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Localization of *cis*-Acting Sequences in the Latency-Related Promoter of Bovine Herpesvirus 1 Which Are Regulated by Neuronal Cell Type Factors and Immediate-Early Genes

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Bovine herpesvirus 1 (BHV-1) establishes a latent infection in sensory ganglionic neurons of cattle. During a latent infection, a single latency-related (LR) transcript is expressed. This observation suggested that DNA sequences in the LR promoter are positively regulated by neural cell type factors. The regulation of the LR gene was examined in neural cells as well as nonneural cells in transient assays. A 258-bp *Xba*I-*Sph*I fragment from the LR promoter *cis* activated the herpes simplex virus type 1 thymidine kinase promoter in rat pheochromocytoma (PC-12) cells and differentiated human (HCN1A) neurons. In contrast, *cis* activation was not observed with rat (Rat-2) fibroblasts, undifferentiated HCN1A cells, or bovine turbinate cells. Treatment of PC-12 cells with nerve growth factor increased transcriptional activity of the *Xba*I-*Sph*I fragment. Exonuclease III footprinting experiments suggested that nuclear factors bind to the *Xba*I-*Sph*I fragment. The immediate-early genes of BHV-1 *trans* activated the LR promoter, and DNA sequences 5′ to the *Xba*I-*Sph*I fragment were necessary for maximal stimulation. These results imply that neural-cell-type-specific factors and BHV-1 immediate-early genes positively regulate LR gene expression.

Bovine herpesvirus 1 (BHV-1) is a significant viral pathogen of cattle and can cause respiratory disease, abortions, or occasionally encephalitis (for a review, see reference 31). Like all members of the alphaherpesvirus subfamily, BHV-1 establishes a latent or persistent infection in sensory ganglionic neurons of the infected host (1, 10, 21, 22). The virus can persist in a latent state for the lifetime of the infected host or can periodically reactivate and cause extensive damage to the host. In contrast to the 70 to 80 viral genes expressed during a lytic infection of bovine cells, viral gene expression is severely impaired during a latent infection. One small region of the genome is transcriptionally active in latently infected neurons, and this region is designated the latency-related (LR) gene (14, 19–21). Transcripts originating from the LR gene accumulate in the nuclei of sensory neurons which are latently infected (14, 19–21). The LR transcript is also detected in lytically infected cells and accumulates late after infection (14).

The transcriptional promoter which regulates expression of the LR gene is contained within a 980-bp *Pst*I fragment (12). In primary cultures of rabbit neurons, the LR promoter is 10-fold more active than the simian virus 40 early promoter and enhancer. Viral infection also positively regulates LR promoter activity. Consequently, it was hypothesized that *cis*-acting sequences within the LR promoter are responsible for neuronal cell type expression and *trans* activation by viral or virus-induced factors.

In this study, two regions within the LR promoter which were necessary for efficient *trans* activation by BHV-1 immediate-early (IE) genes were identified. A 258-bp *Xba*I-*Sph*I fragment *cis* activated the herpes simplex virus type 1 (HSV-1) thymidine kinase (TK) promoter in cells of neuronal origin but not other cell types. However, this fragment does not have promoter activity and was not necessary for *trans* activation by BHV-1 IE genes. If neuronal cells were treated with nerve growth factor (NGF), transcriptional activity was stimulated slightly. If the *Xba*I-*Sph*I fragment was digested with *Alu*I, *cis* activation of the TK promoter was observed in neuronal cells only after treatment with NGF. When the *Xba*I-*Sph*I fragment was incubated with nuclear extracts from rat cells, protection from exonuclease III (ExoIII) digestion was observed, suggesting that nuclear factors bind sequences in the *Xba*I-*Sph*I fragment. These results indicated that distinct regions of the LR promoter are necessary for neuronal cell type expression and *trans* activation by IE genes.

MATERIALS AND METHODS

Cells. Bovine turbinate and Rat-2 cells were maintained as described previously (11, 12). PC-12 cells (rat pheochromocytoma [9]) were obtained from the American Type Culture Collection and maintained in RPMI media supplemented with 8% horse serum and 4% fetal bovine serum. HCN1A cells (human cortical neurons [23]) were maintained in Earle's modified Eagle medium supplemented with 15% fetal bovine serum. To differentiate HCN1A cells, cultures were treated with 0.5 mM 3-isobutylmethylxanthine (IBMX), 0.5 mM dibutyryl cyclic AMP (cAMP), and 25 ng of NGF per ml for 6 days (23). During differentiation, cells were fed every 3 days. Rat-2, BT, and PC-12 cells were treated with 25 ng of NGF per ml for the indicated times.

Cell transfection. Transfection of BT, Rat-2, and PC-12 cells was carried out by calcium phosphate precipitation as described previously (11, 12). HCN1A cells were transfected by the Polybrene method (18).

CAT assays. Forty hours posttransfection, cells were washed in phosphate-buffered saline and a cell-free lysate was prepared by three freeze-thaw cycles in 0.25 M Tris (pH 7.8). Chloramphenicol acetyltransferase (CAT) enzymatic activity was measured as described previously (11, 12). After

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thin-layer chromatography (TLC), the amount of [^{14}C]chloramphenicol (CM), acetylated or unacetylated, was measured with an Ambis radioanalytical device.

Preparation of nuclear extracts. Nuclear extracts were prepared from mammalian cells by the procedure of Dignam et al. (7). Protein concentrations of the various extracts were measured (4), and the extracts were subsequently frozen at −120°C.

Plasmid constructs. Plasmids p0.95cat/1, p0.95cat/6, pΔXhoI, and p0.5Xba/47 were described previously (12). Plasmids pSV2cat, pA10cat2, and pSV0cat were obtained from Bruce Howard. To construct pAXP/1, the unique *Xho*I site in pΔXhoI was converted to a *Bgl*II site by addition of synthetic *Bgl*II linkers and the 531-bp *Xho*I-*Hin*dIII fragment (nucleotides [nt] 451 to 981) was released by digestion with *Bgl*II-*Hin*dIII and subsequently inserted into a pA10cat2 vector digested with *Bgl*II and *Hin*dIII. Prior to cloning, the pA10cat2 vector was purified in a polyacrylamide gel to eliminate simian virus 40 early promoter sequences. The plasmid pAXS/1 was constructed by digesting pAXP/1 with *Sph*I and *Hin*dIII, filling in the ends with Klenow fragment, and subsequently ligating with T4 ligase. For a summary of these constructs, see Fig. 1.

