JOURNAL OF VIROLOGY, Jan. 1995, p. 239–246
0022-538X/95/$04.00+0

Vol. 69, No. 1

NF-Y Controls Transcription of the Minute Virus of Mice P4 Promoter through Interaction with an Unusual Binding Site

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Received 14 June 1994/Accepted 26 September 1994

Electrophoretic mobility shift assays performed with nuclear extracts from human fibroblasts revealed the formation of two major protein complexes with an oligonucleotide (nucleotides 78 to 107) from the palindromic region located upstream from the minute virus of mice (MVM) P4 promoter. It was shown that this oligonucleotide bound USF at the enhancer E box CACATG. The second complex contained the transcription factor NF-Y, whose association was surprising because its target sequence lacks the canonical CCAAT motif present in all mammalian NF-Y binding sites identified so far. The MVM NF-Y recognition element instead contains the CCAAC sequence. USF and NF-Y had distinct but overlapping sequence requirements for binding, suggesting that their associations with MVM DNA were mutually exclusive. Because of the palindromic nature of MVM DNA terminal sequences, NF-Y associated with the three nucleotide configurations corresponding to the hairpin structure and to the external and internal arms of the extended duplex replication form, respectively. However, owing to the imperfection of the palindrome, the binding of USF was restricted to the internal arm. Point mutations that suppressed the in vitro binding of NF-Y to the internal palindromic arm reduced the activity of the resident P4 promoter, while those preventing complex formation with USF did not, as determined by transient expression assays using the luciferase reporter gene. The data led to the identification of a novel P4 upstream regulatory region capable of interacting with two transcription factors, from which one (NF-Y) appeared to upmodulate the activity of the promoter.

Minute virus of mice (MVM) is an autonomous parvovirus belonging to the family *Parvoviridae*, a group of linear single-stranded DNA viruses that parasitize arthropods, birds, and mammals, including humans (54). Their genomes (approximately 5 kb long) comprise terminal palindromic sequences at both ends which contain critical *cis*-acting elements required for viral DNA replication (15, 50). Whereas members of the adeno-associated virus subgroup of parvoviruses mostly rely on helper viruses (adenoviruses and herpesviruses) for their multiplication (6), the autonomous parvoviruses replicate during the S phase in appropriate host cells (17). Yet, in contrast to DNA tumor viruses, the autonomous parvoviruses are unable to force quiescent host cells into the S phase of the cell cycle.

Like most autonomous parvoviruses of vertebrates, MVM and the closely related H-1 virus contain two overlapping transcription units, the promoters of which are located near the left-hand site (map unit 4) and middle (map unit 38) of the viral genome (41) and drive the production of mRNA species by using the same polyadenylation signals (13). The left-hand promoter (P4) directs the synthesis of the R1 and R2 transcripts, which are translated into the nonstructural proteins NS-1 and NS-2, respectively (16). Both nonstructural proteins are essential for optimal virus multiplication in cells from their natural host (33, 38). Besides its role in viral DNA replication (15), the NS-1 protein *trans*-activates the middle promoter (P38), which controls the structural transcription unit (23, 24, 45), thus establishing the sequential program of parvoviral gene expression (14). In addition, NS-1 *trans*-modulates a number of heterologous promoters (27, 48, 55), a property that may be relevant to the cytotoxic activity of this protein (10, 32, 40, 46). It should also be stated that parvoviruses have oncosuppressive properties that may rely, at least in part, on the observed stimulation of their replication and/or cytotoxicity in many transformed cells (49). Accordingly, cell susceptibility to MVM and H-1 virus lytic growth is modulated as a function of cell differentiation and proliferation (17). One of the steps in the life cycle of a parvovirus which is dependent on the physiological state of the host cell is transcription, in particular, transcription from the pivotal P4 promoter (53). The functioning of this promoter therefore constitutes a key issue for the understanding of parvovirus-host cell interactions.

The only functional determinants of promoter P4 of MVM identified so far consist of a TATA box and a proximal GC-rich sequence that interacts with nuclear proteins of the SP1 family of transcription factors (1). Yet, additional upstream elements appear to positively contribute to P4 activity, as shown by mutational analyses of this promoter (1, 26, 52). In this report, we focus our attention on a P4 region that is located in the internal arm of the left-hand terminal palindrome of MVM DNA and contains the sequence CACATGGTTGGT (nucleotides [nt] 91 to 102 according to reference 3). This sequence is about 100 nt upstream from the transcription start site and is potentially interesting in several respects.

On the one hand, the CACATG motif (nt 91 to 96) belongs to the group of CANNTG elements referred to as E boxes (37). Typically, E boxes are recognition sites for a class of regulatory proteins characterized by a basic DNA binding

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region and a helix-loop-helix domain (37). On the other hand, the considered region of promoter P4 comprises the sequence 5′-TGGTTGGT-3′ (nt 95 to 102), partly overlapping the E element. This motif is overrepresented in MVM DNA (8) and is found as a 6- to 8-of-8-nt match at similar positions in the left- and right-hand palindromes and internal promoter from other autonomous parvoviruses (3, 4, 7, 21, 30, 35, 43, 44, 47). This octamer and its inverted form 5′-ACCAACCA-3′ will be referred to as the parvovirus repetitive element (PRE). The PRE motif was suggested to mediate upregulation of the P4 promoter by NS-1 because of its resemblance to the *trans*-activation region of the P38 promoter (8, 23). A region centered around the PRE was protected from UV (in vivo) or DNase (in vitro) attack, suggesting that nuclear proteins bind to this DNA sequence (26).

Results presented in this paper indicate that upon incubation with nuclear extracts from simian virus 40-transformed human cells, the above-mentioned E box associated in vitro with the transcription factor USF. This protein contains a basic DNA binding domain as well as helix-loop-helix and leucine zipper dimerization motifs (28). Moreover, the region comprising the E box and PRE was found to constitute an unconventional binding site for the transcription factor NF-Y (12, 25). The physiological relevance of the association of NF-Y with the P4 promoter-enhancer was supported by the fact that suppression of this interaction, as a result of point mutations in target DNA, correlated with a reduced promoter activity.

