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ras Oncogene-Dependent Activation of the P4 Promoter of Minute Virus of Mice through a Proximal P4 Element Interacting with the Ets Family of Transcription Factors

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The P4 promoter of parvovirus minute virus of mice (MVMp) directs transcription of the genes coding for nonstructural proteins. The activity of promoter P4 is regulated by several *cis*-acting DNA elements. Among these, a promoter-proximal GC box was shown to be essential for P4 activity (J. K. Ahn, B. J. Gavin, G. Kumar, and D. C. Ward, J. Virol. 63:5425–5439, 1989). In this study, a motif homologous to an Ets transcription factor-binding site (EBS), located immediately upstream from the GC box, was found to be required for the full activity of promoter P4 in the *ras*-transformed rat fibroblast cell line FREJ4. In normal parental FR3T3 cells, the transcriptional function of P4 EBS was insignificant but could be restored by transient cell transfection with the c-Ha-*ras* oncogene. P4 EBS may thus contribute to the stimulation of promoter P4 in *ras*-transformed cells. Electrophoretic mobility shift assays using crude extracts from FREJ4 cells revealed the binding of a member(s) of the Ets family of transcription factors to the P4 EBS, as well as the interaction of two members of the Sp1 family, Sp1 and Sp3, with the adjacent GC box. When produced in *Drosophila melanogaster* SL2 cells, Ets-1 and Sp1 proteins acted synergistically to transactivate promoter P4 through their respective cognate sites.

Autonomous parvoviruses are small, nuclear replicating DNA viruses which infect a variety of vertebrates, including humans. Because of their low genetic complexity, parvoviruses depend extensively on host cell factors for all steps of their life cycles (for a review, see reference 15). The availability of these factors depends largely on the proliferative activity and the differentiation state of the host cell. These requirements are likely to account for the specificity of parvovirus infections, both in vivo and in cell cultures (2, 25, 55). The genome of the prototype strain of minute virus of mice (MVMp), an autonomous parvovirus that can productively infect cells of rodent and primate origin, consists of a linear, single-stranded DNA of 5,149 nucleotides (nt) containing two overlapping transcription units. The left-hand promoter, P4, directs the synthesis of a transcript whose spliced derivatives encode the nonstructural proteins NS-1 and NS-2. The internal promoter, P38, regulates the production of the viral capsid proteins VP1 and VP2 encoded by alternatively spliced mRNAs (14, 15, 38, 48).

The above-mentioned dependence of the outcome of parvovirus infections on the physiological state of host cells is exemplified by the oncotropism of these agents and may underlie their ability to prevent the appearance or cause the regression of a variety of tumors (for a review, see reference 50). In addition, oncogenic transformation of a number of host cells (fibroblasts and epithelial cells of human or rodent origin) is accompanied by an increase in their sensitivity to the killing effect of MVMp (12, 51, 57), raising the possibility that the antitumor activity of this agent in vivo (28) involves an oncolytic component. In this respect, it is worth mentioning that NS-1 not only is involved in parvovirus DNA replication and gene expression (16, 20, 23, 52, 56) but also has been shown to be cytotoxic (9, 10, 39). It was suggested that parvoviral oncolysis may result, at least in part, from the greater accumulation of NS-1 in transformed than in normal cells (51, 57). Previous reports have shown that this increase in NS-1 production is controlled, in particular, at the level of initiation of transcription. Indeed, the activity of promoter P4 was found to be up-modulated in the c-Ha-*ras*-transformed rat fibroblast line FREJ4 (54). Hence, the regulation of this promoter constitutes a key issue for the understanding of parvovirus-host cell interactions and may provide insights into the mechanism(s) by which neoplastic transformation exacerbates the parvoviral lytic effect.

The regulation of transcription initiation in eukaryotic cells is governed by a combination of proteins that bind to *cis*-acting DNA elements. At least a fraction of these elements is typically found within a 50- to 200-nt-long region located upstream from the transcription start site (43), as was shown for the P4 promoter (1, 22). Various regulatory motifs, in particular proximal GC and TATA boxes (1, 49), upstream E and Y boxes (27), and cyclic AMP (cAMP) response elements (47), have been reported to contribute to P4 promoter activity. The present study aimed at analyzing another putative P4 promoter element that is located immediately upstream from the GC box and consists of the sequence 5′-AAGGAAG-3′. This element is highly homologous to the core consensus motif (C/A)GG A(A/T) that constitutes an Ets transcription factor-binding site (EBS). The generic name Ets designates a large family of regulatory proteins, of which about 30 members have been identified. This family is characterized by the conservation of a specific DNA-binding domain that comprises 85 amino acids and is made of the juxtaposition of two regions with α-helix and basic properties, respectively (59). Although the DNA sequences flanking the core EBS consensus exert an influence on the type of Ets factor that is able to interact, target selectivity of the various Ets members does not appear to be determined in a simple way by their DNA recognition capacity (44,

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58). There is growing evidence that another level of binding selectivity is achieved by protein-protein interactions between Ets and adjacently bound transcription factors (for a review, see reference 34). It is also worth noting that EBS motifs were shown to mediate promoter activation by several nonnuclear oncoproteins, including c-Ha-Ras, v-Src, and v-Mos, that have been shown to enhance Ets DNA-binding activities (11, 45, 60).

Results presented in this report indicate that the above-mentioned EBS motif of MVMp contributes to the activation of promoter P4 in *ras*-transformed FREJ4 rat cells. The functionality of this sequence correlates with its ability to interact with proteins of the Ets family of transcription factors. In cotransfection experiments using *Drosophila melanogaster* SL2 cells, promoter P4 was found to be transactivated in a synergistic way by the prototype member of this family, Ets-1, and the Sp1 factor that binds to a GC box flanking the EBS motif. EBS appears to play little role in P4 activity in normal rat fibroblasts unless cells are transfected with the *ras* oncogene, implicating Ets proteins as effectors of the modulation of promoter P4 by some oncoproteins.

MATERIALS AND METHODS

Cells and recombinants plasmids. Parental Fisher rat fibroblasts (cell line FR3T3) and the c-Ha-*ras*-transformed derivative cell line FREJ4 (51, 57) were grown in Dulbecco's modified Eagle's minimum essential medium supplemented with 10% donor calf serum and 1% sodium pyruvate at 37°C. Mouse A9 cells were grown in Eagle's minimum essential medium supplemented with 5% fetal calf serum and 1% sodium pyruvate at 37°C. *D. melanogaster* SL2 cells (53) were a gift from R. Paro and maintained in Schneider medium supplemented with 10% fetal calf serum and 1% glutamine at 25°C.