For construction of enhancer plasmids, a CAT construct containing the HSV-1 TK promoter was used (pBLcat/4 [26]). Plasmid p0.95cat/1 was digested with *Xba*I, which released nt 523 to 981 as an *Xba*I fragment. This was possible since the *Sma*I-*Pst*I sequences of the pUC-19 polylinker, including an *Xba*I site, are adjacent to nt 981 of the LR promoter. nt 523 to 981 of the LR promoter were cloned into pBLcat/4 at the *Xba*I site, and a clone with nt 981 adjacent to the TK promoter was selected by restriction mapping. This construct was digested with *Sph*I, which released the *Xba*I-*Sph*I site of the LR promoter (523 to 781) by virtue of an *Sph*I site in the polylinker of pBLcat/4. The resulting linearized plasmid was recircularized with T4 ligase and designated pBL/Sph−. The released *Sph*I fragment (*Xba*I-*Sph*I) was cloned into the *Sph*I site of pBLcat/4, and the resulting construct was designated pBL/Sph+. The orientation of the fragment was determined by digestion with *Xba*I. The *Hin*dIII-*Xba*I fragment of the LR promoter (1 to 523) was cloned into the *Hin*dIII-*Xba*I sites of pBLcat/4 and designated pBL/H-Xba. For a summary of these constructs, see Fig. 3. All DNA fragments were purified in 5% polyacrylamide gels and electroeluted. Plasmids were purified from bacteria by alkaline lysis followed by two CsCl density centrifugations (25).

Plasmids containing the BHV-1 IEtu1 region (c601) and E2.6 were obtained from M. Schwyzer (28, 29). To prepare a construct that expressed only IE4.2, c601 was digested with *Sal*I plus *Hin*dIII, the ends were made blunt with Klenow fragment, the large fragment was purified by agarose gel electrophoresis, and the fragment was ligated with T4 DNA ligase. For a summary of these constructs, see Fig. 2.

Binding assays for ExoIII digestion. The *Xba*I-*Sph*I fragment was labeled at its 5′ phosphate ends by incubating with polynucleotide kinase and [γ-^{32}P]ATP. The fragment was subsequently digested with *Alu*I, and the individual fragments were purified in polyacrylamide gels as described above. Binding volumes for ExoIII digestion were 50 μl. The reaction mixtures contained the following components: 0.5 to 1 ng of end-labeled DNA (10,000 to 20,000 cpm), 45 mM KCl, 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.8), 0.1 mM EDTA, 0.5 mM dithiothreitol, 3 μg of poly(dI-dC), and 15 μg of nuclear extract. After the reaction was incubated for 20 min at 30°C, $MgCl_2$ was added to a final concentration of 5 mM and 375 U of ExoIII was also added. This is the amount of ExoIII that yielded little or no background when the fragments were incubated with just bovine serum albumin (data not shown). Reaction mixtures were incubated for an additional 20 min at 30°C, and reactions were subsequently terminated by addition of phenol-$CHCl_3$-isoamyl alcohol (50:49:1). After ethanol precipitation, the products were separated on a 6% denaturing polyacrylamide gel.

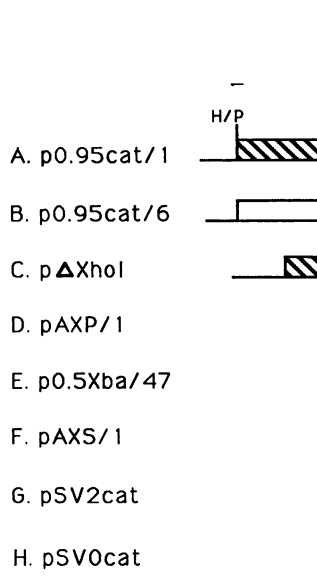
For competition footprinting assays with the respective oligonucleotides, 20 or 200 ng of the double-stranded oligonucleotide was incubated with the nuclear extract mixture for 20 min at 37°C. The labeled fragment was subsequently added, and the reaction mixture was incubated as described above.

PC-12, Rat-2, or HCN1A cells were transfected with the designated plasmids (10 μg). Twenty-four hours posttransfection, some cultures of Rat-2 cells or PC-12 cells were treated with 25 ng of NGF per ml since this concentration was shown to induce differentiation of PC-12 cells as well as induce early-response genes (5, 9, 24). For differentiation of HCN1A cells, cultures were treated with NGF (25 ng/ml), IBMX (0.5 mM), and forskolin (0.5 mM) for 6 days prior to transfection (23). At 40 h posttransfection, total cell lysates were prepared and CAT enzymatic activity was measured in the presence of [^{14}C]CM (40 μg of protein, 1 h, 37°C). For HCN1A cells, extracts derived from an equivalent number of cells (2×10^4) were used to measure CAT activity (4 h, 37°C). The various forms of CM were separated by TLC and quantified.

The 21-bp oligonucleotide which contains three AP-1 or tetradecanoyl phorbol acetate-responsive elements (TRE) is 5′-TGAGTCATGAGTCATGAGTCA-3′ (2). The 39-bp oligonucleotide which contains three cAMP-responsive elements (CRE) is 5′-GATCTGACGTCATGACTGACGTCAT GACTGACGTCATCA-3′ (17). The 36-bp oligonucleotide which contains three octamer (Oct)-binding sites is 5′-GAT CATGCAAATGATCATGCAAATGATCATGCAAAT-3′ (8).