MATERIALS AND METHODS

Cells and recombinant plasmids. The simian virus 40-transformed human cell line NB-E (51), which is fully permissive for MVM, was cultivated in minimal essential medium supplemented with 5% fetal calf serum. Exponentially growing cultures were used for all experiments.

Plasmid pP4-luc, which contains the firefly luciferase gene under control of the P4 promoter and upstream regulatory region from the fibrotropic variant of parvovirus MVM, was produced from pP4-cat (53) by substituting the luciferase cDNA (20) for the chloramphenicol acetyltransferase gene. Point mutations in the upstream region were introduced by replacing the 185-bp *Afl*III-*Nco*I fragment of MVM DNA with its equivalent carrying mutations in the E box, NF-Y binding site, or both elements. Mutated fragments were obtained by PCR, using the wild-type right-hand primer 5′-GGGCCATGGTTAGTTGGTTACT CTC-3′ and one of the following left-hand primers:

5′-GTCACACGTCACTTACGTTTCACATGGTTGGTCAGTTC-3′ (wild type)
5′-GTCACACGTCACTTACGTTTCACATGGTGTGTCAGTTC-3′ (99/100)
5′-GTCACACGTCACTTACGTTTCACAGTGTTGGTCAGTTC-3′ (95/96)
5′-GTCACACGTCACTTACGTGAACATGGTTGGTCAGTTC-3′ (GA)
5′-GTCACACGTCACTTACGTGAACATGGTGTGTCAGTTC-3′ (GA99/100)

Mutated nucleotides are underlined. The final constructs were confirmed by DNA sequencing.

Transfection and transient expression assays. Cells were transfected with 2 μg of reporter plasmid (pP4-luc or mutated derivatives) by the standard calcium phosphate precipitation technique. Forty-three hours after transfection, cells were collected and luciferase assays were performed as described elsewhere (20). Enzyme activities were determined from equal amounts of total proteins (20 μg), as determined by the Bradford method (Bio-Rad).

Electrophoretic mobility shift assays (EMSAs). Crude nuclear extracts were prepared essentially according to the method of Dignam et al. (22). All buffers contained a cocktail of proteinase and phosphatase inhibitors (0.5 mM phenylmethylsulfonyl fluoride; 1 μg each of leupeptin, antipain, and pepstatin per ml; 2 mM benzamidine; 10 mM β-glycerophosphate; 2 mM levamisole; and 10 mM orthovanadate). Binding reactions were performed for 10 min on ice with 1 ng of radiolabelled DNA probe and 4 μg of nuclear extract in 14 μl of 10% glycerol–10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.5)–50 mM NaCl–10 mM $MgCl_2$–1 mM dithiothreitol–0.1% Nonidet P-40 in the presence of 1 μg of sonicated salmon sperm DNA and 1.5 μg of poly(dI-dC). For competition experiments, the nuclear extract was preincubated with various excesses of unlabelled double-stranded competitor oligonucleotide for 5 min prior to the addition of the ^{32}P-labelled probe. Samples were loaded on a 6% native polyacrylamide gel, run for 4 h at 180 V in order to well separate the NF-Y and USF complexes. The gels were examined by autoradiography after exposure of the dried gel to a Kodak X-AR film at −70°C with an intensifying screen.

Synthetic double-stranded oligonucleotides were used as probes and competitors. MVM-specific oligonucleotides corresponded to upstream regions of the P4 promoter and to various mutated derivatives thereof, as depicted in Fig. 1, 5, and 6. The Eα-Y oligonucleotide 5′-ATTTTTCTGATTGGTTAAAAGT-3′ contained the consensus Y box from the mouse Eα promoter (5). The UE oligonucleotide (5′-GGTGTAGGCCACGTGACCGGGTGT-3′) harbors a strong USF binding motif, from the adenovirus 2 major late promoter (36). The binding motifs in both oligonucleotides are underlined.

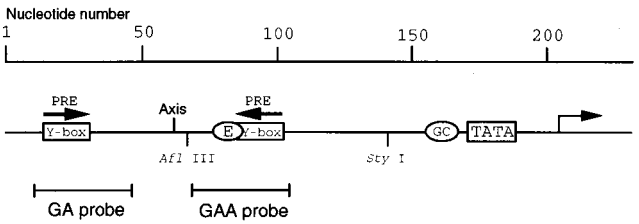

FIG. 1. Location of *cis*-acting elements and probes within the MVM P4 promoter. The scheme depicts the left-hand portion of MVMp DNA, up to the start site for P4-directed transcription (angled arrow). The positions of the axis of symmetry of the extended terminal palindrome, the *Afl*III and *Sty*I restriction sites, and the GAA and GA oligonucleotide probes used in this work are shown. *cis*-acting regulatory elements (TATA box, GC-rich motif, and putative E and Y boxes) are boxed. Arrows show the location and orientation of the PREs (5′-ACCAACCA-3′) on either strand. Nucleotide numbering is according to the work of Astell et al. (3).

For antibody supershifts in EMSAs, 0.1 to 0.5 μl of antiserum or ascites fluid was preincubated for 30 min on ice with 4 μg of nuclear extract prior to the addition of probe. αNF-YA is a monoclonal antibody that recognizes the NF-YA subunit, while αNF-YB is a rabbit antibody directed against the B chain of NF-Y (34). Rabbit anti-USF sera 1-310 and 18-105 were directed against the 310 N-terminal amino acids and to a peptide encompassing amino acids 18 to 105 of the 43-kDa component of USF, respectively (42).