Plasmid P4wtLuc, which contains the firefly luciferase gene under control of the P4 promoter of MVMp, was constructed as previously described (22). The *Bgl*II recognition motif (AGATCT) was then substituted by site-directed mutagenesis for a sequence of equivalent length within either the EBS (nt 153 to 158; P4mut23Luc), the GC box (nt 159 to 164; P4mut24Luc), a motif interacting with an as yet unidentified factor(s) (nt 114 to 119; P4mut17Luc) (22), or a P4 region that does not appear to be involved in specific protein interactions (nt 126 to 131; P4mut19Luc) (22). Another derivative of P4wtLuc, P4mEBSLuc, was constructed by site-directed mutagenesis, introducing a G-to-T substitution at nt 154 in the EBS. Recombinant constructs were verified by DNA sequence analysis using the dideoxy-chain termination method.

The *D. melanogaster* expression plasmids, under the control of the *Drosophila* actin 5C promoter, were generous gifts of R. Tjian (pPac-Sp1) (17), G. Suske (pPac-Sp3) (31), and J. Ghysdael (pPac-Ets-1) (26). The control plasmid pBLCAT5, expressing the chloramphenicol acetyltransferase gene under control of the herpes simplex virus thymidine kinase promoter, was kindly provided by G. Schütz (8).

Transfection and transient expression assays. Transfection of 10^5 (FR3T3, FREJ4, and A9) or 10^6 (SL2) cells was performed with the LipofectAMINE reagent (Gibco BRL) as recommended by the manufacturer. Each set of transfections was performed in triplicate and repeated at least four times with different plasmid preparations.

FREJ4 and A9 cells were cotransfected with 0.1 μg of reporter plasmid (P4wtLuc or mutated derivatives) and 0.1 μg of a standard plasmid expressing human growth hormone under the control of the herpes simplex virus thymidine kinase promoter (pTKhGH; Nichols Institute). At 36 h posttransfection, luciferase activities were measured as described previously (18) and normalized for human growth hormone expression as determined by means of a growth hormone detection kit (Nichols Institute). FR3T3 cells were cotransfected with 0.5 μg of reporter plasmid (P4wtLuc or mutated derivatives), various amounts of a c-Ha-*ras* expression plasmid (pSVneoEJ) (57), and, as a standard, 1 μg of a chloramphenicol acetyltransferase expression plasmid under control of the herpes simplex virus thymidine kinase promoter (pBLCAT5) (8). Total DNA was kept constant by the addition of pSVneo vector. At 24 h posttransfection, cells were washed and further incubated for 24 h in medium containing 0.5% donor calf serum. Subsequently, luciferase activities were determined and adjusted for transfection efficiencies with the help of the cotransfected standard plasmid.

Drosophila SL2 cells were cotransfected with 1 μg of reporter plasmid (P4wtLuc or mutated derivatives) and various amounts of plasmids pPac-Sp1, pPac-Sp3, and/or pPac-Ets-1. Total DNA was kept constant by addition of the nonrecombinant vector pPac-0. At 48 h posttransfection, luciferase activities were determined and normalized for the total amounts of proteins measured by the Bradford method (Bio-Rad).

Cell extract preparation and EMSA. Extracts were prepared from FREJ4 cells as described previously (6). Briefly, cells were harvested from culture dishes, rinsed with phosphate-buffered saline, and resuspended in 1.5 volumes of lysis buffer (20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.9], 0.4 M NaCl, 25% glycerol, 1 mM EDTA, 2.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride). The lysate was kept on ice for 20 min and subsequently frozen at −70°C. After thawing on ice, the suspension was vigorously vortexed and centrifuged for 10 min at 15,000 rpm in an Eppendorf centrifuge. The supernatant was frozen in liquid nitrogen and used as the crude cell extract for electrophoretic mobility shift assays (EMSAs).

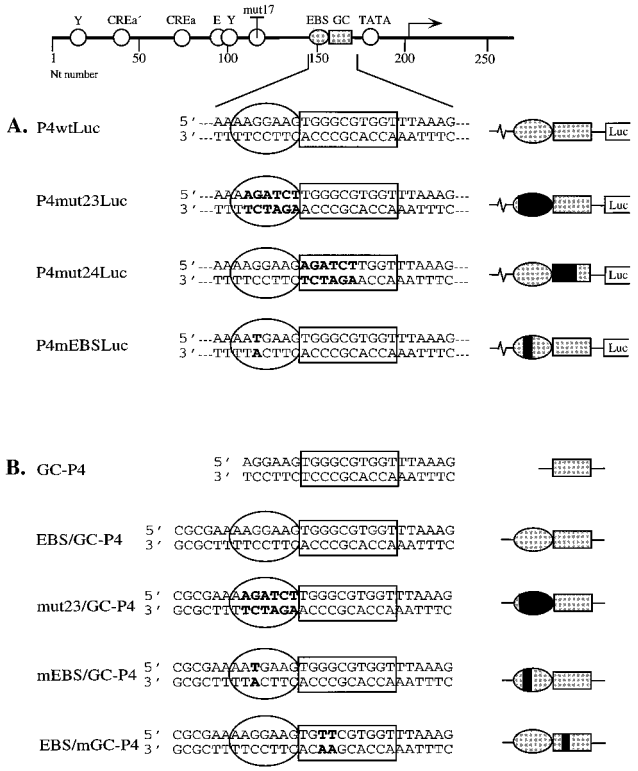

FIG. 1. Nucleotide sequence and schematic representation of P4 promoter constructs and P4-related oligonucleotides used in this study. The upper line depicts the MVMp P4 promoter and transcription start site (arrow). Open circles represent cell protein-binding sites (22), some of which are identified as indicated: TATA box (TATA) (1), Y boxes (Y) (27), E box (E) (27), and cAMP response elements (CREa and CREa′) (47). mut17 indicates a site which is mutated in the P4mut17Luc construct (see Fig. 6B) and interacts with as yet unidentified proteins. Base numbering is according to Astell et al. (4). The sequence elements analyzed in this study are framed by a rectangle (GC box) (1) and an oval (EBS) and are aligned beneath the line diagram of the promoter. Boldface type (sequences) and filled areas (right-hand schemes) indicate mutations that were introduced in corresponding elements. (A) P4 promoter constructs driving expression of the luciferase (Luc) reporter gene. (B) P4 promoter-related synthetic oligonucleotides used in EMSAs.

