated extract; H, heat-treated extract.

doublet and bound the majority of the probe. Comparison of binding affinity to the wild type and 2D mutant probe detected a greater affinity for the wild-type sequence. Probe T was completely bound by protein, resulting in no free probe, in contrast to the situation observed with probe TM. Competition of each probe with nonlabeled probes revealed that T could compete with itself and TM more effectively, confirming its stronger affinity for the binding factors. Since complex 1 is seen only with probe T but is competed for by both T and TM, and since probe T contains an additional 14 bases of thymidine, complex 1 may be composed of a multimer of the proteins responsible for complex 2 and 3 binding. The binding of all the probe T primarily by complexes 2 and 3 and the inability to demonstrate complete self competition indicates that these proteins are present in great abundance.

T_{23}-binding protein has properties of the HMG I(Y) protein. The low apparent molecular weight and T oligomer affinity of complexes 2 and 3 (Fig. 4) suggested that the proteins present in these complexes may be related to HMG I(Y) proteins (19, 55). HMG molecules bind DNA in the minor groove (51) and are heat stable (58, 60). To test the heat stability of the T_{23}-binding activity, EMSA was performed with heat-treated extracts. The T_{23} binding activity was heat stable (Fig. 5A). In contrast, heat treatment of the extract eliminated Sp1 binding (Fig. 5B). Minor-groove-binding drugs block DNA binding by HMG proteins (60), and thus we tested the effect of two minor-groove-binding drugs on the T_{23} binding activity (Fig. 5). Both diminazene aceturate and distamycin inhibited T_{23} binding activity effectively (Fig. 5A), although distamycin had a greater inhibitory effect at these concentrations. In contrast, distamycin had no effect on binding by Sp1 (Fig. 5B), a major-groove-binding protein. The heat stability and minor-groove properties of complexes 2 and 3 further suggest that they contain factors related to HMG proteins. To determine whether the T_{23}-binding activity was immunologically related to HMG I(Y), we tested an anti-HMG I(Y) antiserum (55) for its ability to block the formation of any of the complexes seen with the T_{23} oligomer. Addition of anti-HMG I(Y) antiserum

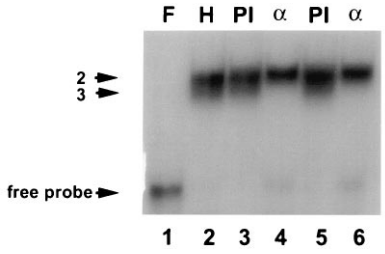

FIG. 6. Antiserum to HMG I(Y) blocks formation of complex 3. EMSA was performed with heat-treated nuclear extract (lanes 2 to 6). Anti-HMG I(Y) antiserum (lanes 4 and 6) or preimmune serum (lanes 3 and 5) was added either before (lanes 3 and 4) or after (lanes 5 and 6) incubation of the radioactively labeled poly(T) stretch probe with heat-treated nuclear extract. F, free probe; H, heat-treated extract; PI, preimmune serum; α, anti-HMG I(Y) antibody.

either before or after incubation with heat-treated nuclear extracts prevented the formation of complex 3 (Fig. 6), in contrast to the addition of preimmune serum, which had no effect on complex formation. The blocking of complex 3 formation with anti-HMG I(Y) antibody suggests an antigenic similarity between HMG I(Y) and the binding activity seen with complex 3.

HMG I(Y) facilitates Sp1 binding to the LAP2 promoter. HMG proteins are not transcriptional activators themselves but appear to function by assisting the binding of activators by altering DNA conformation. We tested the ability of HMG I(Y) to facilitate Sp1 binding to the LAP2 promoter by incubating a labeled LAP2 promoter fragment containing both the HMG and Sp1 sites with limiting amounts of Sp1 protein in the presence of poly(dG-dC) and titrating the concentration of a recombinant GST-HMG I(Y) fusion protein. Under these conditions, Sp1-DNA complex formation is barely detectable in the absence of added GST-HMG I(Y) (Fig. 7, lane 1). As increasing concentrations of GST-HMG I(Y) were added, the formation of the Sp1-containing complexes (complexes 1 and 2) was greatly increased. Stimulation of Sp1 binding requires the HMG I(Y) moiety, since added GST had no effect on Sp1 binding (Fig. 7, lane 11). Thus, the binding of HMG I(Y) to the