In situ Cu-orthophenanthroline footprinting. The *Afl*III-*Sty*I fragment of MVM DNA (nt 75 to 140) was 3′ end labelled on either strand by using the Klenow enzyme and incubated with nuclear extracts under the same conditions as described for the EMSAs, except for a twofold scaling up of the reaction. After electrophoresis, the gel was soaked for 10 min in 50 mM Tris (pH 8.0)–0.08 mM orthophenanthroline–0.09 mM $CuSO_4$–6 mM mercaptopropionic acid, and then 2.8 mM neocuproin was added to stop the reaction (31). After autoradiographic exposure of the wet gel, the complexes and free probe were excised, eluted in 10 mM Tris (pH 8.0)–1 mM EDTA–500 mM NaCl, ethanol precipitated, and loaded onto a denaturing polyacrylamide gel. Base positions were determined by A+G sequencing, according to the method of Maxam and Gilbert.

RESULTS

Association of NF-Y and USF transcription factors with an upstream sequence of promoter P4. The left-hand terminus of MVM DNA consists of an imperfect palindromic sequence whose axis of symmetry is depicted in Fig. 1. Previous in vivo and in vitro footprinting experiments showed that a region centered around nt 100 was protected from DNase digestion and UV damage (26), suggesting that nuclear proteins bind to the inner arm of the terminal palindrome. As illustrated in Fig. 1, this region contains the above-mentioned E box and PRE motif. Moreover, PRE is part of a sequence (5′-GACCAAC CAT-3′) that overlaps the E box and is an 8-of-10-nt match with the Y box (5′-PuPuCCAATCAG-3′), a *cis*-regulatory element common to all class II major histocompatibility complex promoters (for a review, see reference 5). Since the terminal palindrome lies upstream from the initiation site for promoter P4-driven transcription (Fig. 1) and is involved in the resolution of MVM DNA replicative intermediates (15), its interaction with proteins is of potential physiological relevance and was further analyzed by EMSA.

A radiolabelled duplex oligonucleotide (nt 78 to 107), designated GAA and encompassing the putative E and Y boxes (Fig. 1), was incubated with nuclear extracts from NB-E cells. As shown in Fig. 2, three major retarded complexes were detected (lane 1), the formation of which could be inhibited by adding an excess of the homologous unlabelled oligonucleotide

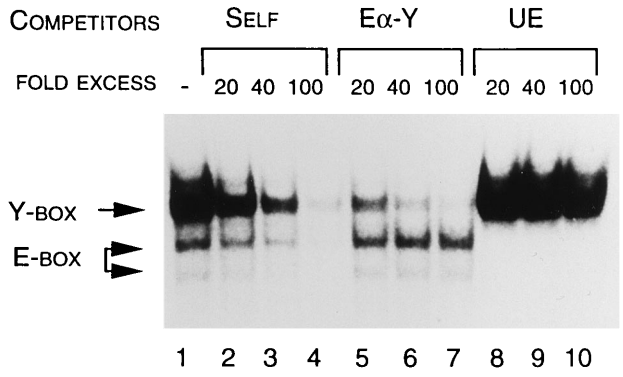

FIG. 2. Competitive EMSAs of the association of nuclear proteins from NB-E cells with the MVM GAA oligonucleotide probe. Nuclear extracts were incubated with the ^{32}P-labelled probe in the absence (lane 1) or presence (lanes 2 through 10) of indicated molar excesses of unlabelled competitor DNA. The competitor oligonucleotide either was homologous to the probe (SELF, lanes 2 through 4) or contained a prototype Y box (Eα-Y, lanes 5 through 7) or E box (UE, lanes 8 through 10). The upper arrow and lower pair of arrows indicate the positions of the NF-Y complex and the USF-containing complexes, respectively. An autoradiogram of the region of retarded complexes is shown.

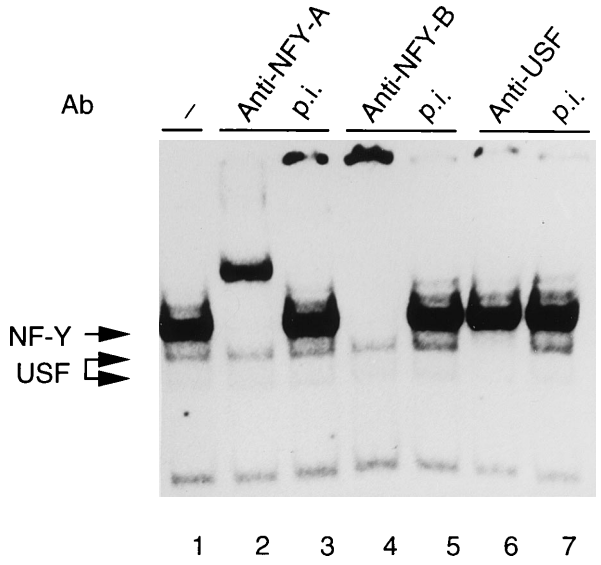

FIG. 3. Effects of antibodies directed against USF and NF-Y on the electrophoretic mobilities of DNA-protein complexes. The radiolabelled MVM GAA probe was incubated with NB-E nuclear extracts alone (lane 1) or with monoclonal antibodies specific for the A chain of NF-Y (lane 2), rabbit antisera directed against the B chain of NF-Y (lane 4), or the 43-kDa component of USF (serum 1-310; lane 6) and the respective preimplantation peritoneal fluid (lane 3) and preimmune sera (lanes 5 and 7) (p.i.). The free probe and retarded complexes were identified by EMSAs and revealed by autoradiography. The arrows point to the NF-Y- and USF-containing complexes.

