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Characterization of Two Novel YY1 Binding Sites in the Polyomavirus Late Promoter

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NF-D is a ubiquitous nuclear factor that has been shown to bind specifically to a DNA element in the polyomavirus regulatory region. In this report, we demonstrate that NF-D is either identical or very similar to a transcription factor that has been variously named YY1, δ, NF-E1, UCRBP, or CF1. Moreover, we show the presence in the polyomavirus genome of a second DNA motif, located 40 bp from the first, which binds YY1/NF-D with high affinity. Both sites lie downstream of the major late transcription initiation sites. By site-directed mutagenesis, we demonstrate that both elements contribute positively to the activity of the late promoter, probably by a cooperative mechanism. We also demonstrate that the requirement of the YY1/NF-D function for late promoter activity varies with the cell line.

The late transcription of polyomavirus (Py) is an example of temporally regulated transcription. Early after infection of permissive mouse cells, the predominant viral messages are transcribed from the early strand of the viral genome, which encodes the three T antigens: large T (LT), middle T, and small T. During this phase of infection, early messages predominate over late messages by a factor of 4 to 10 (17, 34). After the onset of replication, messages from the late strand of the virus, encoding the virion structural proteins VP1, VP2, and VP3, can exceed early messages by a factor of 20 to 80 (17, 34). The two opposite transcription units are separated by an intragenic region containing most of the elements responsible for their regulation. In particular, the enhancer region consists of four functionally redundant domains, A, B, C, and D (36). Each domain comprises several sequence motifs constituting binding sites for multiple transcription factors. The transcription factors (identified in 3T3 cells) specifically binding the Py regulatory region are indicated in Fig. 1 (1, 18, 21, 23, 24, 27, 30, 37).

The Py late promoter lacks a consensus TATA box or a CAAT box, and late mRNAs are initiated at multiple sites. The principal region, in which more than 90% of these sites are found, is located between nucleotides (nt) 5075 and 5155 (9, 35). The Py late promoter can be active in the absence of viral early proteins, and DNA replication does not appear to be required for efficient late promoter function (2, 4, 19). The Py late promoter contains multiple elements, and certain sequences reported to influence early promoter have been found to affect also the late promoter (2, 4, 19). Transcription studies performed in cultured cells demonstrate that there is no single element which, upon its removal, totally abolishes the late promoter activity, suggesting that the elements are functionally redundant. Three main regulatory regions, two positive and one negative, have been identified. The first positive element coincides with the enhancer A element (4, 19). There is strong evidence that the transcription factors PEA1 and PEA3, by binding to this element, can function as initiators of late transcription (41). The second positive element is contained entirely between nt 4900 and 5023, downstream of, and across, the late splice donor site. The negative element is composed of 89 bp located on the early side of the origin of replication (nt 56 to 144) (4).

In a previous study, we identified a DNA binding site that specifically interacted with a ubiquitous nuclear factor called NF-D (6). In the present report, we demonstrate that NF-D is very similar or identical to the transcription factor YY1 (32). Moreover, we show that an additional DNA motif, centered on nt 5003, is able to bind NF-D with high affinity. We have used site-directed mutagenesis to assess the relative contributions of the two NF-D binding elements in Py late transcription. Our results indicate that both elements contribute positively to the activity of the late promoter, probably by a cooperative mechanism. We also demonstrate that the functional importance of NF-D in the late promoter activity varies with the cell type.

MATERIALS AND METHODS

Plasmids. The construct pPB-CAT was obtained by cloning the Py A2 sequence (GenBank accession no. JO2288) spanning from the *Bam*HI site at nt 4634 to the *Pvu*II site at nt 5132 into the *Hin*dIII site of pSVO-CAT (11). The *Bam*HI and *Pvu*II sites were converted to *Hin*dIII sites by using 8-bp *Hin*dIII linkers, thus placing the VP2 and the VP3 initiation codons in phase with the chloramphenicol acetyltransferase (CAT) initiation codon.