RESULTS

Localization of the minimal LR promoter. To localize regions of the LR promoter necessary for transcriptional activity, restriction fragments from the LR promoter were inserted at the 5′ terminus of a plasmid carrying a promoter but not CAT and transcriptional activity was measured in bovine turbinate (BT) cells, rat pheochromocytoma (PC-12) cells, or rat (Rat-2) fibroblasts. Previous results indicated that the LR promoter was an efficient promoter in sensory neurons but not other cell types (12). However, these studies did not localize elements in the LR promoter which were necessary for efficient transcription in neural cells. As previously demonstrated, LR promoter activity had strict orientation preferences in all cell lines tested since p0.95cat/1 was an efficient promoter but p0.95cat/6 was not (Fig. 1). In BT and Rat-2 cells, the simian virus 40 early promoter and enhancer (pSV2cat) was five to six times more efficient as a promoter than was p0.95cat/1. In contrast, p0.95cat/1 had higher promoter activity in rat neuroblastoma (PC-12) cells. A construct containing the *Xho*I-*Pst*I fragment (nt 451 to 981; pAXP/1) had promoter activity similar to that in pΔXhoI in BT, PC12, and Rat-2 cells. If the *Xho*I-*Xba*I fragment was removed (nt 451 to 523; p0.5Xba/47), promoter activity was abolished. When sequences between *Sph*I-*Pst*I (nt 812 to 981) were deleted, promoter activity was eliminated. These



	%acetylated CM		
	BT	Rat-2	PC-12
A. p0.95cat/1	12.5 ± 4.5	10.2 ± 4.1	64.2 ± 19.2
B. p0.95cat/6	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.1
C. pΔXhoI	59.2 ± 12.3	6.3 ± 1.9	39.5 ± 19.2
D. pAXP/1	54.4 ± 29.8	7.8 ± 3.5	33.6 ± 13.5
E. p0.5Xba/47	0.5 ± 0.3	0.2 ± 0.1	6.4 ± 1.4
F. pAXS/1	0.7 ± 0.3	0.3 ± 0.1	0.2 ± 0.1
G. pSV2cat	65.8 ± 15.7	68.4 ± 19.2	49.7 ± 13.4
H. pSV0cat	0.2 ± 0.1	0.2 ± 0.1	0.5 ± 0.2

FIG. 1. Localization of LR promoter activity. Cells were transfected with various plasmids (10 μg). At 40 h posttransfection, total cell lysates were prepared and CAT activity was measured in the presence of [^{14}C]CM (40 μg of protein, 1 h, 37°C). The various forms of CM were separated by TLC and quantified. Restriction enzyme sites are: *Pst*I (P), *Xho*I (X), *Alu*I (A), *Xba*I (Xb), *Ssp*I (Ssp), *Sph*I (S), and *Hin*dIII (H). The arrow indicates the direction of LR transcription and the approximate start site. Nucleotide numbering is from the published sequence (14). Results are the averages from three independent experiments.

results indicated that in bovine cells and rat cells (neural or nonneural), the minimal LR promoter was localized to the *Xho*I-*Pst*I fragment (nt 451 to 981).

***trans* Activation of LR promoter by BHV-1 IE genes.** To analyze the ability of BHV-1 IE genes to regulate LR promoter activity, cotransfection experiments were performed with BT cells. Previous experiments demonstrated that LR promoter activity and steady-state levels of LR RNA accumulate at late times after infection, suggesting that viral genes or virus-induced factors were responsible for activation (12, 14). BT cells were cotransfected with the respective LR promoter constructs and a plasmid containing one or more of the BHV-1 IE genes (for a summary of the BHV-1 IE genes used in this study, see Fig. 2). A plasmid containing IEtul (c601) or just IEtul/4.2 positively regulated the intact LR promoter (p0.95cat/1) sixfold. If the *Xho*I fragment (nt 307 to 452; pΔXhoI) or *Pst*I-*Xho*I fragment (nt 1-452; pAXP/1) was deleted, *trans* activation by c601 and IEtul/4.2 was reduced almost threefold. In contrast, E2.6 *trans* activated pΔXhoI as efficiently as the intact LR promoter. However, transactivation of pAXP/1 by E/2.6 was reduced more than twofold. These results demonstrated that the *Xho*I fragment of the LR promoter was necessary for *trans* activation by IEtul/4.2 and that sequences within the *Pst*I-*Xba*I region were necessary for *trans* activation by E/2.6.

***cis* Activation of the TK promoter by LR promoter fragments.** To determine whether regions of the LR promoter have transcriptional enhancer activity, DNA fragments which spanned the LR promoter were inserted adjacent to an HSV-1 TK promoter construct (pBLcat/4) and transcriptional activity was measured in PC-12, BT, or Rat-2 cells. The PC-12 (rat pheochromocytoma) cell line has features which are similar to those of neurons, can be differentiated into neuronlike cells by numerous agents, and has widely been used to study neural-cell-type-specific events (5, 9, 24). Rat-2 cells are a fibroblast cell line (11) and served as a nonneural cell type to test for cell-type-specific *cis*-acting sequences. In PC-12 cells, the *Xba*I-*Sph*I fragment of the LR promoter (pBL/Sph+) enhanced TK promoter activity 15-fold (Fig. 3). Constructs containing either the *Sph*I-*Pst*I fragment (nt 812 to 981; pBL/Sph−) or the *Hin*dIII-*Xba*I fragment (nt 1 to 523; pBL/H-Xba) had promoter activity that was at least threefold higher than that of pBLcat/4 in PC-12 cells. In Rat-2 cells, however, promoter activities of pBL/Sph+ and pBL/Sph− were lower than that of pBLcat/4. In BT cells, promoter activity of pBL/Sph+ was slightly higher than that of pBLcat/4. These results demonstrated that an *Xba*I-*Sph*I fragment *cis* activated the TK promoter in rat pheochromocytoma (PC-12) cells but not in nonneural (Rat-2 or BT) cells.

Effect of NGF on LR enhancer activity. To examine the effect NGF has on LR enhancer activity, cells were transfected with the various enhancer constructs, in the presence of NGF, and promoter activity was measured. In PC-12 cells, promoter activity of pBL/Sph+ was slightly higher after cells were treated with NGF (Table 1). Promoter activity of the other enhancer constructs was not altered in Rat-2 or PC-12 cells by NGF treatment. These results demonstrated that NGF stimulated enhancer activity of the *Xba*I-*Sph*I fragment in PC-12 cells but not Rat-2 cells.