(lanes 2 through 4). The upper (most slowly migrating) specific complex was inhibited by competition in a dose-dependent manner by Eα-Y, an oligonucleotide carrying a consensus Y box (Fig. 2, lanes 5 through 7), while the lower ones were suppressed by the E-box-containing oligonucleotide UE (lanes 8 through 10). No significant cross-reaction was observed in competition experiments, suggesting that the protein components of the upper and two lower complexes might consist of Y- and E-box binding factors, respectively. The ability of the consensus Y-box-containing competitor to trap factors associating with the MVM probe was unexpected. Indeed, Y-box recognition in higher eukaryotic cells is considered to require an internal CCAAT motif (5, 25), while the corresponding MVM sequence consists of CCAAC instead. The competing capacity of the consensus Y box was first confirmed under conditions in which the *Afl*III-*Sty*I restriction fragment of MVM DNA (nt 75 to 140 [Fig. 1]) was used as a probe for complex formation (data not shown). It should also be stated that oligonucleotides containing the Eα-Y element competed more efficiently for protein binding to the MVM probe than did the parvovirus sequence itself (Fig. 2, lanes 2 through 7), suggesting that the latter constitutes a low-affinity Y box.

In order to back up the conclusions drawn from competition experiments, antibodies directed against the Y-box binding factor NF-Y (34) were tested for their effects on the pattern of GAA probe retardation. NF-Y is a factor that is composed of two distinct polypeptide chains referred to as A and B (29). As shown in Fig. 3, the retardation of the upper complex (lane 1) became more pronounced after treatment with monoclonal antibodies specific for the A chain of NF-Y (lane 2). Moreover, a polyclonal antiserum that recognized the B chain of NF-Y inhibited formation of the complex (34), which led to aggregation of material at the top of the gel (Fig. 3, lane 4). Given that the antibodies used were monospecific and failed to alter the modality of the unrelated lower complexes (Fig. 3, lanes 2 and 4), and that corresponding control sera had no detectable effect (lanes 3 and 5), these data strongly argue that NF-Y is the protein component of the upper complex with the GAA probe. On the other hand, the transcription factor USF/MLTF/UEF was reported to associate with DNA containing the same E box as MVM contains (36, 42). Moreover, purified USF forms two characteristic retarded complexes with a probe from the adenovirus major late promoter (28, 42). Accordingly, the polyclonal antiserum directed against USF selectively supershifted both faster-migrating complexes formed with the GAA probe (Fig. 3, lane 6), under conditions in which the preimmune serum was ineffective (lane 7). Similar results were obtained with serum 18-105, which recognizes another USF epitope (data not shown). It was concluded from these observations that the NF-Y and USF transcription factors were capable of independently interacting in vitro with an oligonucleotide that was derived from the internal arm of the left-hand MVM palindrome and encompassed overlapping Y-like and E boxes.

Respective involvement of Y and E boxes in NF-Y and USF binding to MVM DNA. The MVM DNA sequence interacting with NF-Y was first defined by copper orthophenanthroline footprinting. In order to avoid the interference of USF binding to the DNA probe, the NF-Y-specific upper complex from EMSAs (see previous section) was treated with copper-orthophenanthroline in the gel, eluted, and analyzed by sequencing gel electrophoresis. As illustrated in Fig. 4, protection against chemical cleavage was given by NF-Y to a DNA stretch (nt 85 to 109) that encompassed both E and Y boxes. This footprint was slightly out of center with regard to the latter element. Therefore, the importance of the MVM Y box for NF-Y recognition was further assessed by using GAA oligonucleotide probes that carried point mutations or a small deletion within this region, as depicted in Fig. 5A. Bound NF-Y was revealed by EMSAs in the form of the above-mentioned slowly migrating complex that could be supershifted by NF-Y-specific antibodies. As shown in Fig. 5B (lanes 1 to 4), the conversion of the MVM Y box into the reported consensus (mut97/103) led to a threefold increase in the yield of NF-Y binding to the probe. Similarly, transformation of the CCAAC core into the canonical CCAAT sequence was reported to enhance the affinity of HAP2/3, the yeast homolog of NF-Y, for the Y box

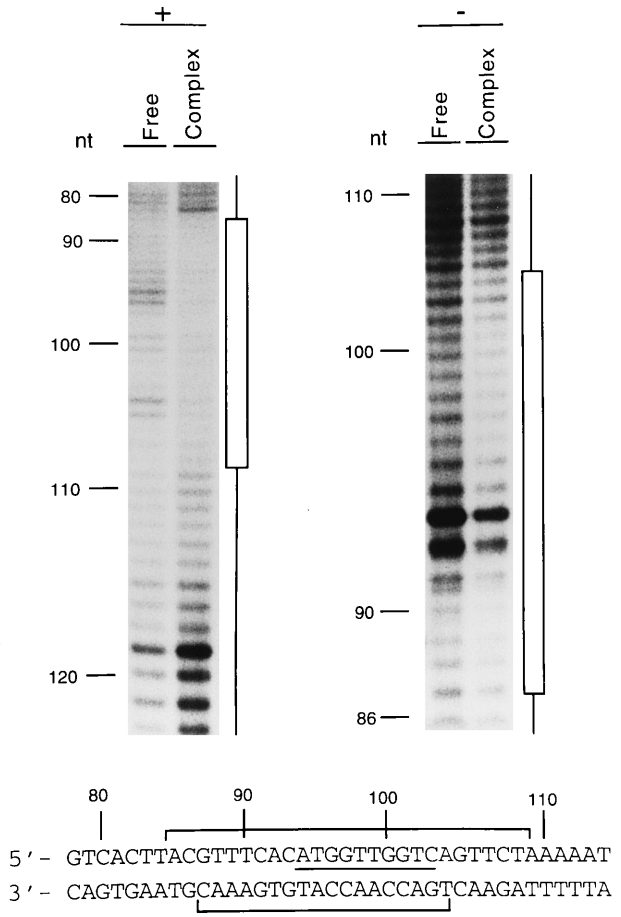

FIG. 4. Location of the proximal NF-Y binding site of promoter P4 by in vitro footprinting. The *Afl*III-*Sty*I DNA restriction fragment (Fig. 1) was end labelled on either the plus (left panel) or minus (right panel) strand and incubated with nuclear extracts from NB-E cells. Binding reaction products were subjected to electrophoretic separation and digested by treating the gel with copper-orthophenanthroline. The free probe (lanes marked "Free") and NF-Y complex (lanes marked "Complex") were eluted and analyzed by sequencing gel electrophoresis, with reference to an A+G ladder. Protected zones are indicated by open boxes on the right-hand site of the autoradiograms. Below the autoradiograms, protected regions are shown located on the MVMp sequence. The MVM Y box is underlined.