The constructs pPBxD1-CAT, pPBxD2-CAT, and pPBxD1/xD2-CAT, which contain mutated NF-D binding sites, were obtained from plasmid pPB-CAT by site-directed mutagenesis of the NF-D1, the NF-D2, and both sequence motifs, respectively. The mutations were introduced by the PCR procedure described by Stappert et al. (33). The oligonucleotides TCAGAAGATCTCGGAGGGCCTC CAACACAG and GCGGCTCGCATGCTGAAAAT were used as internal primers; the underlined bases are mismatches with the template and produce the xNF-D1 and xNF-D2 mutations. Other primers used were TGCCATCCAGCC ATTCAGTAACGGTGGTATATCCAGTG (tagged flanking primer), TGCCA TCCAGCCATTCAGT (tag), and CACCGCATATGGTGCACTCTCAG (external primer). The final constructs were checked by nucleotide sequencing.

The Py LT expression construct p91023LT contains the Py LT coding region under the control of the adenovirus major late promoter (8). The vector p91023 has been described previously (40). Plasmid pCMVβ contains the β-galactosi-

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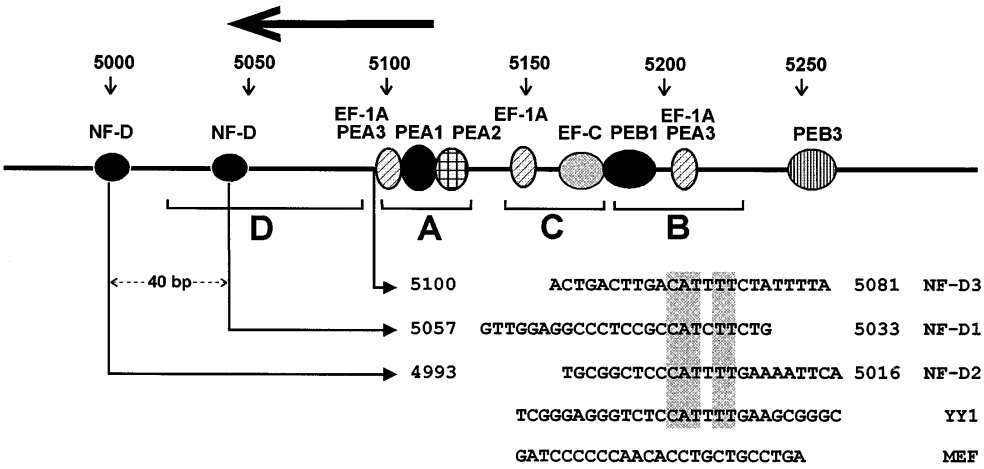


FIG. 1. Structure of the Py A2 regulatory region. The D, A, C, and B enhancer domains are indicated. The interactions of transcription factors from NIH 3T3 fibroblasts with each of the enhancer domains are represented. The NF-D binding site located between nt 4993 and 5016 was identified and characterized in this work. The large arrow indicates the direction of late transcription. The sequences of the DNA oligonucleotides used in mobility shift assays are shown at the bottom. The nucleotide coordinates of the NF-D1, NF-D2, and NF-D3 sequences and their locations within the Py regulatory region are indicated. The YY1 oligonucleotide carries the YY1 binding site from the adeno-associated virus P5 promoter; the MEF oligonucleotide sequence from the muscle creatine kinase enhancer contains an E-box DNA motif. The oligonucleotide sequences are aligned so as to demonstrate the homology between the YY1 and the NF-D binding sites.

dase gene under the control of the cytomegalovirus promoter (Clontec no. 6177-1).