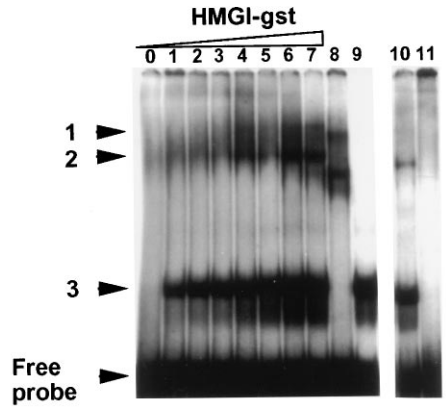

FIG. 7. HMG I(Y) facilitates Sp1 binding to the LAP2 promoter. Sp1 protein (1 footprint unit) was incubated with labeled LAP2 promtoter fragment (−189 to −39) containing the T_{23} region as well as the downstream CT element that binds Sp1 (lanes 1 to 8 and 10). Increasing concentrations of purified GST-HMG I(Y) fusion protein were included in the binding reactions (2 to 128 ng in twofold increments [lanes 2 to 7]). The mobility of the Sp1-DNA complex is shown in lane 8, from which poly(dC-dG) · poly(dC-dG) was omitted. The reaction mixture in lane 9 contained 8 ng of GST-HMG I(Y) and no Sp1 protein. Reaction mixtures containing equivalent concentrations of Sp1 protein and either 8 ng of GST-HMG I(Y) or 8 ng of GST protein were resolved in lanes 10 and 11, respectively.

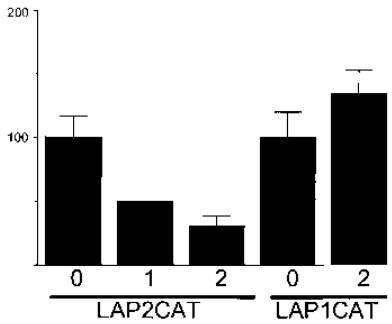

FIG. 8. Inhibition of the LAP2 promoter by antisense HMG I(Y). Vero cells were transfected with 1 μg of LAP1- or LAP2-CAT reporter plasmids as indicated and either 0, 1, or 2 μg of HMG I(Y) antisense plasmid as indicated. CAT activities are expressed as a percentage of the reporter activity in the absence of the antisense plasmid. All assays were performed in triplicate, and transfection efficiency was normalized to a cotransfected *lacZ* reporter. Error bars indicate one standard deviation.

LAP2 promoter facilitates the binding of Sp1, suggesting a mechanism for HMG I(Y) stimulation of LAP2 function.

Expression of antisense HMG I(Y) RNA specifically inhibits LAP2 promoter function. The results of the DNA-binding studies presented above indicate that the recombinant HMG I(Y) protein facilitated the binding of Sp1 to the LAP2 promoter. To test whether HMG I(Y) proteins affect LAP2 promoter function in vivo, the ability of the LAP2 promoter to drive the expression of the CAT gene was measured in cells in which the expression of the HMG I(Y) proteins was inhibited by antisense RNA. Earlier studies with this HMG I(Y) antisense plasmid demonstrated its ability to block viral induction of the human beta interferon gene. Full-length HMG I(Y) coding sequence was expressed in the antisense orientation in Vero cells that were cotransfected with 1 μg of either LAP2-CAT or LAP1-CAT reporter plasmid. Increasing concentrations of the HMG I(Y) antisense expression plasmid inhibited the activity of the LAP2 promoter as judged by the decrease in CAT activity in transfected cells (Fig. 8). Transfection efficiency was normalized to the expression of β-galactosidase from a separate reporter plasmid. Inhibition by the HMG I(Y) antisense plasmid was specific to the LAP2 promoter, since no inhibition of LAP1-CAT reporter plasmid was observed. This experiment provides direct evidence that the expression of the HMG I(Y) proteins is required for LAP2 promoter function in vivo.