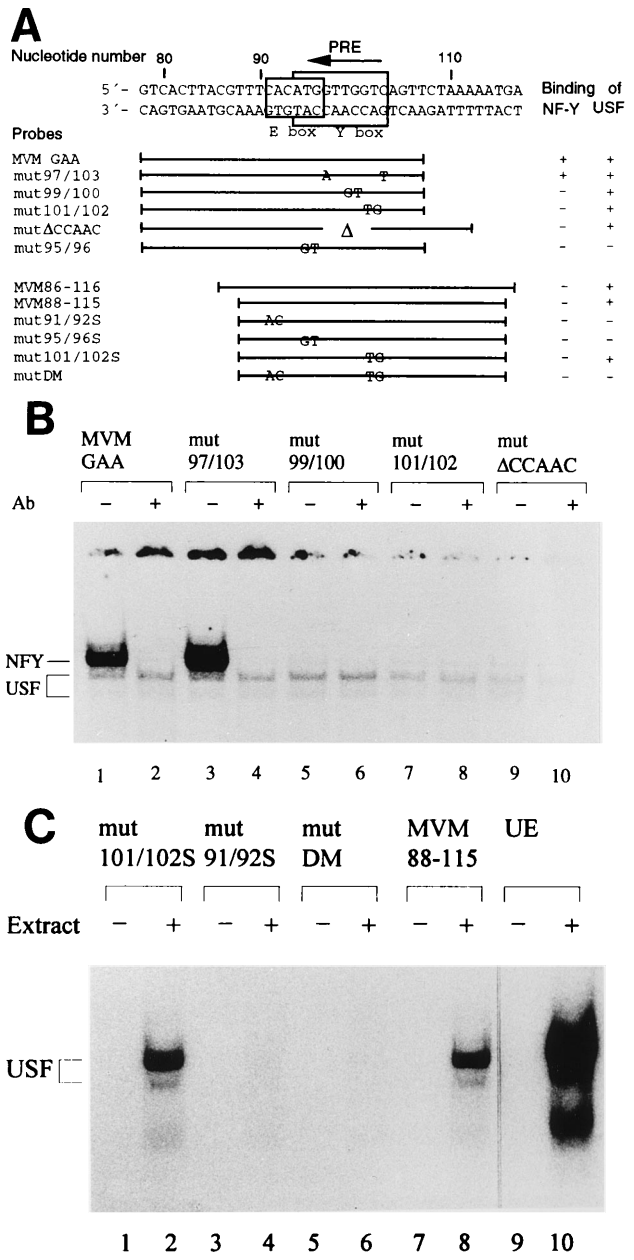

FIG. 5. Mutational analysis of the binding sites of the NF-Y- and USF-responsive elements upstream of promoter P4. Oligonucleotides corresponding to the inner arm of the extended terminal palindrome and mutated derivatives thereof were used as probes in EMSAs with nuclear extracts from NB-E cells. (A) Location of oligonucleotide probes and mutations within the P4 upstream region. The regulatory boxes (E and Y) and PRE sequence are shown by boxes and a horizontal arrow, respectively. The deletion of CCAAC is indicated by a Δ. The capacities (+) or inabilities (−) of the various probes to associate with NF-Y and USF are given on the right. (B and C) Band shift analysis of the MVM probes GAA (B) and 88-115 (C) and mutated derivatives. Only retarded complexes are shown. For panel B, rabbit antibodies (Ab) directed against the NF-Y B chain were added (+) or not added (−) to the reaction mixture. For panel C, the UE probe containing a prototype USF recognition site was included for comparison. + indicates the addition and − indicates the absence of nuclear extracts in the reaction mixture. The NF-Y- and USF-containing complexes are indicated.

of the CYC1 promoter (39). In contrast, mutations causing the MVM Y box to diverge from the consensus sequence (mut99/100, mut101/102, and mut95/96), or a deletion of the CCAAC motif (mutΔCCAAC), prevented NF-Y from interacting with the DNA probes (Fig. 5A and panel B, lanes 5 through 10). Taken together, these data indicated that the CCAAC core was necessary (mut99/100 and mutΔCCAAC) but not sufficient (mut95/96) for NF-Y binding, thereby showing that the atypical MVM Y box fulfilled the reported requirements of the core pentanucleotide (25) and specific flanking sequences (12) for constituting an NF-Y-responsive element. It should also be stated that nucleotides beyond the MVM Y box appeared to exert a longer-range influence on its recognition by NF-Y. Indeed, NF-Y failed to form a complex with MVM oligonucleotides 86-116 and 88-115, which extended only to 8 and 6 nt upstream from the Y box, respectively (Fig. 5A and panel C, lanes 7 and 8). This feature may be related to the shift of the NF-Y-induced footprint towards the 5′ side of the MVM Y box (Fig. 4), but it contrasted with the reported need of only 6 nt upstream from the Eα-Y Y box to allow NF-Y binding (25).