EMSA binding reactions were carried out for 10 min at room temperature in a final volume of 10 μl containing 250 pmol of ^{32}P-end-labeled probe (synthetic double-stranded oligonucleotides), 0.5 μg of salmon sperm DNA, 2 mM $MgCl_2$, 10% glycerol, and either 3 to 6 μg of crude cell extract or 0.1 footprint unit of affinity-purified human Sp1 protein (Promega Corp.) in the presence of 5 μg of bovine serum albumin. For competition studies, unlabeled double-stranded oligonucleotides were included in the reaction mixture before addition of the probe. The MVMp-specific oligonucleotides (corresponding to upstream regions of promoter P4) and mutated derivatives are depicted in Fig. 1B. Sequences of oligonucleotides bearing consensus EBS, GC box, and NF-κB-binding sites (EBSc, GCc, and NFBSc) and point-mutated versions thereof (mEBSc and mGCc) were as follows: 5′-CTTCGAGCAGGAAGTTCGA-3′ (EBSc), 5′-CTTCGAGCA**T**GAAGTTCGA-3′ (mEBSc), 5′-ATTCGATCGGGGCGGGGCGA-3′ (GCc), 5′-ATTCGATCG**TT**GCGGGGCGA-3′ (mGCc), and 5′-ACAAGGGACTTTCCGCTGGGGACTTTCCAG-3′ (NFBSc). Consensus binding sites are underlined, and mutations are shown in boldface. Protein-DNA complexes were resolved by 4.5% native polyacrylamide gel electrophoresis at 4°C in 0.3× TBE (11 mM Tris-HCl, 41 mM H_3BO_3, 2 mM EDTA [pH 8.3]) for 90 min at 280 V. Gels were fixed, dried, and exposed for autoradiography at −70°C with an intensifying

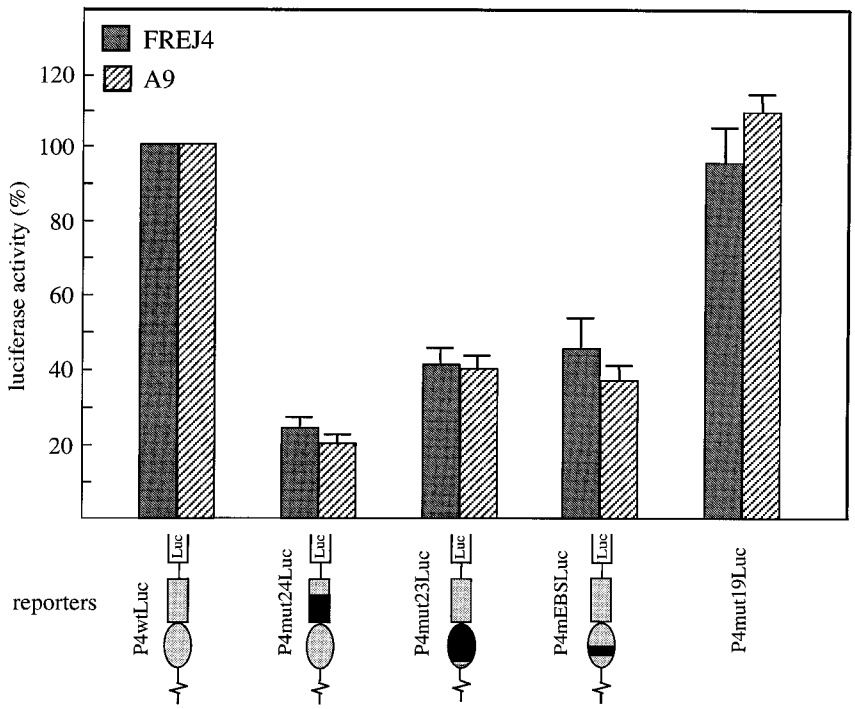

FIG. 2. Effects of mutations within the GC box or the EBS on P4 promoter-driven gene expression. FREJ4 and A9 cells were transfected with plasmids expressing the reporter luciferase gene from the whole wild-type P4 promoter (P4wtLuc) or from derivatives carrying mutations in the GC box, the EBS motif (see Fig. 1A for the corresponding sequences), or a region that is not involved in DNA-protein interactions (P4mut19Luc). Normalized luciferase activities achieved by the mutants are given as percentages of the expression driven by the wild-type promoter. Average values from at least four independent experiments, each performed in triplicate, are shown with standard deviation bars. Luciferase activities were at least 15 times higher than background.

screen. For supershift assays, 1 μl of polyclonal antiserum, monoclonal antibody, or control nonimmune serum was added to the binding reaction mixture, and incubation was continued for 50 min on ice before loading of the gel. The monoclonal antibody directed against Sp1 (IC6) was a generous gift of S. P. Jackson. The rabbit polyclonal anti-Sp3 antiserum (31) was kindly provided by G. Suske.

RESULTS

A putative EBS contributes to the activity of the MVMp P4 promoter in FREJ4 cells. The MVMp P4 promoter and upstream left-hand terminal sequences direct transcription initiation at nt 204. Proximal elements (GC and TATA boxes) proved to play a major role in P4 activity in human, mouse, and rat cells, yet efficient transcription appears to require additional upstream sequences that were shown to interact with cellular proteins (1, 22, 27, 47). As illustrated in Fig. 1A, a putative regulatory motif (EBS) is found immediately upstream from the GC box, on the basis of the high homology of its sequence (AAGGAAG, nt 152 to 158) with the DNA binding site of the Ets family of transcription factors (40, 59). The functionality of this motif was tested by measuring the effect of EBS mutation on the expression of a reporter gene programmed by the MVMp P4 promoter in transient transfection assays performed with *ras*-transformed FREJ4 cells. P4wtLuc, a plasmid containing the luciferase reporter gene under control of the complete P4 promoter, was used to this end.