To further explore the effects of NGF on LR enhancer activity, the human cortical neuron cell line, HCN1A, was transfected with the various constructs and promoter activity was measured in undifferentiated or differentiated cells. HCN1A cells were obtained from an individual with unilateral megalencephaly (23). These cells express neuron-specific but not glia-specific markers and can be differentiated with dibutyryl cAMP, IBMX, and NGF. After differentiation, cells display a mature neuronal morphology with numerous long, extensively branched processes with spines and varicosities (11a, 23). In undifferentiated HCN1A cells,

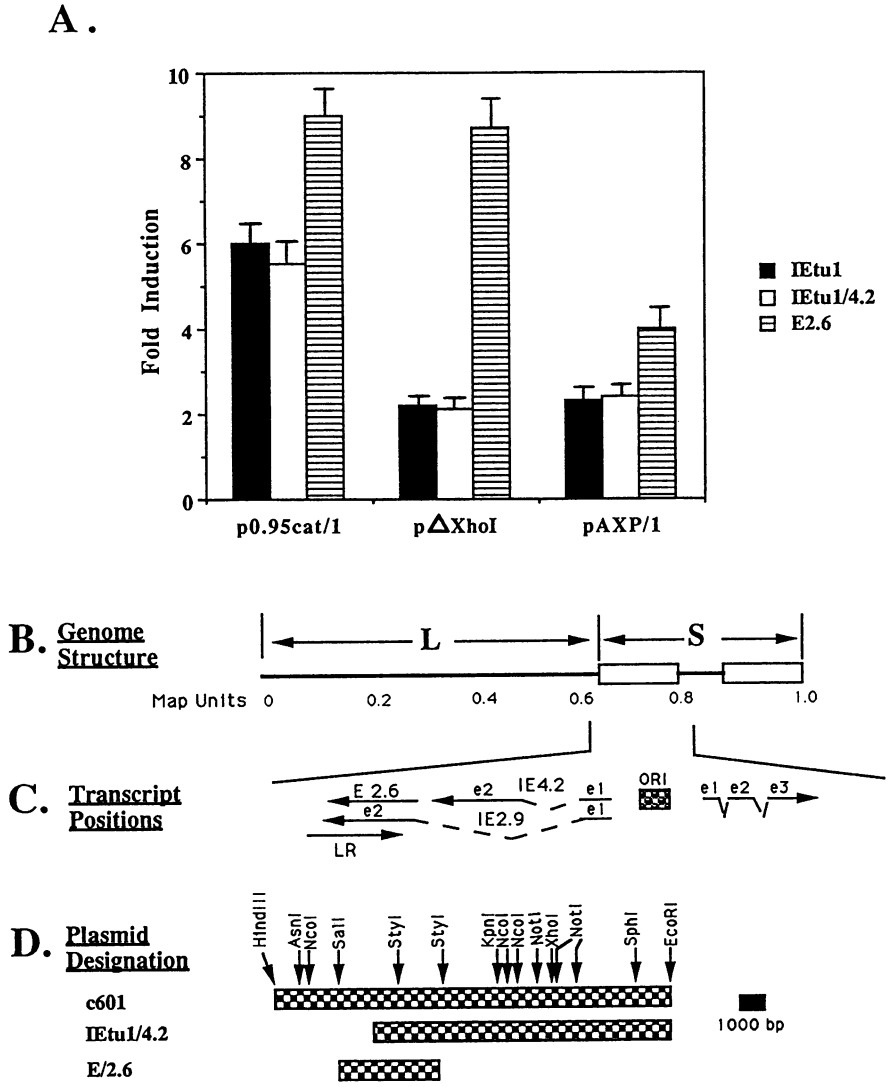


FIG. 2. *trans* Activation of the LR promoter by BHV-1 genes. (A) BT cells were transfected with 10 μg of the respective promoter-CAT plasmids and 8 μg of a plasmid containing BHV-1 IE genes. At 48 h posttransfection, CAT enzymatic activity was measured. Values are percents acetylated CM in samples cotransfected with LR promoter constructs and the designated IE plasmids divided by control values (without any IE construct). Values are averages for five independent experiments. (B) Diagram of BHV-1 genome. (C) Positions of IE genes. IE4.2 is the 4.2-kb RNA transcript derived from IEtu1. IE2.9 is the 2.9-kb transcript. Solid lines in the transcript position map represent exons (e1 or e2). Dashed lines indicate the positions of introns and arrows indicate the direction of transcription. (D) IE containing plasmids used in this study. Restriction map of the IEtu1 region is derived from reference 29. E/2.6 is an early 2.6-kb transcript which encodes a protein identical to IE/2.9 (28).

pBLcat/4 had higher activity compared with those of LR enhancer constructs (Table 1). In differentiated HCN1A cells, pBL/Sph+ had 10-fold-higher promoter activity than pBLcat/4. Thus, in differentiated human neurons, the TK promoter was *cis* activated by the *Xba*I-*Sph*I fragment.

Localization of elements in the *Xba*I-*Sph*I fragment which are necessary for enhancer activity. To begin to understand which regions of the *Xba*I-*Sph*I fragment were necessary to *cis* activate the TK promoter in PC-12 cells, the fragment was digested with *Alu*I and individual fragments were tested for enhancer activity. The results indicated that the *Alu*I-*Sph*I fragment (pBL/Sph2A) stimulated TK promoter activity approximately twofold in PC-12 cells as well as Rat-2 cells compared with pBLcat/4 (Fig. 4). The *Xba*I-*Alu*I fragment (pBL/Xbal1C) had little, if any, effect on TK promoter activity in PC-12 or Rat-2 cells. When PC-12 cells were treated with NGF, promoter activities of pBL/Sph2A and pBL/Xba1C were stimulated at least twofold compared with those for normal cells. If Rat-2 cells were treated with NGF, promoter activity of pBL/Sph1A and pBLcat/4 was reduced approximately twofold compared with those for normal cells. Individual *Alu*I fragments were not efficient enhancer elements in PC-12 cells compared with the intact *Xba*I-*Sph*I fragment, suggesting that sequences near the *Alu*I site are necessary for transcriptional enhancer activity in PC-12 cells.