The MVM probe contained an E box identical to an established binding site for the transcription factor USF (36, 42). This element was therefore likely to be responsible for the above-mentioned association of the MVM oligonucleotide

with USF. This was confirmed by showing that mutations disrupting the E box alone (mut91/92S) or both the E and Y boxes (mut95/96, mut 95/96S, and mutDM) abolished USF binding, as measured by EMSAs (Fig. 5A and panel C, lanes 3 through 6). Yet, the E box of MVM proved to constitute a low-affinity USF-responsive element in comparison with the E box CACGTG from the reference UE oligonucleotide, as apparent from the relative abundances of the respective complexes (Fig. 5C, compare lanes 8 and 10). Interestingly, USF was able to associate with oligonucleotides 86-116 and 88-115, which were not recognized by NF-Y although they contained the Y box (Fig. 5A and panel C, lane 8). This may be indicative of the lesser requirement of upstream sequences for the stabilization of USF versus NF-Y complexes with corresponding DNA boxes. It is noteworthy that mutations specifically disrupting the MVM Y box (mut99/100, mut101/102, mutΔ CCAAC, and mut101/102S) did not prevent the formation or alter the mobility of USF-containing complexes (Fig. 5A; panel B, lanes 5 through 10; and panel C, lane 2). Together with the fact that USF and NF-Y complexes were insensitive to antibodies directed against NF-Y and USF, respectively (Fig. 3), our observations indicated that the associations of these factors with the DNA probe were independent and mutually exclusive.

Influence of the structure of the MVM left-hand palindrome on its association with NF-Y and USF. The left-hand terminal sequence of MVM virion single-stranded DNA consists of an imperfect palindrome (4). The intracellular replication of this genome involves the formation of double-stranded molecules in which the left-hand palindrome is either folded into a hairpin or copied and extended (2, 17). As depicted in Fig. 6A, the palindromic stem contains a mismatched "bubble" (GAA/GA) leading to a sequence difference between the inner and outer arms of the extended duplex form. This feature deserves consideration in at least two respects. On the one hand, the first replicative intermediates produced result from the extension of the 3′ end of the folded hairpin, which bridges the complementary strands and contains the bubble. The processing of these duplex DNA molecules is thought to require their prior expression into nonstructural proteins, raising the question of the influence of the bubble on the activity of promoter P4. On the other hand, the resolution of replicative intermediates appears to involve their asymmetrical nicking on either side of the extended palindrome (15), suggesting that sequence heterogeneities between the inner and outer arms may direct strand recognition.

Since the bubble overlapped the E box and was close to the Y box (Fig. 6A), its effects on the recognition of these elements were determined by EMSAs with oligonucleotide probes corresponding to the outer (GA) and inner (GAA) palindromic arms, or to the mismatched hairpin ("Bubble"). As shown in Fig. 6B, NF-Y was able to associate with all three probes in the form of complexes that could be inhibited by competition with Eα-Y, an oligonucleotide harboring an NF-Y binding site. A mutation disrupting the Y box (mut99/100 [Fig. 5A]) abolished these interactions (Fig. 5B, lanes 5 and 6, and data not shown). Therefore, the three structures of the bubble region appeared to meet the above-mentioned requirement of flanking sequences for the binding of NF-Y to the MVM Y box, pointing to this factor as a potential regulator of both extended and hairpin-folded replicative intermediates. In contrast, neither the bubble probe nor the GA probe showed a detectable interaction with USF, which only associated with the GAA oligonucleotide (inner arm) in a UE competitor-sensitive manner (Fig. 6B). This result could be explained by the mismatch-related absence of a complete E box in the hairpin

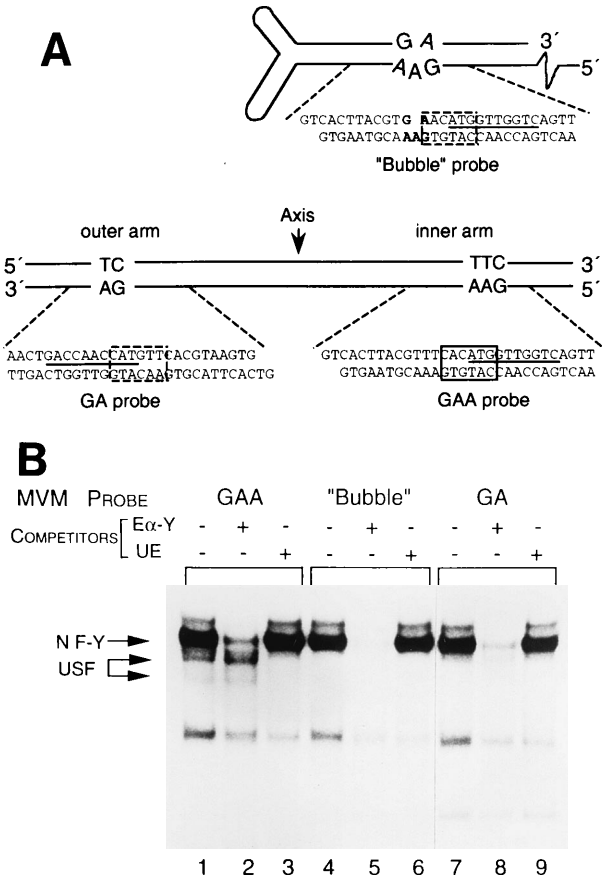

FIG. 6. Influence of terminal palindrome structures on their interactions with NF-Y and USF. (A) Scheme of the left-hand palindrome in its hairpin (upper diagram) and extended-form (lower diagram) configurations. The MVM oligonucleotide probes encompassed the mismatched region of the hairpin stem or corresponding sequences from either arm of the palindrome unfolded around its axis of symmetry. The Y box is underlined, while complete and incomplete E boxes are shown by solid and dashed boxes, respectively. (B) EMSAs performed with the indicated ^{32}P-labelled probes in the absence (−) or presence (+) of a 100-fold excess of Y-box or UE competitor oligonucleotides. Specific NF-Y- and USF-containing complexes are indicated by arrows. The region of retarded complexes is shown.

and outer palindromic arm and raised the possibility that USF may participate in processes discriminating between the two arms of the palindrome.