As a first approach, the *Bgl*II recognition motif (AGATCT; Fig. 1A) was substituted for a sequence of equivalent length within the putative EBS motif of promoter P4 (P4mut23Luc). For comparison, a similar substitution was introduced in the neighboring GC box (P4mut24Luc) or, as a negative control, within a P4 region which does not appear to be involved in specific interactions with polypeptides (P4mut19Luc) (22). Normalized luciferase activities achieved by these constructs are presented in Fig. 2. P4mut19Luc and P4wtLuc sustained similar levels of expression of the luciferase reporter gene, indicating that the *Bgl*II recognition motif had no intrinsic effect on P4 promoter activity. In contrast, the disruption of the GC box (P4mut24Luc) led to a four- to fivefold decrease of P4 promoter activity, in agreement with previous results (1, 22). Similarly, replacement of most of the EBS motif by the unrelated *Bgl*II site (P4mut23Luc) reduced P4-driven luciferase gene expression to 40%, suggesting that the EBS element contributed to P4 promoter activity in FREJ4 cells. This was ascertained by using another P4 promoter construct that carries a single point mutation (nt 154, G to T) within the EBS (P4mEBSLuc; Fig. 1A). An equivalent mutation was previously shown to inactivate a genuine EBS (42). Like the mut23 substitution, the point mutation from P4mEBSLuc resulted in a twofold reduction of luciferase gene expression (Fig. 2), confirming the importance of the EBS region for the full activity of promoter P4 in FREJ4 cells. Similar results were obtained with murine A9 cells (Fig. 2), indicating that the EBS motif is also involved in P4 activation in permissive cells from the natural host of MVMp.

The MVMp GC box interacts with Sp1 and Sp3 proteins. The GC box of the MVMp P4 promoter has high sequence homology to the consensus binding site for the Sp1 family of transcription factors (30, 35, 36) and has been found to constitute a high-affinity binding site for Sp1-like proteins (1, 49). To identify the Sp1 family members that interact with the GC box of promoter P4 in FREJ4 cells, EMSAs were performed (Fig. 3). Upon incubation of oligonucleotide GC-P4 (Fig. 1B) with whole extracts from FREJ4 cells, four retarded complexes (referred to as C2, C3, C5, and C6) were detected (Fig. 3, lane

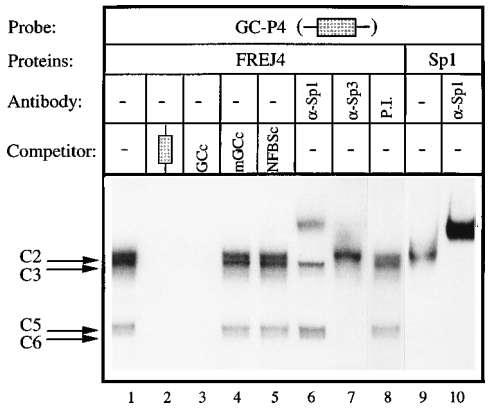

FIG. 3. Association of FREJ4 cell proteins with the GC box of promoter P4. Whole extracts from FREJ4 cells were incubated with ^{32}P-end-labeled oligonucleotide GC-P4 (see Fig. 1B) in the absence (lane 1) or presence of either a 100-fold molar excess of unlabeled competitors (lanes 2 to 5) or antibodies (lanes 6 to 8). Affinity-purified human Sp1 protein was incubated with the GC-P4 probe in the absence (lane 9) or presence (lane 10) of antibodies. Oligonucleotide competitors consisted of GC-P4 itself (lane 2), intact (lane 3) or mutated (lane 4) consensus GC box, and consensus NF-κB-binding sites (lane 5). Immunoshift assays were performed with monoclonal antibodies directed against Sp1 (lanes 6 and 10), anti-Sp3 polyclonal antibodies (lane 7), or preimmune serum (P.I.; lane 8). The binding reaction products were submitted to native polyacrylamide gel electrophoresis under conditions allowing specific DNA-protein complexes (arrows) to be resolved and the free probe to run out of the gel. Retarded complexes were revealed by autoradiography.

1). These complexes proved to be specific, since their formation could be inhibited by the homologous oligonucleotide (Fig. 3, lane 2) but not by a heterologous oligonucleotide that harbors a consensus NF-κB-binding motif (Fig. 3, lane 5). In addition, a heterologous oligonucleotide containing a bona fide GC box interfered with the formation of all four complexes (Fig. 3, lane 3), while a point-mutated derivative known to be unable to bind Sp1 (33) failed to compete (Fig. 3, lane 4). Furthermore, when the bona fide GC box oligonucleotide was used as a probe, we detected four DNA-protein complexes which were similar in electrophoretic mobility and binding specificity to those formed with the GC-P4 probe (data not shown). Taken together, these observations confirmed that the GC-rich proximal element of promoter P4 was recognized by the same proteins as a bona fide GC box.

To identify these proteins, immunoshift assays were performed with antibodies directed against different members of the Sp1 family. As illustrated in Fig. 3 (lane 6), a monoclonal antibody directed against Sp1 (IC6) specifically enhanced retardation of complex C2, indicating the involvement of Sp1 in this complex. In agreement with a previous report (49), purified human Sp1 protein was found to associate with the GC-P4 probe, giving rise to a complex that comigrated with C2 (Fig. 3, lane 9) and was supershifted by the monoclonal anti-Sp1 antibody IC6 (Fig. 3, lane 10). Together, these observations implicated Sp1 as the sole protein constituent of the C2 complex. In contrast, addition of another antiserum that was shown to specifically suppress the formation of Sp3-DNA complexes without causing the appearance of supershifted bands (31) inhibited the formation of C3, C5, and C6 but not C2 complexes (Fig. 3, lane 7). A preimmune serum failed to affect any of these DNA-protein associations (Fig. 3, lane 8).

Sp3 is thought to exist in three different isoforms (31), which would explain the involvement of this protein in three distinct complexes. EMSAs performed with purified Sp3 proteins showed similar retardation patterns consisting of three retarded species (36), suggesting that Sp3 was the only protein component of complexes C3, C5, and C6. Together, these data indicate that Sp1 family members, in particular Sp1 and Sp3, can bind in a mutually exclusive way to the GC box of the MVMp P4 promoter.

The EBS motif of the MVMp P4 promoter is recognized by members of the Ets family of transcription factors. The putative EBS motif AAGGAAG, which has been shown to contribute to P4 activity (see above), was analyzed in order to identify the interacting protein(s). To this end, the oligonucleotide EBS/GC-P4, which included both the putative EBS motif and the GC box (Fig. 1B), was subjected to gel retardation analysis. As shown in Fig. 4A, incubation of EBS/GC-P4 with FREJ4 whole cell extracts led to the formation of five retarded complexes designated C1, C2, C3, C5, and C6 (Fig. 4A, lane 1). All five complexes proved to be specific, as demonstrated by competition experiments using homologous (Fig. 4A, lane 2) and heterologous (NFBSc; Fig. 4A, lane 9) oligonucleotides.