Binding of cellular factors to the LR enhancer fragment. ExoIII footprinting assays were conducted to determine

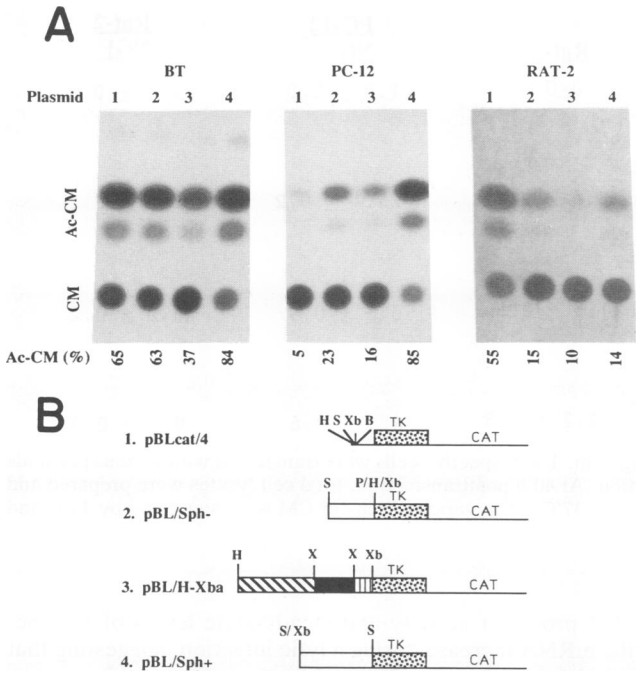


FIG. 3. Localization of DNA sequences in the LR promoter with transcriptional enhancer activity. (A) Cells were transfected with various plasmids (10 μg). At 40 h posttransfection, total cell lysates were prepared and CAT activity was measured in the presence of [^{14}C]CM (40 μg of protein, 1 h, 37°C). The various forms of CM were separated by TLC, percents acetylated CM were quantified, and these values are given. The numbers above the lanes refer to the plasmids presented in panel B. (B) Diagram of the plasmids used in this study. Restriction sites are *Hin*dIII (H), *Sph*I (S), *Xba*I (Xb), *Bam*HI (B), and *Xho*I (X).

whether cellular factors bound to the *Xba*I-*Sph*I fragment. ExoIII is a 3′ to 5′ exonuclease which has been used successfully to analyze interactions between regulatory elements and DNA binding proteins in crude nuclear extracts (30). When nuclear extracts from PC-12 or Rat-2 cells were incubated with the *Xba*I-*Alu*I fragment, at least three distinct regions were protected (Fig. 5; for a summary of binding, see Fig. 6). If the *Xba*I-*Alu*I fragment was incubated with nuclear extracts treated with NGF, enhanced binding was observed in PC-12 cells but not in Rat-2 cells. Oligonucleotides containing consensus sites for Oct, CRE, or TRE were incubated with nuclear extracts (20 min) prior to addition of the radiolabeled *Xba*I-*Alu*I fragment to determine whether these respective transcription factors interact with the labeled *Xba*I-*Alu*I fragment. Addition of the CRE oligonucleotide had no effect on the ExoIII protection pattern in Rat-2 or PC-12 cells. The Oct oligonucleotide inhibited binding slightly to regions A and B in PC-12 and Rat-2 cells. The TRE oligonucleotide also reduced binding slightly to region C in nuclear extracts prepared from PC-12 cells but not Rat-2 cells. An examination of the sequence (Fig. 6) did not reveal a TRE in region C or an Oct-binding site in region A or B, suggesting that the inhibition observed was nonspecific or that factors that bind to sequences in the *Xba*I-*Alu*I fragment also have an affinity for a TRE consensus or Oct consensus.

When the *Alu*I-*Sph*I fragment was incubated with nuclear extracts prepared from PC-12 or Rat-2 cells, protection from ExoIII digestion could be observed in three separate domains (Fig. 5 and 6). NGF-inducible binding to the entire fragment was observed for PC-12 and Rat-2 cells. However, qualitative differences in ExoIII protection patterns and the protection patterns obtained with nuclear extracts prepared from untreated cells were not obvious. When the *Alu*I-*Sph*I fragment was incubated with nuclear extracts prepared from PC-12 cells, subtle differences in ExoIII protection profiles were observed in regions E and F compared with results for Rat-2 cells (Fig. 5; denoted by asterisks). Even after treatment with NGF, the novel protection pattern was detected in region F. Within region E, two Oct-like elements are flanked by putative TATA boxes (region F) and a CCAAT box (Fig. 6). When an oligonucleotide containing a consensus Oct-binding site was used as a competitor in footprinting experiments, binding to region E was diminished compared with ExoIII protection patterns in control samples. In Rat-2 cells, the Oct oligonucleotide inhibited binding to two Oct-like elements in region E as well as surrounding sequences. In PC-12 cells, inhibition of binding to region E by the Oct oligonucleotide was confined to the two overlapping Oct-like elements. Oligonucleotides containing a consensus TRE or CRE did not alter the ExoIII protection pattern in region D, E, or F in the *Alu*I-*Sph*I fragment. In summary, binding of nuclear factors to the *Xba*I-*Sph*I fragment was complex, and subtle differences in ExoIII protection profiles were observed for PC-12 cells versus Rat-2 cells.

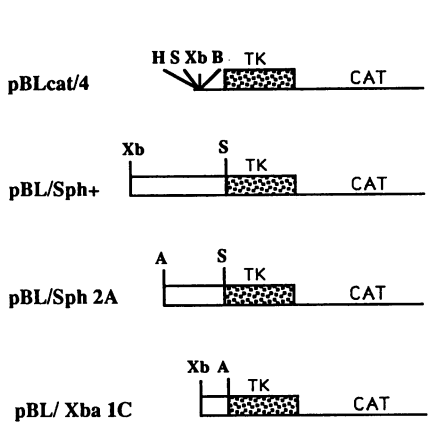
DISCUSSION

In this study, we identified a 258-bp *Xba*I-*Sph*I fragment from the LR promoter which *cis* activated the HSV-1 TK promoter in cells of neuronal origin. NGF stimulated transcription in neuronal cells but not other cell types. The *Xba*I-*Sph*I fragment did not have promoter activity in any cell type tested, suggesting that other regions of the LR promoter were required for promoter activity. ExoIII footprinting experiments indicated that nuclear factors inter-

TABLE 1. Effect of NGF on LR enhancer activity[a]

Plasmid	Mean (SD) for cell type:					
	PC-12	PC-12 treated with NGF	Rat-2	Rat-2 treated with NGF	HCN1A	
					Undifferentiated	Differentiated
pBLcat/4	1.0 ± 0.3	1.0 ± 0.4	1.0 ± 0.2	1.0 ± 0.3	1.0 ± 0.3	1.0 ± 0.5
pBL/Sph−	3.2 ± 1.8	2.6 ± 1.3	0.2 ± 0.1	0.3 ± 0.2	0.2 ± 0.1	1.2 ± 0.8
pBL/Sph+	17.2 ± 4.8	23.1 ± 3.1	0.6 ± 0.4	0.2 ± 0.3	0.5 ± 0.2	10.5 ± 2.9
pBL/H-XbaI	2.6 ± 0.8	2.2 ± 0.9	0.4 ± 0.1	0.5 ± 0.2	0.9 ± 0.3	1.2 ± 0.4

[a] Values given are a comparison of promoter activity in the respective enhancer constructs to that in pBLcat/4. Each value represents the average from at least three independent experiments. For details, see Materials and Methods.