Functional role of the Y and E boxes of promoter P4. The contribution of the combination of NF-Y- and USF-responsive elements to P4 activity was assessed by transfecting NB-E cells with wild-type and mutated derivatives of pP4-luc, a plasmid containing the firefly luciferase reporter gene under the control of P4 proximal and upstream sequences in the extended configuration. As diagrammed in Fig. 7, pP4-luc was modified by site-directed mutagenesis to disrupt the proximal Y box (mut99/100; TG→GT [Fig. 5A]), the E box (mutGA; TTC [nt 89 to 91]→GA), or both motifs (mut95/96; TG→GT [Fig. 5A]) from the inner palindromic arm. As shown in Fig. 5A and B and 6B, these mutations prevented the Y (mut99/100 and mut95/96) and E (mutGA and mut95/96) boxes from being recognized by NF-Y and USF, respectively.

The abilities of the various clones to direct the production of luciferase were compared (Fig. 7). The inactivation of the Y box alone (pP4-99/100-luc) reduced P4-driven luciferase expression to about 40% of the level achieved by pP4-luc. In

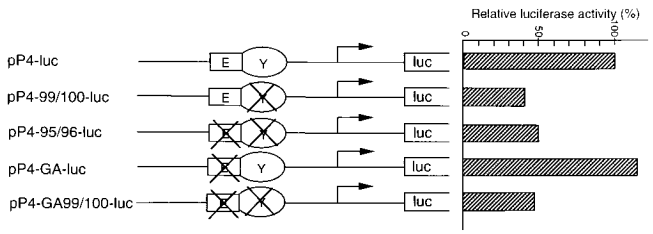

FIG. 7. Effect of mutations in the E and Y boxes of the inner palindromic arm on the activity of promoter P4. NB-E cells were transfected with plasmids expressing the reporter luciferase gene from the whole wild-type P4 promoter (pP4-luc) or from derivatives carrying mutations in the E and Y boxes as indicated (see Materials and Methods for the corresponding sequences). Luciferase activities are expressed as percentages of the level obtained by pP4-luc. The data shown are average values from four to five independent experiments (standard deviations were less than 25%). Arrows, transcriptional start site.

contrast, an E-box-specific mutant (pP4-GA-luc) was not impaired and rather was slightly stimulated in this respect. Moreover, the E box did not appear to contribute to the residual activity of pP4-99/100-luc, since mutations disrupting both E and Y motifs (pP4-GA 99/100-luc and pP4-95/96-luc) were not more inhibitory than those affecting the Y box alone (pP4-99/100-luc) with regard to the yield of luciferase expression. Because the USF E-box interaction was not impeded by some of the mutations suppressing the NF-Y Y-box complex, our results indicated that the Y box participated in the activation of promoter P4 in NB-E cells, while the E box was ineffective in this respect. Since the protein occupancies of neighboring E and Y motifs were found to be mutually exclusive (see above), E-specific mutations may release this promoter region from its not-very-functional interaction with USF and make it more available for other transcription factors, in particular, NF-Y. This might tentatively explain the slight increase in promoter P4 strength, which resulted from the inactivation of the E box.

DISCUSSION

An upstream region of promoter P4, located within the terminal left-hand palindrome of MVM at about 100 nt from the transcription start site, was found to contain binding sites for two transcription factors. One of these was the USF/MLTF/UEF factor, a protein with basic, helix-loop-helix, and leucine zipper domains (28), which associated with an E box (CACATG) in MVM DNA. The other transcription factor was NF-Y, also designated CP1, CBF, and YEBP, a protein (references 12, 29, and 56 and references therein) which bound to a Y-box-related motif (GACCAACCAT) overlapping the PRE and E box. USF and NF-Y are both ubiquitous nuclear factors that proved to be involved in the regulation of a variety of cellular and viral genes (references 5, 12, 19, 29, 34, 36, and 42 and references therein). The mutational analysis presented in this paper suggested that USF did not appreciably contribute to P4 promoter activity in NB-E cells, while NF-Y was physiologically relevant in this respect. Yet, it is quite possible that USF may *trans*-activate promoter P4 in other host cells and/or participate in the resolution of the terminal palindrome during the process of MVM DNA replication (15). However that may be, our results indicated that besides the previously reported functional TATA- and CG-rich proximal motifs (1), at least one additional *cis*-acting upstream element appeared to modulate the functioning of promoter P4. Interestingly, NF-Y binding was conserved in the hairpin configuration of the terminal palindrome, pointing to this factor as the possible regulator of early parvoviral gene expression. In the extended configuration, the 3′ end of MVM DNA contains two NF-Y binding sites located in the inner and outer palindromic arms, respectively. For technical reasons, the present mutational analysis was restricted to the inner Y box, which proved to be functional. Whether the outer NF-Y binding site also participates in the regulation of promoter P4 within monomer- and/or dimer-length replicative forms remains to be determined.

Atypical NF-Y binding site in the MVM left-hand palindrome. To the best of our knowledge, all NF-Y binding sites identified so far upstream from mammalian genes contain the internal 5′-CCAAT-3′ motif initially identified as the NF-Y core binding sequence (25). In the Y box of the MVM promoter P4, C is found instead of T at the 3′-terminal position of the core sequence. This natural substitution did not prevent NF-Y from associating with MVM DNA, yet it reduced the binding affinity from the level observed with a mutated MVM probe in which the consensus Y box was restored. In contrast, an experimental T→C substitution in the Y-box core of the β-globulin promoter abolished complex formation (19). Therefore, the tolerance of NF-Y with regard to the 3′-terminal pyrimidine of the Y-box core sequence appeared to depend on flanking nucleotides. Accordingly, the shortening of the MVM probe upstream from the Y box made it unrecognizable for NF-Y. It should also be stated that a CCAAC core of the MVM type is also present in the UAS2 promoter element of the yeast *CYC1* gene and associates with HAP2/3, the yeast homolog of NF-Y (39). The conversion of this sequence to the canonical CCAAT motif enhanced both HAP2/3 binding to the UAS2 element and promoter activity (39). It is presently a matter of speculation why C was conserved instead of T in the Y-box core sequence of MVM, in spite of its apparent disadvantage insofar as DNA-protein complex formation is concerned. A possible clue may lie in the subtle balance of the transcriptional and putative replicational regulatory activities of NF-Y, as discussed below.