Transfections and CAT assays. NIH 3T3 fibroblasts and HeLa cervix carcinoma cells were grown in Dulbecco's modified Eagle's medium containing 10% bovine serum. F9 teratocarcinoma cells were grown in Dulbecco's modified Eagle medium containing 10% fetal calf serum. Then 10^5 NIH 3T3 cells or 5×10^4 F9 and HeLa cells were transfected with 4 μg of the CAT plasmid and 0.5 μg of plasmid pCMVβ by the calcium phosphate method (39). Cells were incubated for 16 h with the calcium phosphate-precipitated DNAs and then fed with fresh growth medium. Forty hours later, cell extracts were prepared and equal volumes were assayed for CAT activity as described by Gorman et al. (11). Acetylated and nonacetylated chloramphenicol fractions were separated by thin-layer chromatography on silica gel-coated plates (Macherey-Nagel) developed in 95% chloroform–5% methanol. The dried chromatograms were exposed for 4 h to a phosphor storage screen and quantified with a Molecular Dynamics PhosphorImager. CAT activity was calculated as the percentage of chloramphenicol conversion to the acetylated forms. The CAT activity was normalized for β-galactosidase activity, calculated as described by Herbomel et al. (15). Each experiment was done in duplicate and repeated at least three times.

In transfections of F9 cells, plasmid pCMVβ was not used because of its low level of expression. In this case, equal amounts of protein from each cell extract, measured by the Bradford method (3), were used for the assays.

Electrophoretic mobility shift assays. The sequences of the oligonucleotides used in mobility shift experiments are shown in Fig. 1. Single-stranded oligonucleotides were end labeled with [γ-^{32}P]ATP (6,000 Ci/mmol) by T4 polynucleotide kinase to a specific activity of greater than 5×10^6 cpm/pmol and annealed with the unlabeled complementary strand as previously described (3). For 20 μl of DNA binding reaction mixture, 2 μg of poly(dI-dC) was incubated with 10 μg of F9 whole cell extract prepared as described by Pagano et al. (25) in 15 mM *N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES; pH 7.9)–40 mM KCl–1 mM EDTA–0.5 mM dithiothreitol–5% glycerol. Where indicated, 0.2 to 2 μl of antiserum was also added. After 10 min at 25°C, 10 fmol of labeled oligonucleotide probe was added, and the incubation was continued for additional 15 min. The DNA-protein complexes were resolved by electrophoresis on 5% nondenaturing polyacrylamide gels in 0.5× Tris-borate-EDTA buffer at 10 V/cm at room temperature.

The rabbit polyclonal antibody against YY1 (C-20) was purchased from Santa Cruz Biotechnology (Santa Cruz, Calif.).

RESULTS

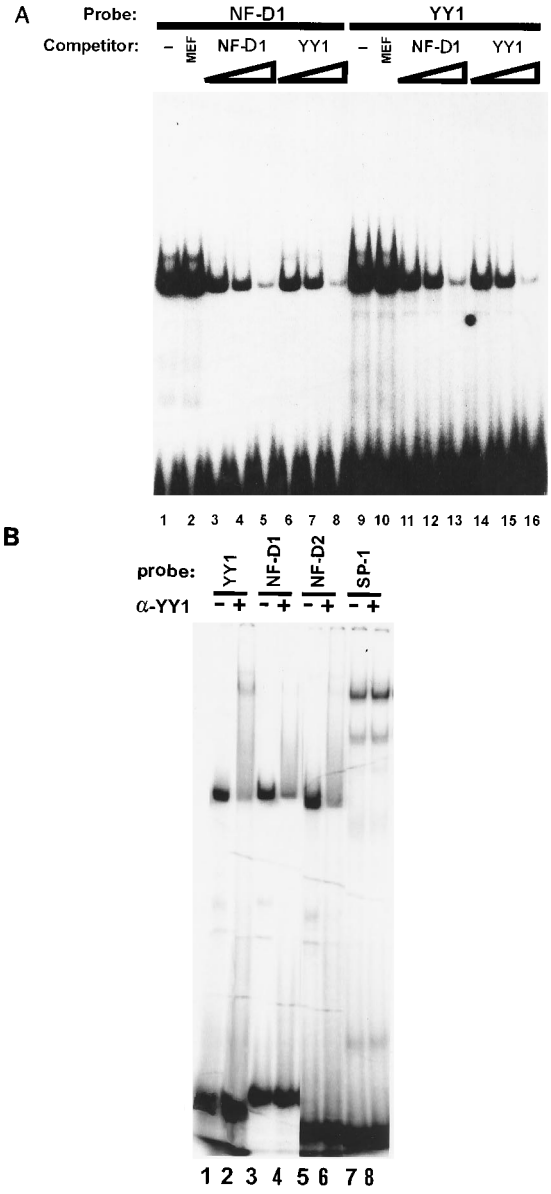
NF-D and YY1 are the same nuclear factor. The Py regulatory region comprises four functional domains containing the binding sites for multiple transcription factors, as depicted in Fig. 1. In this region, we have previously identified a DNA binding site that specifically interacts with a ubiquitous nuclear factor that we called NF-D. The minimal recognition sequence of NF-D is contained in the D domain of the Py enhancer, lying between nt 5038 and 5045 (6). In this report, we will refer to this DNA recognition sequence as NF-D1.