	PC-12	Rat-2	PC-12 NGF	Rat-2 NGF
pBLcat/4	1.0±0.3	1.0±0.4	1.0 ± 0.3	1.0 ± 0.3
pBL/Sph+	17.2 ± 2.8	0.6 ± 0.4	23.1 ± 0.2	0.5 ± 0.1
pBL/Sph 2A	2.6 ± 0.6	1.9 ± 0.5	6.5 ± 0.9	0.9 ± 0.2
pBL/ Xba 1C	1.4 ± 0.5	1.2 ± 0.5	4.1 ± 0.6	0.9 ± 0.3

FIG. 4. Enhancer activity of *Alu*I subclones derived from *Xba*I-*Sph*I fragment. The respective cells were transfected with various plasmids (10 μg). In some samples, NGF (25 ng/ml) was added at 16 h posttransfection. At 40 h posttransfection, total cell lysates were prepared and CAT activity was measured in the presence of [^{14}C]CM (40 μg of protein, 1 h, 37°C). The various forms of CM were separated by TLC and quantified.

acted with the *Xba*I-*Sph*I fragment, and subtle differences were observed for PC-12 cells versus Rat-2 cells. LR promoter sequences 5′ to the *Xba*I-*Sph*I fragment were required for *trans* activation by BHV-1 IE genes. These data suggested that a transcriptional enhancer in the LR promoter is active in neuronal cells and that other sequences near the 5′ terminus of the promoter were *trans* activated by BHV-1 IE genes.

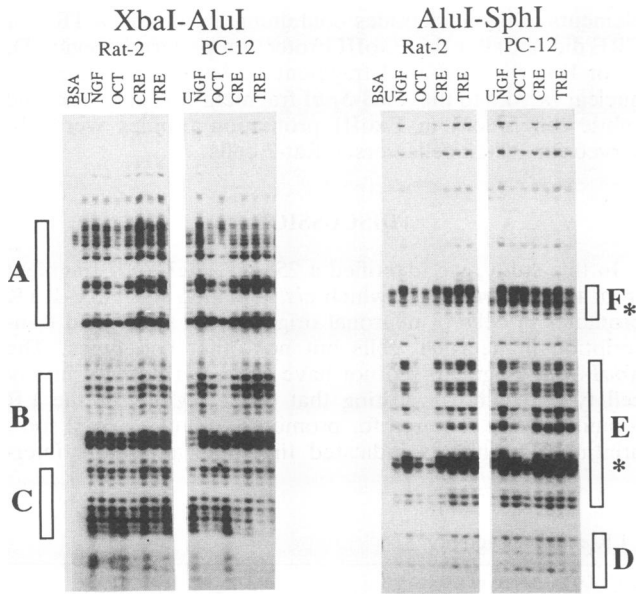


FIG. 5. Analysis of nuclear proteins which interact with *Xba*I-*Sph*I fragment by ExoIII digestion. The respective *Alu*I fragments were incubated with 15 μg of bovine serum albumin (BSA), nuclear extract from Rat-2 or PC-12 cells (U), or nuclear extract prepared from cells treated with NGF (25 ng/ml, 24 h [NGF]). For competition experiments, synthetic oligonucleotides (20 or 200 ng) were incubated for 20 min with nuclear extracts prepared from cells treated with NGF and the radioactive fragment was subsequently added. ExoIII footprinting was conducted as described in Materials and Methods.

LR promoter activity and steady-state levels of LR-specific mRNA increase during a lytic infection, suggesting that a viral gene or a virus-induced factor mediates this process (12, 14). This study indicated that IEtu1 stimulated LR promoter activity (Fig. 2). Sequences within the *Xho*I-*Xho*I fragment were necessary for *trans* activation by IEtu1/4.2. The *Xho*I-*Xho*I fragment is a negative regulatory element in bovine cells, suggesting that other factors, in addition to IEtu1/4.2, mediate LR transcription by interacting with DNA sequences in the fragment. The *Pst*I-*Xba*I fragment (nt 1 to 307) was necessary for *trans* activation by E/2.6, implying that *trans* activation by IE genes was mediated by at least two independent DNA sequences. These results suggested that IEtu1 products coordinate LR gene expression during a lytic infection cycle and that neuronal factors direct high levels of expression during a latent infection.

Attempts to localize a LR promoter fragment which was active in PC-12 cells but not BT or Rat-2 cells were not successful, suggesting that requirements for basal promoter activity were similar for all cell types. In PC-12 cells and differentiated human (HCN1A) neurons, the *Xba*I-*Sph*I fragment increased TK promoter activity more than 10-fold. The *Sph*I-*Pst*I fragment (nt 812 to 981; pBL/SphI−) and *Hin*dIII-*Xba*I fragment (nt 1 to 523; pBL/H-Xba) also increased TK promoter activity three- to fourfold in PC-12 cells (Fig. 3). However, these fragments did not *cis* activate the TK promoter in BT cells, Rat-2 cells, or undifferentiated HCN1A cells. The finding that treatment of HCN1A and PC-12 cells with NGF led to higher promoter activity of pBL/Sph+ but not pBLcat/4 strengthens the hypothesis that cell-type-dependent interactions were crucial for *cis*-acting functions of the *Xba*I-*Sph*I fragment since NGF is critical for neuron differentiation and maintenance of neuron phenotype (for a review, see reference 15). In summary, the *Xba*I-*Sph*I fragment in the LR promoter was a strong transcriptional enhancer in cells of neuronal origin when linked to the TK promoter. The finding that other regions of the LR promoter have weak enhancer activity in PC-12 cells implied that expression of the LR gene in neural cells is not entirely dependent on one *cis*-acting sequence.