Mutually exclusive binding activities of NF-Y and USF to the MVMp upstream promoter element. The sequence of the inner arm of the MVMp left-hand palindrome shows an overlap of 3 nt between the E box and the 10-nt-long MVM Y box. Accordingly, neither NF-Y nor USF was able to bind to a mutant (mut95/96) affected with a double substitution (TG→GT) in the overlapping region. This substitution concerned nucleotides that were shown to be essential to both a functional E box and NF-Y contacts with the Y box (12, 25). The overlap of the E and Y boxes in MVM DNA raised the question of a possible competition between NF-Y and USF for the occupancy of this regulatory region. The NF-Y complex detected in this study did not contain USF, just as the USF complexes had no NF-Y component. This was shown by means of specific competitors, antibodies, and mutated probes that altered the formation or migration of one type of complex while inducing no qualitative or major quantitative change in the other. These results showed that NF-Y and USF could bind independently to MVM DNA and suggested that this association was mutually exclusive, as no DNA complex with both factors was identified. It cannot be ruled out, however, that such ternary complexes were unstable under the experimental conditions used. Assuming a mutually exclusive binding, a greater versatility in the control of promoter P4 may be achieved by varying the relative proportions of competing NF-Y and USF proteins. Accordingly, the functional tests performed in this work indicated that NF-Y binding contributed to the *trans*-activation of the promoter, while USF was ineffective in this respect.

Sequence	Virus	Reference
18-**CAGTTGGT**CAGTTTAAAG-1	PPV	43
21-**TGGTTGGT**CAGTTCTAAAAAT-1	MVM	3
22-**TGGTTGGT**CAGTTCTAAAAATG-1	H-1	47
24-**TGGTTGGT**TAGTTCTAAAAATGAT-1	LuIII	21
21-**TGGTTGGT**CAGTTCTAAAAAT-1	KRV	4
15-**CAGTTGGT**TCTAAAG-1	FPV	35
18-**CAGTTGGT**TCTAAAGAAT-1	CPV	44
62-**CAGTTGGT**TCTAAAGAATGAT-42	MEV	30
19-**ATATTGGT**TGAGAATTAAT-1	ADV	7

FIG. 8. Location of PRE-like elements at the left-hand termini of various parvoviral genomes. PREs are indicated by boldface letters. Nucleotide numbering is according to the references listed. For the terminal residue at the 3′ end of MVM, see also reference 15. PPV, porcine parvovirus; KRV, Kilham rat virus; FPV, feline panleukopenia virus; CPV, canine parvovirus; MEV, mink enteritis virus; ADV, Aleutian mink disease parvovirus.

Conservation of PRE-like sequences in parvoviral promoters and replication origins. Among more than 500 vertebrate promoters screened, about 30% were found to contain the canonical Y-box core sequence CCAAT, while none possessed the MVM equivalent CCAAC (9). In contrast, the CCAAC motif is overrepresented in MVM DNA (8). Moreover, the comparison of presently available genomic sequences of several related autonomous parvoviruses of vertebrates revealed 6- to 8-of-8-nt matches of PREs near to the resolution sites of the left-hand palindrome (Fig. 8), in the internal promoter (3, 21, 43, 47), and in the right-hand palindrome (21, 30, 43, 47, 50). Among the 3′ termini listed in Fig. 8, only the PRE of Aleutian mink disease parvovirus comprises the CCAAT instead of the CCAAC sequence (Fig. 8). Since PRE appeared from the present work to be able to associate with transcriptionally active NF-Y, as did the corresponding cellular motif (34), this sequence divergence is puzzling and may tentatively be traced back to an additional role of the parvoviral element in the control of DNA replication. Indeed, the terminal palindromes contain the *cis* determinants for the initiation of DNA replication and the processing of replicative intermediates (2, 15). The resolution of these intermediates requires the endonucleolytic cleavage of palindromic ends through a process that involves the virus-encoded nonstructural protein NS-1 (15) and eventually leads to the covalent linkage of this product to the 5′ end of both viral and complementary strands (18). Although the equivalent nonstructural proteins (Rep) of the defective members of the family *Parvoviridae* (the adeno-associated viruses) proved able to directly interact with the terminal palindrome (11), the mechanism by which NS-1 targets DNA is the object of current investigations. Interestingly, the PRE from promoter P4 was suggested to constitute a direct or indirect binding site for the transactivating NS-1 protein (23). Owing to their unique position in the vicinity of palindromic ends, PREs are candidates for *cis*-acting elements of this interaction that could be mediated or antagonized by NF-Y. It is worth mentioning in this respect that limited deletions removing one or both of the putative NF-Y binding sites of the right-hand palindrome severely inhibited the replication of MVM DNA (50). It is therefore tempting to speculate that the tight conservation of an atypical Y-box core sequence in parvoviral DNA may reflect its dual involvement in the regulation of both transcription and replication and may result from constraints imposed on the responsive element so that it can directly or indirectly interact with replication factors besides NF-Y. Further studies are required to test this working hypothesis by assessing, in particular, the influence of mutations within the Y-box region on the yield of parvoviral DNA replication.

ACKNOWLEDGMENTS

We are grateful to R. Roeder and P. Pognonec and to D. Mathis, C. Benoist, and R. Mantovani for providing antisera to USF and NF-Y, respectively. We thank L. Deleu for providing the pP4-luc construct. We also thank J. C. Jauniaux for help with the DNA sequence analyses.

This work was supported by grants from the European Union (Belgium and Germany) and the Association pour la Recherche sur le Cancer (France).

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