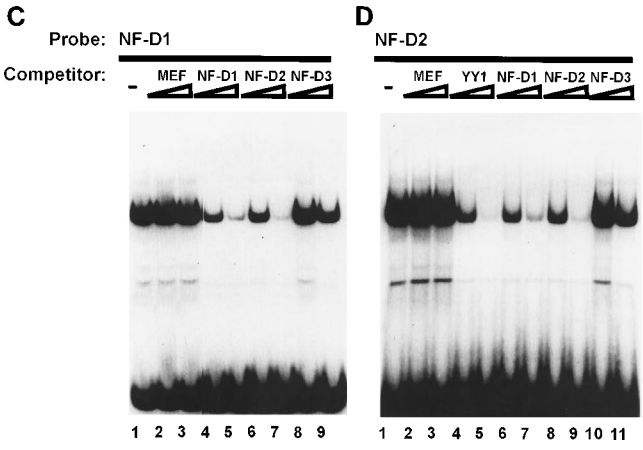
The NF-D1 sequence was analyzed by computer-assisted analysis and found to bear close sequence homology to the binding site for the transcription factor YY1 (32), as can be observed in Fig. 1. The nuclear factor YY1 is a ubiquitous transcription factor that has been variously named δ, NF-E1, UCRBP, or CF1 (10, 14, 26, 28).

The homology between the binding sites suggested that NF-D and YY1 might be either the same or highly related transcription factors. To verify this hypothesis, we used two approaches. First, we tested the ability of the NF-D and YY1 recognition sequences to cross-compete for binding to nuclear factors. Two double-stranded oligonucleotides, one containing the NF-D1 sequence from the Py late promoter and the other containing the YY1 binding site located at +1 in the promoter of the adeno-associated virus P5 (32), were chemically synthesized. The sequences of these oligonucleotides are shown in Fig. 1. Each oligonucleotide was labeled and used as a substrate in a DNA mobility shift assay performed with whole cell extracts prepared from murine F9 teratocarcinoma cells.

The results in Fig. 2A show that the bands produced by using either one of the two DNA substrates migrated identically (lanes 1 and 9). Moreover, the shifted band generated by the NF-D1 probe could be reduced by increasing amount of either unlabeled NF-D1 or YY1 oligonucleotide (lanes 3 to 8) but not by the unrelated MEF sequence from the muscle creatine kinase enhancer (lane 2) (38). Similarly, the YY1 oligonucleotide probe generated a DNA-protein complex that was competed for with the same efficiency by either unlabeled YY1 or NF-D1 oligonucleotide (lanes 11 to 16).