Several discrete regions within the *Xba*I-*Sph*I fragment were bound by nuclear factors as judged by ExoIII footprint-

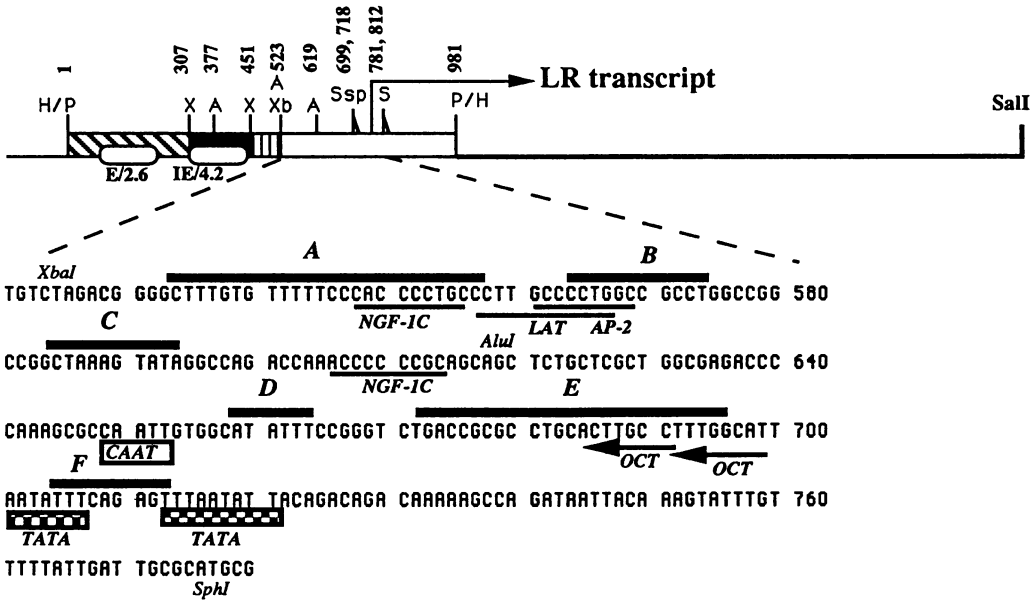


FIG. 6. Summary of ExoIII protection experiments from LR enhancer region. (A) Restriction map of the LR promoter region and approximate start site of LR transcription. Regions of the LR promoter which were necessary for *trans* activation by E/2.6 and IE/4.2 are indicated. Restriction enzyme sites within the LR promoter are *Pst*I (P), *Hin*dIII (H), *Xho*I (X), *Xba*I (Xb), *Alu*I (A), *Ssp*I (Ssp), and *Sph*I (S). (B) DNA sequence of the LR promoter from the coding strand of the LR transcript was derived from the published sequence (14). The letters A to F above the sequence correspond to regions protected from ExoIII digestion after incubation with nuclear extracts prepared from PC-12 cells treated with NGF. Shown is the position of the NGF 1C binding site (NGF-1C; GCGGGGGCG [5]). Both NGF-1C sites contain seven of nine matches and are present on the complementary DNA strand. The AP-2 site matches the consensus (GCCNNNGGC [27]). Positions of the Oct-like elements have an arrow in the direction of the consensus, are located on the complementary DNA strand, and contain two mismatches from an Oct-1 consensus (ATGCAAAT; for a review, see reference 8). A region of the HSV-1 latency-associated transcript promoter which is within the neuron-specific enhancer (LAT [3]) has 9 of 11 bases matching but is 3′ to 5′ with respect to the HSV-1 sequence. Positions of the TATA-like elements and the CAAT box are denoted by the boxes. Regions of the LR promoter which are regulated by BHV-1 IE genes are also depicted.

ing experiments (Fig. 5). However, only minor differences in ExoIII protection patterns were observed when the *Xba*I-*Sph*I fragment was incubated with nuclear extracts from PC-12 cells versus Rat-2 cells. In PC-12 cells, NGF induces a family of genes which are designated the early-response genes (5). A member of this family, NGF-1C, is a transcriptional activator that specifically binds the sequence GCGGGGGCG (5). Within the *Xba*I-*Sph*I fragment, two regions are present with seven of nine bases matching NGF-1C (Fig. 6). A region which contains a consensus AP-2 binding site was protected from ExoIII digestion after incubation with nuclear extracts (Fig. 5). AP-2 plays an important role in neural crest development and as such may be important with respect to transcriptional activity of the *Xba*I-*Sph*I fragment in neuronal cells (16). The presence of an Oct-like element in the *Xba*I-*Sph*I fragment is interesting since neuron-specific Oct proteins that bind HSV-1 IE promoters and inhibit transcription have been identified (13). These neuronal Oct-2 factors also differ in their binding specificity and function (6), suggesting that this element or other similar factors can influence the activity of the *Xba*I-*Sph*I fragment. The HSV-1 latency-associated transcript promoter has a single motif which is a strong enhancer in neuronal cells (3). An 11-base sequence in the *Xba*I-*Sph*I fragment (CAGGGGCAagG; nt 567 to 557) is the only region in the LR promoter which resembles the HSV-1 sequence. Since the *Xba*I-*Alu*I fragment did not have strong enhancer activity, this sequence alone does not appear to be crucial for transcriptional enhancer activity. Considering the dramatic difference in enhancer activity in the two cell lines, the data implied that factors in PC-12 cells bind sequences in the *Xba*I-*Sph*I fragment and activate transcription. In contrast, nuclear factors in Rat-2 cells bind similar DNA sequences but were unable to activate transcription. Current studies are aimed at identifying which factors interact with the *Xba*I-*Sph*I fragment and how these interactions affect transcriptional activity.

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REFERENCES

1. **Ackerman, M., E. Peterhans, and R. Wyler.** 1982. DNA of bovine herpes virus type 1 in the trigeminal ganglia of latently infected calves. Am. J. Vet. Res. **43**:36–40.
2. **Angel, P., M. Imigawa, R. Chiu, B. Stein, R. J. Imbra, H. J. Rhamsdoerf, C. Jonat, P. Herrlich, and M. Karin.** 1987. Phorbol ester inducible genes contain a common cis element recognized by a TPA modulated trans-acting factor. Cell **49**:729–739.
3. **Batchelor, A. H., and P. O'Hare.** 1992. Localization of *cis*-acting sequence requirements in the promoter of the latency-associated transcript of herpes simplex virus type 1 required for cell-type-specific activity. J. Virol. **66**:3573–3582.
4. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.