DISCUSSION

Using DNase I footprinting, we have identified four regions in LAP2, designated FP1 through FP4, that bind to nuclear factors. Substitution mutations in these four regions were constructed and tested in transfection experiments. In all cases, mutations in the footprinted regions were found to confer changes in the transcriptional activity of LAP2, thus validating this approach to identify *cis*-acting elements. Mutations is FP1 (mutants 1A and 1C), FP2 (mutants 2B and 2C), and FP4 (mutant 4C) caused between two- and eightfold reductions in transcription of the LAP2-CAT reporter gene, indicating that these sequences function as activation elements. Two mutations, 3 and 4A, caused a significant increase (10- and 2-fold, respectively) in transcription, indicating that these sequences act as repression elements. Footprinting experiments with nuclear extracts from a neuroblastoma cell line yielded similar regions of protection. Thus, we were unable to identify sequences that might account for the transcription regulation observed in neuronal cells.

The identities of two of the *trans*-acting factors that bind to and regulate the LAP2 promoter have been determined. First, the GC-rich nature of this region led us to test the ability of the Sp1 transcription factor to bind to LAP2. Purified Sp1 bound to sequences in FP1, FP3, and FP4. The pattern of DNase I protection by purified Sp1 in FP1 was not identical to that observed with nuclear extract, suggesting that other protein factors were also bound to these regions. Evidence that Sp1 activates LAP2 transcription comes from experiments with *Drosophila* Schneider SL2 cells, in which transfection of a Sp1 expression plasmid caused an increase in LAP2 transcription (21). FP4 is uniquely protected by Sp1 (Fig. 2). Mutation 4C resulted in a fivefold decrease in transcription. This mutation had the strongest effect on transcription of any of the regions that bind to Sp1, perhaps as a result of its proximity to the start site of LAP2. Sp1 sites that are close to the transcription start site have previously been shown to have a stronger effect on transcription than do more distal Sp1 sites (48, 63). Sp1 is important for full induction of promoter activity in many cases (35, 42, 61, 62) and may also be involved in maintaining expression from constitutive promoters (4, 24, 38). Sp1 may maintain promoter function by constitutively binding to its site, thereby preventing methylation of the promoter (4, 24, 38) and elimination of methylation repression (6, 9, 25). Previous work has suggested that the HSV-1 genome is partially methylated during latency (17) and that the LAP2 region possesses many CpG islands (11), although the role of methylation in the function of the LAPs during latency remains to be elucidated.

Sp1 also bound to FP3 in vitro; however, mutation of this region resulted in a significant increase in LAP2 transcription. The increased expression observed with this mutant suggests that region 3 functions as a *cis*-acting repressor element. Factors other than Sp1 may be responsible for the footprint seen with nuclear extract. These factors may function by blocking Sp1 binding at region 3 and/or region 4, as has been described with Sp1 (1, 10, 20, 64) and other transcription factors (31). Alternatively, region 3 may function by directly recruiting a repressor protein (28).

The second *trans*-acting factor to be identified as a regulator of LAP2 was HMG I(Y). The LAP2 promoter in the KOS strain contains a stretch of 23 consecutive thymidine residues (T_{23}). This region of LAP2 even as naked DNA is not cut by DNase I, and therefore it was not possible to determine by DNase I protection assays if this region was bound by proteins in the HeLa nuclear extract. However, mutation of the T_{23} element resulted in a greater than 20-fold reduction in promoter activity (Fig. 3). To determine if this region was bound by a nuclear factor, EMSA was performed with a T_{23}-containing DNA oligonucleotide. An abundant nuclear factor that showed a greater affinity for the wild-type T_{23} sequence over the 2D mutant sequence was detected (Fig. 4), demonstrating a correlation between transcriptional activity and the ability of this *cis* element to bind specific proteins. We have shown that the DNA-binding properties of factors which bind to the T stretch include heat stability, binding competition with poly(dA-dT) · poly(dA-dT), and inhibition by the minor-groove-binding drugs distamycin and berenil (Fig. 5). These properties are shared by a number of proteins in the HMG class. Finally, one of the T_{23}-binding factors (complex 3) is blocked by a polyclonal antiserum specific for HMG I(Y) (Fig. 6). Taken together, these properties strongly suggest that one the factors that binds to the T stretch is related or identical to HMG I(Y).