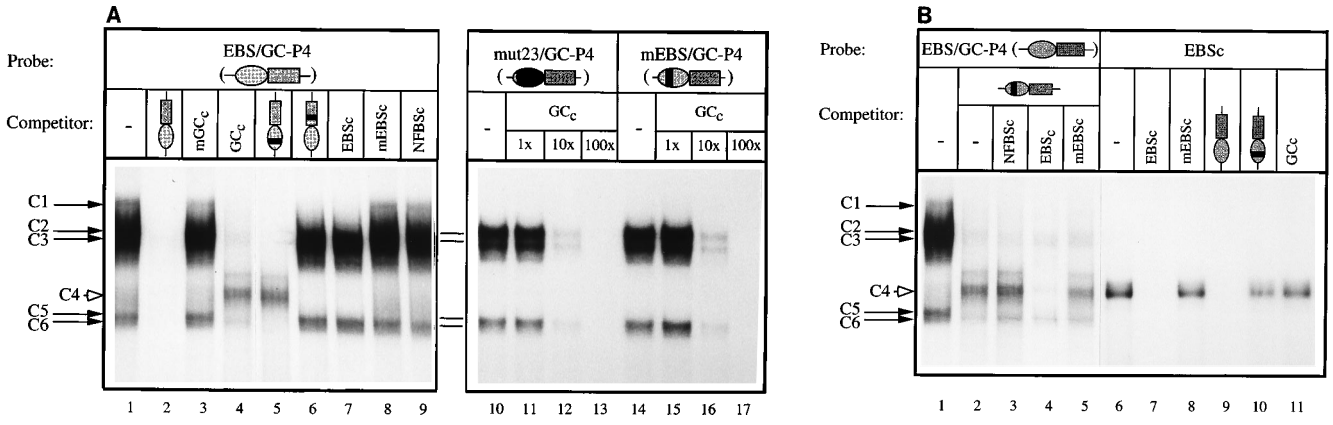

FIG. 4. Interaction of cellular proteins with the EBS-GC region of promoter P4. Whole FREJ4 cell extracts were incubated with ^{32}P-end-labeled oligonucleotide EBS/GC-P4, mut23/GC-P4, mEBS/GC-P4 (see Fig. 1B), or EBSc (consensus EBS binding site), as indicated, in the absence or presence of unlabeled competitors. The molar excess of unlabeled competitor oligonucleotides was 100-fold except where otherwise stated. Competing oligonucleotides either derived from P4 (EBS/GC-P4 and corresponding EBS or GC mutants schematized according to Fig. 1B) or consisted of the following consensus motifs: intact (GCc) and mutated (mGCc) GC box, intact (EBSc) and mutated (mEBSc) EBS, and NF-κB-binding site (NFBSc). Specific DNA-protein complexes (arrows) were fractionated by EMSA and revealed by autoradiography. The free probe ran out of the gel.

Complexes C2, C3, C5, and C6 were equivalent to the Sp1/3-containing complexes formed with the GC-P4 probe, as shown by competition analysis and immunoshift assays. The formation of these complexes was inhibited by consensus (Fig. 4A, lane 4) and P4 (Fig. 4A, lane 5) GC boxes but not by mutated derivatives thereof (Fig. 4A, lanes 3 and 6). C2, C3, C5, and C6 were also insensitive to the Ets protein-binding competitor (Fig. 4A, lane 7).

In contrast, the more slowly migrating complex C1 was abolished by oligonucleotides harboring either an intact GC box (Fig. 4A, lanes 4 and 5) or an intact EBS (Fig. 4A, lanes 6 and 7), irrespective of whether consensus or P4-derived competitors were used. Mutations known to suppress the recognition of consensus GC box and EBS by Sp1-like and Ets proteins, respectively, were found to impair the abilities of these elements to act as competitors and inhibit the formation of complex C1 (Fig. 4A, lanes 3 and 8). Furthermore, complex C1 did not form when the mutated mut23/GC-P4 (Fig. 4A, lane 10), mEBS/GC-P4 (Fig. 4A, lane 14), and EBS/mGC-P4 (data not shown) oligonucleotides were used as probes. Together, these data strongly suggest that C1 results from the concomitant interaction of the EBS/GC-P4 oligonucleotide with at least two types of proteins which can be trapped by one or the other consensus motif and may thus belong to the Sp1 and Ets families of transcription factors.

The Sp1 constituents of the presumably ternary C1 complex await further identification, since immunoshift experiments using the above-mentioned antibodies failed to show a displacement of C1, possibly because of epitope masking or confusion caused by the comigration of C1 with other complexes supershifted in the presence of anti-Sp1 antibodies (Fig. 3).

It is worth noting that the formation of an additional complex, C4, was observed with the EBS/GC-P4 probe when the binding reaction took place in the presence of competition oligonucleotides that specifically interacted with GC box-binding proteins (Fig. 4A, lanes 4 and 5). Complex C4 could be abolished by the EBSc oligonucleotide containing a consensus EBS but neither by the mEBSc derivative bearing a point mutation, previously shown to impair Ets protein binding (42), nor by the NFBSc oligonucleotide (Fig. 4B, lanes 1 to 5). Furthermore, incubation of radiolabeled EBSc oligonucleotide with FREJ4 whole cell extracts led to the formation of a complex which comigrated with complex C4 and could be abolished by consensus and P4 EBSs but not by mutated derivatives or the consensus GC box (Fig. 4B, lanes 6 to 11). These data indicate that complex C4 is specific and is likely to result from the association of the P4 EBS with an Ets-related protein(s). In keeping with this possibility, complex C4 (and the presumably ternary complex C1) did not form when the mut23/GC-P4 and mEBS/GC-P4 oligonucleotides were used as probes in EMSAs, while the Sp1/3-containing complexes C2, C3, C5, and C6 were not affected by the EBS mutation (Fig. 4A, lanes 10 to 17). The nature of the Ets protein constituent(s) of C1 and C4 is unknown, since these complexes could not be supershifted or suppressed by a panel of antibodies directed against specific members of the Ets family.