5. **Crosby, S. D., J. J. Puetz, K. S. Simburger, T. J. Fahrner, and J. Milbrandt.** 1991. The early response gene NGFI-C encodes a zinc finger transcriptional activator and is a member of the GCGGGGGCG (GSG) element-binding protein family. Mol. Cell Biol. **11**:3835–3841.
6. **Dent, C. L., K. A. Lillycrop, J. K. Estridge, N. S. B. Thomas, and D. S. Latchman.** 1991. The B-cell and neuronal forms of the octamer-binding protein Oct-2 differ in DNA-binding specificity and functional activity. Mol. Cell. Biol. **11**:3925–3930.
7. **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**:1475–1489.
8. **Falkner, F. G., R. Moickat, and H. G. Zachau.** 1986. Sequences closely related to an immunoglobulin promoter/enhancer element occur upstream of other eukaryotic and of prokaryotic genes. Nucleic Acids Res. **13**:7847–7863.
9. **Greene, L. A., and A. S. Tischler.** 1976. Establishment of a nonadrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc. Natl. Acad. Sci. USA **73**:2424–2468.
10. **Homan, E. J., and B. C. Easterday.** 1983. Experimental latent and recrudescent bovine herpesvirus 1 infections in calves. Am. J. Vet. Res. **44**:309–313.
11. **Jones, C.** 1989. The minimal transforming fragment of HSV-2 is a complex transcriptional promoter element. Virology **169**:346–353.

11a. **Jones, C.** Unpublished data.

12. **Jones, C., G. Delhon, A. Bratanich, G. Kutish, and D. Rock.** 1990. Analysis of the transcriptional promoter which regulates the latency-related transcript of bovine herpesvirus 1. J. Virol. **64**:1164–1170.
13. **Kemp, L. M., C. L. Dent, and D. S. Latchman.** 1990. Transcriptional repression of herpes simplex virus immediate early genes and of octamer containing cellular promoters in neuronal cells is mediated by the octamer DNA element. Neuron **4**:215–222.
14. **Kutish, G., T. Mainprize, and D. Rock.** 1990. Characterization of the latency-related transcriptionally active region of the bovine herpesvirus 1 genome. J. Virol. **64**:5730–5737.
15. **Lo, D. C.** 1992. NGF takes shape. Curr. Biol. **2**:67–69.
16. **Mitchell, P. J., P. M. Timmons, J. M. Hebert, P. W. J. Rigby, and R. Tjian.** 1991. Transcription factor AP-2 is expressed in neural crest cell lineages during mouse embryogenesis. Genes Dev. **5**:105–119.
17. **Montminy, M. R., K. A. Sevarino, J. A. Wagner, G. Mandel, and R. H. Goodman.** 1986. Identification of a cAMP responsive element within the rat somatostatin gene. Proc. Natl. Acad. Sci. USA **83**:6682–6686.
18. **Rhim, J. S., J. Fujita, P. Arnstein, and S. A. Aaronson.** 1986. Neoplastic conversion of human keratinocytes by adenovirus 12-SV40 virus and chemical carcinogens. Science **232**:385–388.
19. **Rock, D., J. Lokensgard, T. Lewis, and G. Kutish.** 1992. Characterization of dexamethasone-induced reactivation of latent bovine herpesvirus 1. J. Virol. **66**:2484–2490.
20. **Rock, D. L., S. L. Beam, and J. E. Mayfield.** 1987. Mapping bovine herpesvirus type 1 latency-related RNA in trigeminal ganglia of latently infected rabbits. J. Virol. **61**:3827–3831.
21. **Rock, D. L., W. Hagemoser, F. Osorio, and D. E. Reed.** 1986. Detection of bovine herpesvirus type 1 RNA in trigeminal ganglia of latently infected rabbits by in situ hybridization. J. Gen. Virol. **67**:2515–2520.
22. **Rock, D. L., and D. E. Reed.** 1982. Persistent infection with bovine herpesvirus 1: rabbit model. Infect. Immun. **35**:371–373.
23. **Ronnett, G. V., L. D. Hester, J. S. Nye, K. Connors, and S. H. Snyder.** 1990. Human cortical neuronal cell line: establishment from a patient with unilateral megalencephaly. Science **248**:603–605.
24. **Salton, S. R. J., D. J. Fischberg, and K.-W. Dong.** 1991. Structure of the gene encoding VGF, a nervous system-specific mRNA that is rapidly and selectively induced by nerve growth factor in PC12 cells. Mol. Cell. Biol. **11**:2335–2349.
25. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, p. 1:21–1:50. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. **Stein, B., H. J. Rahmsdorf, A. Steffen, M. Litfin, and P. Herrlich.** 1989. UV-induced damage is an intermediate step in UV-induced expression of human immunodeficiency virus type 1, collagenase, c-*fos*, and metallothionein. Mol. Cell. Biol. **9**:5169–5181.
27. **Williams, T., and R. Tjian.** 1991. Analysis of the DNA binding and activation properties of the human transcription factor AP-2. Genes Dev. **5**:670–682.
28. **Wirth, U. V., C. Fraefel, B. Vogt, Č. Vlček, V. Pačes, and M. Schwyzer.** 1992. Immediate-early RNA 2.9 and early RNA 2.6 of bovine herpesvirus 1 are 3′ coterminal and encode a putative zinc finger transactivator protein. J. Virol. **66**:2763–2772.
29. **Wirth, U. V., B. Vogt, and M. Schwyzer.** 1991. The three major immediate-early transcripts of bovine herpesvirus 1 arise from two divergent and spliced transcription units. J. Virol. **65**:195–205.
30. **Wu, C.** 1985. An exonuclease protection assay reveals heat shock element and TATA box binding proteins in crude nuclear extracts. Nature (London) **317**:84–87.
31. **Wyler, R., M. Engels, and M. Schwyzer.** 1989. Infectious bovine rhinotracheitis/vulvovaginitis (BHV-1), p. 1–72. *In* G. Wittman and Y. Becker (ed.), Herpesvirus diseases of cattle, horses, and pigs. Developments in veterinary virology ser. Kluwer Academic Publishers, Boston.