The HMG I and HMG Y proteins are closely related splice variants that have been shown to interact with several promoters (19, 61, 62). Site-directed mutagenesis studies have re-

vealed that elimination of HMG I(Y)-binding sites results in diminished promoter activity (61, 62). HMG I(Y) has no intrinsic transcriptional activation activity (55), rather, it has been shown to transactivate promoters through mechanisms which facilitate the activity of other transcription factors. For example, HMG I(Y) is associated with nuclear scaffolding regions (51) and can mobilize the transcriptional repressor histone H1 from scaffold-associated regions in vitro (66), resulting in derepression of transcription from those elements. Also, binding of HMG I(Y) alters the structure of the bound DNA (18). This structural perturbation may result in bending of the DNA, bringing distant transcription factors already bound to the promoter into closer proximity, thus facilitating interaction between these factors and bringing more transactivating elements closer to the start of transcription. This increase in the proximity of transcription factors may result in stronger recruitment of the basal transcriptional machinery, resulting in higher levels of transcription. Mutation of the T_{23} element resulted in a more severe decrease in transcription than did mutation of any of the other footprinted regions, suggesting that this mutation may well disrupt synergistic interactions that occur between multiple elements in the LAP2 promoter.

The regulation of both LAP1 and LAP2 is of considerable interest because of their unique ability to escape the transcriptional silencing that occurs during latency. We show here that recombinant HMG I protein facilitates the binding of Sp1 to the LAP2 promoter. Whether this increase in Sp1 binding is related to changes in DNA structure has not been demonstrated but seems likely. The contribution of HMG I(Y) proteins to LAP2 promoter function in vivo was demonstrated by expressing HMG I(Y) antisense RNA. In this experiment, antisense HMG I(Y) inhibited LAP2 promoter function but not LAP1. These results suggest that HMG I(Y) activates LAP2 promoter function by facilitating transactivator binding, possibly Sp1, or by affecting the orientation of other bound factors on LAP2 DNA. A similar mechanism of action for HMG I(Y) has been shown with other transcription factors such as ATF2 and NF-κB (61).

The effect of mutation 2D on expression is much more pronounced than its effect on HMG I(Y) binding. This mutation eliminates 14 bases of the T stretch yet leaves 9 thymidines remaining at the 3′ end. The minimal AT stretch length required for HMG I(Y) binding is 6 bases (51), explaining why HMG I(Y) can still bind the mutant oligomer. However, the ability of HMG I(Y) to recruit transcription factors also depends on the phase of the HMG I(Y) site relative to the binding site of the other factors, including those bound to the regions that make up FP1 and FP4. Also, it is worth noting that the T_{23} stretch present in HSV-1 KOS is not present in other HSV-1 strains. In HSV-1 17, the T_{23} stretch is a smaller T_9 stretch. Our results led to the prediction that the T_9 stretch in HSV-1 17 would also bind HMG I(Y) and that mutation of the T_9 stretch would block both binding by HMG I(Y) and transcriptional regulation by HMG I(Y). Validation of this prediction awaits further experimentation.

The results presented in this study further delineate the sequences of LAP2 that are required for promoter function. Viruses with a precise deletion of LAP2 express the 2-kb LAT RNA during latent infections at a lower level than do wild-type viruses (8). This result suggests that LAP2 either acts as a functional promoter during latency or contributes to the activity of LAP1. Deletion of the 370-bp *Sty*I fragment, which removes much of the upstream LAP2 region (−588 to −218), has little or no effect on LAT expression during latent or lytic growth (39). However, this mutation leaves intact most of the LAP2 proximal promoter and in particular the LAP2 start site, the CT element, and the T_9 tract present in HSV-1 17, which our studies suggest are the critical LAP2 promoter elements. Studies of HSV-2 LAT expression suggest that the LAP2 region is important for enhancing the expression of LAP1 but that LAP2 itself may not be an independent promoter element (65). The DNA sequence of HSV-1 and HSV-2 in the LAP2 region is poorly conserved; however, it is worth noting that the LAP2 sequence in HSV-2, like HSV-1, lacks a recognizable TATA element and includes the CT-rich element but that unlike HSV-1, HSV-2 LAP2 lacks a poly(T) tract (34). It seems possible that an HMG protein with different sequence specificity will contribute to HSV-2 LAP2 activity. Our studies indicate that HSV-1 LAP2 has the ability to act as an independent promoter element in transfection assays and that it can drive the expression of the 2-kb LAT independently of LAP1 during lytic growth. Unraveling the exact role of HSV-1 LAP2 during latency, whether it acts as an independent promoter or as an enhancer of LAP1, will be facilitated by an increased understanding of the *cis*-acting elements that contribute to LAP2 function.