It is noteworthy that C4 could be detected only under conditions such that appropriate competitors removed Sp1-like proteins from the EBS/GC-P4 probe (Fig. 4A, lanes 4 and 5; Fig. 4B, lanes 2 to 5). Also, the consensus Ets recognition element EBSc was more efficient than the EBS/mGC-P4 oligonucleotide in competing with radiolabeled EBSc probe for Ets protein binding (data not shown). These observations, together with the above-mentioned occurrence of the C1 ternary complex, lead to the conclusion that the affinity of Ets proteins for the EBS element of promoter P4 is low but may be increased as a result of the adjacent binding of Sp1-like factors. As apparent from competitive EMSAs, oligonucleotides mut23/GC-P4 and mEBS/GC-P4 could bind Sp1 and Sp3 and were indistinguishable with regard to their affinities for these factors (Fig. 4A, lanes 10 to 17). Furthermore, corresponding mutations impaired the activity of promoter P4 in permissive cells to similar extents (Fig. 2). Therefore, either of these mutants was suitable for the assessment of the specific role of EBS in P4 modulation.

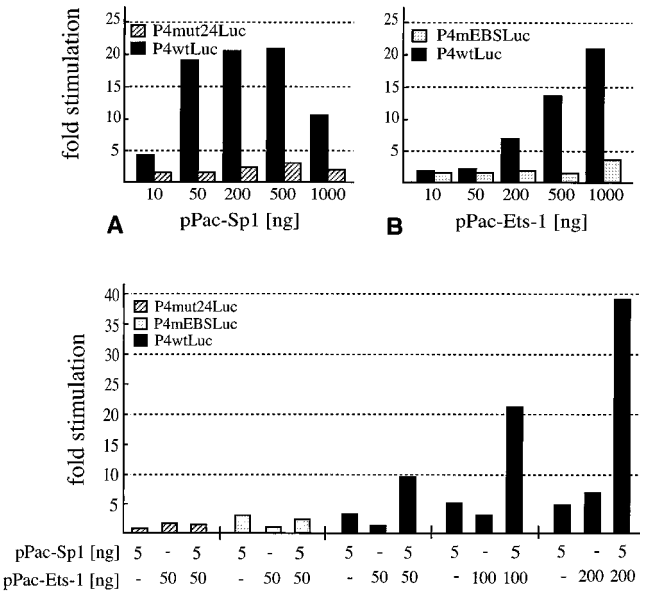

FIG. 5. Effects of Sp1 and Ets-1 transcription factors on promoter P4 activity. *D. melanogaster* SL2 cells were transfected with the luciferase reporter gene driven by wild-type (P4wtLuc), GC box mutant (P4mut24Luc), or EBS mutant (P4mEBSLuc) promoter P4, together with increasing amounts (nanograms per plate) of either Sp1-producing pPac-Sp1 (A) or Ets-1-producing pPac-Ets-1 (B) or both expression plasmids (C). Results are presented as Sp1- and/or Ets-1-induced stimulation of promoter P4 and were calculated as the ratios of luciferase activities in cells transfected with pPac-Sp1 and/or pPac-Ets-1 to luciferase activities in cells transfected with the same amount of empty pPac-0 vector. Luciferase activities were at least 10 times higher than background.

Sp1 and Ets-1 synergistically transactivate promoter P4 in insect cells. Given the evidence of in vitro interaction of Sp1, Sp3, and Ets-related proteins with proximal P4 promoter elements, the respective roles of these factors in P4-driven gene expression were analyzed. To address this question, *Drosophila* SL2 cells, which lack endogenous Sp1-like and Ets-1 activities (17, 19, 31), were cotransfected with P4 promoter constructs and expression vectors encoding Sp1, Sp3, or the prototype member of the Ets family, Ets-1 (29). As illustrated in Fig. 5A, reporter gene expression from the wild-type P4 promoter construct (P4wtLuc) was increased by Sp1 in a dose-dependent manner. This effect was mediated by the P4 GC box, since little stimulation was observed with the P4mut24 derivative that lacked this element. Similarly, Ets-1 production resulted in the dose-dependent activation of the wild-type P4 promoter (P4wtLuc) but not of a derivative (P4mEBS) mutated in the EBS motif (Fig. 5B). The Sp3-producing plasmid had no significant effect on P4 promoter-driven luciferase expression (data not shown), a finding that is consistent with the recently reported transcription-regulating inactivity of Sp3 in SL2 cells (31). It should be stated, however, that Sp3 can act as a transcription regulator of gene expression in mammalian cells (41).

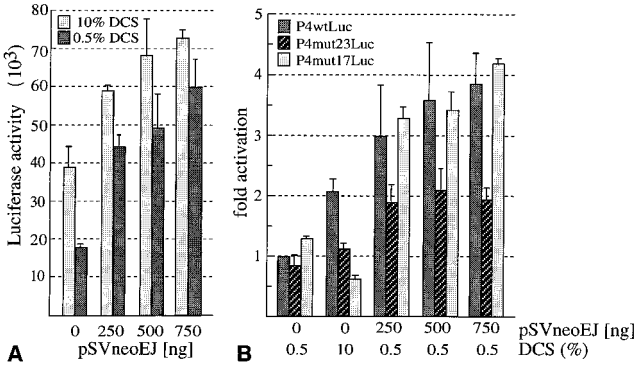

FIG. 6. Effect of Ha-*ras* on promoter P4-driven gene expression. FR3T3 cells were cotransfected with the reporter luciferase gene driven by wild-type (P4wtLuc) (A and B) or mutant (P4mut23Luc and P4mut17Luc) (B) promoter P4 and increasing amounts of *ras* expression plasmid (pSVneoEJ). pBLCAT5 was included as an internal control to correct for variations in transfection efficiencies. The total amount of transfected DNA was kept constant by the addition of *ras*-free vector (pSVneo). Luciferase activities were measured 48 h posttransfection in cells cultivated in the presence of 10 or 0.5% donor calf serum (DCS). In panel B, luciferase gene expression is expressed relative to the level achieved by cells transfected with P4wtLuc in the absence of pSVneoEJ and presence of 0.5% DCS, set as 1.0. Average values from four independent experiments, each performed in triplicate, are given with standard deviation bars.