The fact that the NF-D1 and YY1 binding sites generated shifted bands of identical mobility and exhibited cross-competition strongly argues that the two DNA sequences bind the same factor. To confirm the identity of NF-D and YY1, we tested the ability of an antibody raised against the YY1 protein to interfere with the binding of NF-D to its DNA binding sequence. As shown in Fig. 2B, addition of the YY1 antibody to reaction mixtures containing either labeled YY1 or NF-D1 oligonucleotide and an F9 cell nuclear extract interfered with





the formation of the specific shifted bands (lanes 2 and 4). This inhibition was specific, as demonstrated by the inability of the YY1 antibody to interfere with the formation of protein-DNA complexes generated by the Sp1 recognition sequence (lanes 7 and 8). These results strongly suggest that the NF-D factor is YY1.

The YY1/NF-D factor can bind to other site(s) in the Py regulatory region. Analysis of the DNA sequence adjacent to the Py enhancer D domain revealed the presence of two additional DNA motifs highly homologous to the NF-D1 binding site. These sequences, centered on nt 5003 and on nt 5093, are referred to as NF-D2 and NF-D3, respectively (Fig. 1). To determine whether the NF-D2 and NF-D3 DNA motifs represented additional binding sites for the YY1/NF-D factor, we performed mobility shift assays with synthetic oligonucleotides containing these sequences (Fig. 1). Figure 2C shows that the formation of the shifted band generated by the NF-D1 oligonucleotide could be inhibited either by self-competition, in an assay in which an excess of unlabeled homologous oligonucleotide was included in the reaction mixture, or by competition with the NF-D2 oligonucleotide. In contrast, the unlabeled NF-D3 oligonucleotide competed very weakly for binding with the NF-D1 probe.

These results were confirmed in mobility shift assays performed with the NF-D2 oligonucleotide as a probe and the NF-D1, NF-D2, NF-D3, or YY1 oligonucleotide as a competitor (Fig. 2D). The results indicate that the pattern of shifted bands observed with the NF-D2 oligonucleotide was indistinguishable from that generated by the NF-D1 oligonucleotide. Moreover, the formation of the retarded bands was inhibited to the same extent by the NF-D1, NF-D2, and YY1 unlabeled oligonucleotides but was only slightly affected by the unlabeled NF-D3 oligonucleotide. Thus, both the NF-D1 and NF-D2 sites could bind YY1/NF-D specifically and with high efficiency. In accord with this conclusion, the results in Fig. 2B (lanes 5 and 6) indicated that the complex generated by the NF-D2 probe was disrupted by the YY1 antibody in exactly the same way as the complexes generated by the NF-D1 and YY1 probes.

The competition experiments represented in Fig. 2C suggest that the NF-D3 sequence may represent a low-affinity YY1/NF-D binding site. We tested the binding of cellular proteins to the NF-D3 oligonucleotide probe and observed a faint retarded band corresponding to NF-D binding only upon very long times of exposure (data not shown). In conclusion, our

FIG. 2. (A) Cross-competition for factor binding between the NF-D1 and YY1 oligonucleotides. F9 whole cell extract was added to ^{32}P-labeled DNA oligonucleotides, and following 10 min of incubation at room temperature, protein-DNA complexes were separated from free DNA by electrophoresis on a 5% nondenaturing polyacrylamide gel and visualized by autoradiography. DNA-protein complexes were competed for with a 50-, 100-, or 500-fold molar excess of the indicated unlabeled oligonucleotides. The unrelated MEF oligonucleotide competitor was added at a 500-fold molar excess. Complexes in the absence of any competitor sequence (−) are shown. (B) YY1-specific antibodies prevent binding of the NF-D factor. Labeled YY1, NF-D1, NF-D2, or SP-1 oligonucleotides were incubated with F9 whole cell extract (lanes 1, 3, 5, and 7). Protein-DNA complexes were resolved by electrophoresis on a 5% nondenaturing gel as described for panel A. A YY1-specific antiserum (α-YY1; added prior to the addition of cell extract) blocked the formation of the NF-D- and YY1-specific complexes (lanes 2, 4, and 6). The YY1 antiserum has no effect on the formation of complexes generated by the labeled SP-1 oligonucleotide (lane 8). (C and D) Identification of two novel NF-D binding sites in the Py late promoter region. Probes were incubated with F9 whole cell extract, and complexes were analyzed as described for panel A. The formation of DNA-protein complexes was challenged by the addition of increasing amounts (100- and 500-fold molar excess over the labeled oligonucleotides) of the indicated competitor oligonucleotides. Complexes in the absence of any competitor sequences (−) are shown.