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REFERENCES

1. **Adnane, J., Z. Shao, and P. D. Robbins.** 1995. The retinoblastoma susceptibility gene product represses transcription when directly bound to the promoter. J. Biol. Chem. **270:**8837–8843.
2. **Batchelor, A. H., and P. O. O'Hare.** 1990. Regulation and cell-type-specific activity of a promoter located upstream of the latency-associated transcript of herpes simplex virus type 1. J. Virol. **64:**3269–3279.
3. **Bonham, K., and D. J. Fujita.** 1993. Organization and analysis of the promoter region and 5′ non-coding exons of the human c-src proto-oncogene. Oncogene **8:**1973–1981.
4. **Brandeis, M., D. Frank, I. Keshet, Z. Siegfried, M. Mendelsohn, A. Nemes, V. Temper, A. Razin, and H. Cedar.** 1994. Sp1 elements protect a CpG island from *de novo* methylation. Nature (London) **371:**435–438.
5. **Briggs, M. R., J. T. Kadonaga, S. P. Bell, and R. Tijan.** 1986. Purification and biochemical characterization of the promoter-specific transcription factor. Science **234:**47–52.
6. **Cedar, H.** 1988. DNA methylation and gene activity. Cell **53:**3–4.
7. **Chen, X. C., W. F. Goins, and J. C. Glorioso.** 1996. Unpublished data.
8. **Chen, X. C., M. C. Schmidt, W. F. Goins, and J. C. Glorioso.** 1995. Two herpes simplex virus type 1 latency-active promoters differ in their contributions to latency-associated transcript expression during lytic and latent infections. J. Virol. **69:**7899–7908.
9. **Christy, B. A., and G. A. Scangos.** 1986. Control of gene expression by DNA methylation, p. 3–29. *In* G. M. Malacinski (ed.), Molecular genetics of mammalian cells. Macmillan Publishing Co., New York.
10. **Chung, D. C., S. J. Brand, and L. G. Tillotson.** 1995. Mutually exclusive interaction between factors binding to adjacent Sp1 and AT-rich elements regulate gastrin gene transcription in insulinoma cells. J. Biol. Chem. **270:** 8829–8836.
11. **Coffin, R. S., M. K. Howard, and D. S. Latchman.** 1995. Altered dinucleotide content within the latently transcribed regions of the DNA of alpha herpes viruses—implications for latent RNA expression and DNA structure. Virology **209:**358–365.
12. **Croen, K. D., J. M. Ostrove, L. J. Dragovic, J. E. Smialek, and S. E. Straus.** 1987. Latent herpes simplex virus in human trigeminal ganglia: detection of an immediate early gene "anti-sense" transcript by in situ hybridization. N. Eng. J. Med. **317:**1427–1432.
13. **Deshmane, S. L., and N. W. Fraser.** 1989. During latency, herpes simplex virus type 1 DNA is associated with nucleosomes in a chromatin structure. J. Virol. **63:**943–947.
14. **Devi-Rao, G. B., S. A. Goddart, L. M. Hecht, R. Rochford, M. K. Rice, and E. K. Wagner.** 1991. Relationship between polyadenylated and nonpolyadenylated HSV type 1 latency-associated transcripts. J. Gen. Virol. **65:**2179–2190.
15. **Dignam, J. D., R. M. Lebovitz, and R. G. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated

mammalian nuclei. Nucleic Acids Res. **11:**1475–1485.
16. **Dobson, A. T., F. Sederati, G. Devi-Rao, W. M. Falanagan, M. J. Farrell, J. G. Stevens, E. K. Wagner, and L. T. Feldman.** 1989. Identification of the latency-associated transcript promoter by expression of rabbit β-globin mRNA in mouse sensory nerve ganglia latently infected with a recombinant herpes simplex virus. J. Virol. **63:**3844–3851.
17. **Dressler, G. R., D. L. Rock, and N. W. Fraser.** 1987. Latent herpes simplex virus type 1 DNA is not extensively methylated in vivo. J. Gen. Virol. **68:**1761–1765.
18. **Falvo, J. V., D. Thanos, and T. Maniatis.** 1995. Reversal of intrinsic DNA bends in the IFN beta gene enhancer by transcription factors and the architectural protein HMG I(Y). Cell **83:**1101–1111.
19. **Fashena, S. J., R. Reeves, and N. H. Ruddle.** 1991. A poly(dA-dT) upstream activating sequence binds high-mobility group I protein and contributes to lymphotoxin (tumor necrosis factor-β) gene regulation. Mol. Cell. Biol. **12:**894–903.
20. **Fischer, K.-D., A. Haese, and H. Nowock.** 1993. Cooperation of GATA-1 and Sp1 can result in synergistic transcriptional activation or interference. J. Biol. Chem. **268:**23915–23923.
21. **French, S. W., W. F. Goins, M. C. Schmidt, D. Montesanti, A. J. Kinniburgh, and J. C. Glorioso.** Submitted for publication.
22. **Goins, W. F., L. R. Sternberg, K. D. Croen, P. R. Krause, R. L. Hendricks, D. J. Fink, S. E. Straus, M. Levine, and J. C. Glorioso.** 1994. A novel latency-active promoter is contained within the herpes simplex virus type 1 U_L flanking repeats. J. Virol. **68:**2239–2252.
23. **Ho, D. Y., and E. S. Mocarski.** 1989. Herpes simplex virus latent RNA (LAT) is not required for latent infection in the mouse. Proc. Natl. Acad. Sci. USA **86:**7596–7600.
24. **Holler, M., G. Westin, J. Jiricny, and W. Schaffner.** 1988. Sp1 transcription factor binds DNA and activates transcription even when the binding site is CpG methylated. Genes Dev. **2:**1127–1135.
25. **Holliday, R.** 1987. The inheritance of epigenetic defects. Science **238:**163–170.
26. **Horton, R. M., S. N. Ho, J. K. Pullen, H. D. Hunt, Z. Cai, and L. R. Pease.** 1993. Gene splicing by overlap extension. Methods Enzymol. **217:**270–279.
27. **Ishii, S., X.-H. Xu, and R. H. Stratton.** 1985. Characterization and sequence of the promoter region of the human epidermal growth factor receptor gene. Proc. Natl. Acad. Sci. USA **82:**4920–4924.
28. **Johnson, A. D.** 1995. The price of repression. Cell **81:**655–658.
29. **Kadonaga, J. T., A. J. Courey, J. Ladika, and R. Tjian.** 1988. Distinct regions of Sp1 modulate DNA binding and transcriptional activation. Science **242:**1566–1570.
30. **Kageyama, R., G. T. Merlino, and I. Pastan.** 1989. Nuclear factor ETF specifically stimulates transcription from promoters without a TATA box. J. Biol. Chem. **264:**15508–15514.
31. **Kel, O. V., A. G. Romanschenko, A. E. Kel, E. Wingender, and N. A. Kolchanov.** 1995. A compilation of composite regulatory elements affecting gene transcription in vertebrates. Nucleic Acids Res. **23:**4097–4103.
32. **Kirkness, E. F., and C. M. Fraser.** 1993. A strong promoter element is located between alternative exons of a gene encoding the human γ-aminobutyric acid-type A receptor β3 subunit (GABRB3). J. Biol. Chem. **268:**4420–4428.
33. **Kolluri, R., T. A. Torrey, and A. J. Kinniburgh.** 1992. A CT promoter element binding protein: definition of a double-strand and a novel single-strand DNA binding motif. Nucleic Acids Res. **20:**111–116.