Given that Sp1- and Ets-like proteins are capable of simultaneously binding to the EBS/GC-P4 probe and that Sp1 and Ets-1 both function as transcriptional activators of the MVMp P4 promoter in SL2 cells, experiments were performed to gain further insight into the functional interplay between these factors. As depicted in Fig. 5C, the concomitant production of Sp1 and Ets-1 resulted in reporter gene expression levels that were five to eight times higher than those achieved by each factor alone. This indicates that Sp1 and Ets-1 can cooperate in the activation of P4-driven transcription (5, 32). The GC box and EBS motif were both required for this effect, since mutations in the associated elements (mut 24 and mEBS, respectively) prevented promoter P4 from being synergistically activated by Ets-1 and Sp1 (Fig. 5C).

The EBS contributes to *ras*-mediated activation of promoter P4. The transcriptional activity of promoter P4 is greater in the *ras*-transformed FREJ4 cell line than in the parental FR3T3 cells (54). Since EBS motifs are involved in the *ras*-mediated up-modulation of other promoters (11, 60, 61), the role of the P4 EBS in the activation of promoter P4 by c-Ha-*ras* was investigated. To this end, it was first determined whether promoter P4 could be stimulated by *ras* in transient cotransfection assays using nontransformed FR3T3 cells. As illustrated in Fig. 6A, the *ras* oncogene enhanced the expression of a cotransfected luciferase gene placed under the control of wild-type promoter P4 (P4wtLuc) to a significant but small (about 1.5-fold) extent when cells were maintained in 10% donor calf serum. It has been reported that some of the actions of oncogenic *ras*, mediated in particular by the Ets family of transcription factors, are masked under these culture conditions as a result of the overlapping effect of serum factors (60). Indeed, by reducing the serum concentration from 10 to 0.5%, a lower basal activity and a greater (three- to fourfold) *ras*-dependent induction of promoter P4 were achieved (Fig. 6). Therefore, the latter culture conditions were used to assess the contribution of the P4 EBS motif to the responsiveness of promoter P4 to the *ras* oncogene.

The substitution of an unrelated sequence for the EBS motif (mut23) specifically prevented Ets from binding (Fig. 4A, lanes 10 to 13) and was tested for its effect on the modulation of promoter P4. As shown in Fig. 6B, the activity of the corresponding P4 mutant (P4mut23Luc) was not significantly impaired in *ras* oncogene-free FR3T3 cells, in agreement with the reported little Ets activity present under these conditions (60). Cell cotransfection with increasing amounts of the *ras* oncogene led to the dose-dependent stimulation of wild-type promoter P4 up to fourfold. At the highest *ras* concentration tested, this stimulation was reduced by half as a result of the substitution mutation within the EBS (P4mut23Luc), indicating that part of the *ras*-dependent activation of promoter P4 was mediated by the EBS. This result is consistent with the above-mentioned functional role of the EBS in the activity of the parvoviral promoter in *ras*-transformed FREJ4 cells (Fig. 2). The specificity of this effect was tested by measuring the influence of oncogenic *ras* on the expression of another P4 promoter construct, P4mut17Luc, in which the *Bgl*II site was substituted for nt 114 to 119 in a P4 region previously shown to interact with an as yet unidentified protein(s) (22). While impairing P4 functioning in cells grown with 10% serum, the mut17 substitution had little effect on either constitutive or *ras*-induced P4 activity under low-serum conditions (Fig. 6B). This contrasts with the reduced *ras* responsiveness of the EBS mutant P4mut23, implicating the EBS as a specific *cis* determinant of P4 up-regulation by *ras*. It should be stated, however, that the residual stimulation of the P4 EBS mutant (P4mut23Luc) by *ras* (Fig. 6B) argues for the presence of an additional *ras*-responsive element(s) besides the EBS in the P4 promoter.

DISCUSSION

The activity of the P4 promoter of MVMp is regulated by a number of DNA elements located upstream from the transcription start site (1, 22, 27, 47). In particular, a proximal GC box that constitutes a high-affinity binding site for Sp1-like proteins proved to be essential for P4 promoter activity (1, 49). In the present work, an EBS was identified immediately upstream from this GC box. Though of little functional importance in untransformed FR3T3 cells, the EBS was found to contribute to P4 activation by the *ras* oncogene.

Interaction of members of the Ets and Sp1 families of transcription factors with a proximal element of promoter P4. An oligonucleotide encompassing the EBS-GC box region of P4 took part in distinct DNA-protein complexes when incubated with FREJ4 cell extracts. All of these complexes but one appeared to comprise a single polypeptide component belonging to the Sp1 family of transcription factors, in particular Sp1 or Sp3. In contrast, the most retarded complex contained an additional protein(s) besides the Sp1-like component. This other protein was identified as a member of the Ets family on the basis of its ability to bind to both consensus and P4-derived EBS elements in a sequence-specific way. The association of the P4 EBS with its Ets cognate appeared to be strengthened by concomitant interaction of the adjacent GC box with Sp1/3, since the binding of Ets alone could not be detected unless Sp1-like factors were trapped by competitor oligonucleotides. This observation is in agreement with the reported properties of the Ets family of transcription factors. To date, this family comprises about 30 related proteins characterized by the conservation of the so-called ets DNA-binding domain. Ets proteins bind as monomers to DNA sequences containing the core motif (C/A)GGA(A/T) (21, 40). Sequences flanking this purine-rich core determine the relative affinities of different Ets proteins for target DNA. In addition, transcription factors associating with other promoter regions are thought to assist distinct Ets members in binding to EBS through protein-pro-

tein interactions (for a review, see reference 34). This may explain why in the presence of Sp1-like factors, the association of the EBS-GC box region of P4 with Ets could be detected only in the form of a ternary complex involving both families of proteins.

Cooperation of the EBS and GC box in promoter P4 activity. In agreement with previous reports (1, 22, 49), disruption of the GC box of promoter P4 led to a reduction of transcriptional activity, arguing for the involvement of Sp1-like factors in P4 up-regulation. This possibility was confirmed by showing that exogenous Sp1 supplied to Sp1-free *D. melanogaster* SL2 cells increased their capacity for sustaining P4-driven gene expression. As stated above, the P4 GC box was found to form alternative complexes in vitro with Sp1 and Sp3, two members of a family of proteins characterized by a glutamine-rich activation domain and a DNA-binding region containing three zinc fingers (30, 36). The functional role of Sp3 in P4 activity could not be assessed in the same way as for Sp1, given the lack of intrinsic transcription-regulating potential of Sp3 in *D. melanogaster* SL2 cells (31). It should be stated, however, that Sp3 can play a regulatory role in mammalian cells, possibly in combination with other transcription factors or coactivators that are absent from insect cells (31, 41). Therefore, the possibility that Sp3 contributes to the dynamics of the system by competing with Sp1 for the GC box-mediated control of basal P4 activity needs to be considered. It is noteworthy that within the context of the human immunodeficiency virus type 1 promoter, Sp3 acts as a transcription inhibitor counteracting the activating effect of Sp1 (41).