34. **Krause, P. R., J. M. Ostrove, and S. E. Straus.** 1991. The nucleotide sequence, 5′ end, promoter domain, and kinetics of expression of the gene encoding the herpes simplex virus type 2 LAT. J. Virol. **65:**5619–5623.
35. **Lee, Y.-H., M. Yano, S.-Y. Liu, E. Matsunaga, P. F. Johnson, and F. J. Gonzalez.** 1994. A novel *cis*-acting element controlling the rat CYP2D5 gene and requiring cooperativity between C/EPβ and an Sp1 factor. Mol. Cell. Biol. **14:**1383–1394.
36. **Leib, D. A., K. C. Nadeau, S. A. Rundle, and P. A. Schaffer.** 1991. Promoter of the latency-associated transcripts of herpes simplex virus type 1 contains a functional cAMP-response element: role of the latency-associated transcripts and cAMP in reactivation of viral latency. Proc. Natl. Acad. Sci. USA **88:**48–52.
37. **Lokensgard, J. R., D. C. Bloom, A. T. Dobson, and L. T. Feldman.** 1994. Long-term promoter activity during herpes simplex virus latency. J. Virol. **68:**7148–7158.
38. **Macleod, D., J. Charlton, J. Mullins, and A. P. Bird.** 1994. Sp1 sites in the mouse aprt gene promoter are required to prevent methylation of the CpG island. Genes Dev. **8:**2282–2292.
39. **Maggioncalda, J., A. Mehta, N. W. Fraser, and T. M. Block.** 1994. Analysis of a herpes simplex virus type 1 LAT mutant with a deletion between the putative promoter and the 5′ end of the 2.0-kilobase transcript. J. Virol. **68:**7816–7824.
40. **Margolis, T. P., D. C. Bloom, A. T. Dobson, L. T. Feldman, and J. G. Stevens.** 1993. Decreased reporter gene expression during latent infection with HSV LAT promoter constructs. Virology **197:**585–592.
41. **Mellerick, D. M., and N. Fraser.** 1987. Physical state of the latent herpes simplex virus genome in a mouse model system: evidence suggesting an episomal state. Virology **158:**265–275.
42. **Merika, M., and S. H. Orkin.** 1995. Functional synergy and physical interactions of the erythroid transcription factor GATA-1 with the Kruppel family proteins Sp1 and EKLF. Mol. Cell. Biol. **15:**2437–2447.
43. **Mester, J. C., P. M. Pitha, and J. C. Glorioso.** 1995. Antiviral activity of herpes simplex virus vectors expressing murine α1-interferon. Gene Ther. **2:**187–196.
44. **Rock, D. L., and N. W. Fraser.** 1985. Latent herpes simplex virus type 1 DNA contains two copies of the virion DNA joint region. J. Virol. **55:**849–852.
45. **Rock, D. L., A. B. Nesburn, H. Ghiasi, J. Ong, T. L. Lewis, J. R. Lokensgard, and S. Wechsler.** 1987. Detection of latency-related viral RNAs in trigeminal ganglia of rabbits infect with herpes simplex virus type 1. J. Virol. **61:**3820–3826.
46. **Saito, H., H. Kouhara, S. Kasayama, T. Kishimoto, and B. Sato.** 1992. Characterization of the promoter region of the murine fibroblast growth factor receptor 1 gene. Biochem. Biophys. Res. Commun. **183:**688–693.
47. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
48. **Segal, R., and A. J. Berk.** 1991. Promoter activity and distance constraints of one versus two Sp1 binding sites. J. Biol. Chem. **266:**20406–20411.
49. **Simmons, A., B. Slobeman, P. Speck, J. Arthur, and S. Efstathious.** 1992. Two patterns of persistence of herpes simplex virus DNA sequences in the nervous systems of latently infected mice. J. Gen. Virol. **73:**1287–1291.
50. **Smith, D. B., and K. S. Johnson.** 1988. Single step purification of polypeptides expressed in E. coli as fusions with glutathione S-transferase. Gene **67:**31–40.