The complexity of the regulatory network involving the proximal region of promoter P4 was further documented by showing that mutations introduced in the EBS motif and known to impair Ets protein binding (42) also reduced P4 activity in FREJ4 cells. In addition, Ets-1, the prototype member of the Ets family, was able to transactivate promoter P4 in an EBS-dependent way when expressed in *D. melanogaster* SL2 cells. These observations argue for the functional role of the EBS element and its cognate protein(s) in the up-modulation of P4 activity.

It is noteworthy that the EBS motif is immediately adjacent to the GC box in the proximal region of promoter P4, raising the question of whether Ets and Sp1-like factors may take part in some form of cross talk at the P4 level. Indeed, it has been reported that Sp1 activity can be modulated by factors which recognize DNA elements flanking or overlapping a GC box (24, 46). Moreover, as stated above, the specificity of Ets protein binding appears to be determined in part by transcription factors that interact with neighboring promoter regions (34). The possible interplay of Ets-1 and Sp1 within the context of P4 promoter regulation was tested by cotransfection experiments using *D. melanogaster* SL2 cells that lack endogenous Sp1 and Ets-1 activity (17, 19). When supplied together, Sp1- and Ets-1-producing plasmids induced a 40-fold enhancement of P4-driven gene expression under conditions in which the stimulation achieved by each plasmid alone was only of the order of five- to eightfold. Therefore, in this cell system, Sp1 and Ets-1 appeared to cooperate in the activation of promoter P4. This feature is reminiscent of the recently reported synergistic effect of Ets-1 and Sp1 on transactivation of the human T-cell leukemia virus type 1 (HTLV-1) virus long terminal repeat (LTR) and parathyroid hormone-related protein gene P2 promoter (19, 26). Interestingly, these promoters share with P4 a region that consists of similarly organized Ets-1 and Sp1 recognition sites. Binding of Sp1 and Ets-1 polypeptides to the corresponding region of the HTLV-1 LTR was found to be cooperative (26), in agreement with the above-mentioned involvement of Ets proteins in interactions with nearby transcription factors. It should be stated that Sp1 is able to cooperate not only with Ets-1 but also with other members of this family. In particular, Ets-2 and Fli-1 were shown to act in cooperation with Sp1 in HTLV-1 LTR transactivation (26).

EBS-dependent activation of promoter P4 by the *ras* oncogene. Promoter P4 has been shown to be up-modulated in *ras*-transformed FREJ4 fibroblasts compared with parental FR3T3 cells (54). This promoter activation is likely to contribute to the overproduction of cytotoxic NS-1 proteins as observed in *ras* (57) and other (51) transformants. Indeed, fluorescence-activated cell sorter analysis of NS protein concentration in MVMp-infected cells showed that for equal multiplicities of infection, the proportions of NS-expressing cells were similar, but the NS content per cell was about threefold higher in FREJ4 cells than in FR3T3 cells (unpublished data). Hence, the understanding of the differential regulation of promoter P4 in transformed and normal cells may provide insights into the mechanism(s) by which neoplastic transformation exacerbates the parvoviral lytic effect.

Ets proteins are known to mediate the transcriptional activation of a variety of viral and cellular promoters by growth modulators. In particular, Ets DNA-binding activity was found to be gradually induced in response to serum in the FR3T3 cell line used in the present study (60). It is noteworthy that this serum dependence is removed in *ras*-transformed FR3T3 derivatives which show constitutive Ets DNA-binding activity (60). These observations are consistent with results of the present report, showing that (i) EBS mutations reduced the activity of promoter P4 in FR3T3 cells kept in the presence of 10% donor calf serum while having little influence on P4-driven gene expression under low-serum conditions, and (ii) *ras* was able to stimulate promoter P4 in transiently transfected FR3T3 cells, through a process that was at least partly mediated in *cis* by the EBS motif.

A number of neoplastic transformation-sensitive promoters were found to contain two (or more) distinct enhancer motifs that are recognized by transcription factors with oncogene-inducible activity. Typically, these factors are members of the Ets, Jun/Fos (AP1), or CREB/ATF family of transcriptional activators (for reviews, see references 3, 7, and 59). The combination of multiple control elements of that kind may allow the expression of corresponding genes to be modulated by various signal transduction pathways. Similarly, P4 appears to harbor some other oncogene-responsive element(s) besides EBS, since *ras*-mediated activation of the parvoviral promoter was reduced but not abolished by mutations disrupting the EBS element. It is worth noting in this respect that the recently identified CREB/ATF-binding sites of P4 were also found to act in a *ras*-dependent way (47) and may thus cooperate with EBS in full induction of the promoter by oncoproteins.

Cotransfection experiments of the type performed in this study do not allow NS-1 to be produced in time and concentration conditions mimicking an authentic parvovirus infection. The influence of NS-1, if any, on EBS-mediated P4 activation therefore remains to be assessed by another approach. It would be interesting in this respect to construct recombinant parvoviruses which harbor EBS mutations in promoter P4 and compare them with wild-type particles for their fate after infection of normal and transformed cells. This long-term study should help to correlate the roles of Ets and the P4 EBS in parvoviral oncotropism under physiologically relevant conditions.

In conclusion, the proximal region of promoter P4 appears to be a target for several transcription factors belonging in particular to the Sp1 and Ets families of DNA-binding proteins. These factors interfere both positively and negatively

with each other, bringing a great versatility to the control of P4 activity. Furthermore, some Ets proteins constitute effectors of growth factor and nonnuclear oncoprotein signaling pathways (for reviews, see references 3, 7, and 59) and contribute in this respect to the exquisite responsiveness of promoter P4 to the physiological state of host cells.

The comparison of autonomous parvovirus genomic sequences reveals a high conservation of the proximal P4 promoter region of MVMp, MVMi, H-1, LuIII, and the recently described mouse 1 virus. It is therefore likely that the complex regulation mediated by the GC box and EBS motif represents a general mechanism by which both basal and oncogene-induced gene expression is controlled in the rodent group of autonomous parvoviruses.

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