51. **Solomon, M. J., F. Straus, and A. Varshavsky.** 1986. A mammalian high mobility group protein recognizes any stretch of six A-T base pairs in duplex DNA. Proc. Natl. Acad. Sci. USA **83:**1276–1280.
52. **Stevens, J. G., E. K. Wagner, G. B. Devi-Rao, M. L. Cook, and L. T. Feldman.** 1987. RNA complementary to a herpesviruses α gene mRNA is prominent in latently infected neurons. Science **255:**1056–1059.
53. **Strauss, F., and A. Varshavsky.** 1984. A protein binds to a satellite DNA repeat at three specific sites that would be brought into mutual proximity by DNA folding in the nucleosome. Cell **37:**889–901.
54. **Tamura, T., and K. Mikoshiba.** 1991. Role of a GC-rich motif in transcription regulation of the adenovirus type 2 IVa2 promoter which lacks typical TATA-box element. FEBS Lett. **282:**87–90.
55. **Thanos, D., and T. Maniatis.** 1992. The high mobility group protein HMG I(Y) is required for NF-κB-dependent virus induction of the human IFN-β gene. Cell **71:**777–789.
56. **Timmusk, T., K. Palm, M. Metsis, T. Reintam, V. Paalme, M. Saarma, and H. Persson.** 1993. Multiple promoters direct tissue specific expression of the rat BDNF gene. Neuron **10:**475–489.
57. **Wagner, E. K., G. Devi-Rao, L. T. Feldman, A. T. Dobson, Y. Zhang, W. F. Elanagan, and T. Stevens.** 1988. Physical characterization of the herpes simplex virus latency-associated transcript in neurons. J. Virol. **63:**1194–2002.
58. **Wang, D.-Z., P. Ray, and M. Boothby.** 1995. Interleukin 4-inducible phosphorylation of HMG I(Y) is inhibited by rapamycin. J. Biol. Chem. **270:**22924–22932.
59. **Wechsler, S. L., J. Nesvurn, N. Zwaagstra, and H. Ghiasi.** 1989. Sequence of the latency related gene of herpes simplex virus type 1. Virology **168:**168–172.
60. **Wegner, M., and F. Grummt.** 1990. Netropsin, distamycin, and berenil interact differentially with a high-affinity binding site for the high mobility group protein HMG-I. Biochem. Biophys. Res. Commun. **166:**1110–1117.
61. **Whitley, M. Z., D. Thanos, M. A. Read, T. Maniatis, and T. Collins.** 1994. A striking similarity in the organization of the E-selectin and beta interferon gene promoters. Mol. Cell. Biol. **14:**6464–6475.
62. **Wood, L. D., A. A. Farmer, and A. Richmond.** 1995. HMGI(Y) and Sp1 in addition to NF-κB regulate transcription of the MGSA/GROα gene. Nucleic Acids Res. **23:**4210–4219.
63. **Wu, L., and A. Berk.** 1988. Constraints on spacing between transcription factor binding sites in a simple promoter. Genes Dev. **2:**403–411.
64. **Xu, J., K. L. Thompson, L. B. Shephard, L. G. Hudson, and G. N. Gill.** 1993. T_3 receptor suppression of Sp1-dependent transcription from the epidermal growth factor receptor promoter via overlapping DNA-binding sites. J. Biol. Chem. **268:**16065–16073.
65. **Yoshikawa, T., L. R. Stanberry, N. Bourne, and P. R. Krause.** 1996. Downstream regulatory elements increase acute and latent herpes simplex virus type 2 latency-associated transcript expression but do not influence recurrence phenotype or establishment of latency. J. Virol. **70:**1535–1541.
66. **Zhao, K., E. Kas, E. Gonzalez, and U. K. Laemmli.** 1993. SAR-dependent mobilization of histone H1 by HMG-I/Y in vitro: HMG-I/Y is enriched in H1-depleted chromatin. EMBO J. **12:**3237–3247.
67. **Zwaagstra, J. C., H. Ghiasi, S. M. Slanina, A. B. Nesburn, S. C. Wheatley, K. Lillycrop, J. Wood, K. Latchman, K. Patel, and S. L. Wechsler.** 1990. Activity of herpes simplex virus type 1 latency-associated transcript (LAT) promoter in neuron-derived cells: evidence for neuron specificity and for a large LAT transcript. J. Virol. **64:**5019–5028.