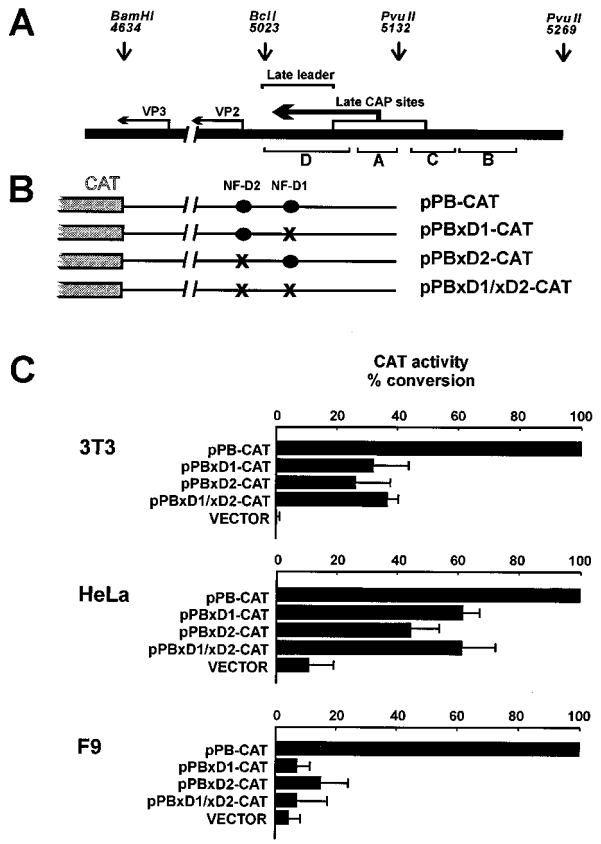


FIG. 3. (A) Organization of the Py genomic region between the *Pvu*II site at nt 5269 and the *Bam*HI site at nt 4634. The D, A, C, and B enhancer domains and the late leader sequence are outlined. The large arrow represents the 5′ heterogeneous late transcription start points. Thin arrows represent the ATG initiation codons of the VP2 and VP3 late proteins. (B) Schematic representation of plasmid constructs used in CAT assays. The Py sequence spanning from the *Bam*HI site at nt 4634 to the *Pvu*II site at nt 5132 was cloned upstream of the CAT reporter gene. In such a construct, transcription of the CAT gene was under the control of the Py late promoter. Mutagenized NF-D1 and NF-D2 binding sites are indicated with solid ovals and crosses, respectively. (C) Functional analysis of the YY1/NF-D binding sites in Py late transcription. 3T3, HeLa, and F9 cells were transfected with 4 μg of the indicated CAT plasmid and 0.5 μg of plasmid pCMVβ. Forty hours later, cell extracts were prepared and equal volumes were assayed for CAT activity, which was then normalized for β-galactosidase activity. In F9 cell transfections, plasmid pCMVβ was omitted because of its low level of expression. In this case, equal amounts of protein from each cellular extract were used. Each experiment was done in duplicate and repeated a minimum of three times; the standard deviations are indicated.

data demonstrate that the nuclear factor NF-D is either the same as or very similar to YY1 and that one low-affinity and two high-affinity YY1/NF-D binding sites are present in the Py late promoter region.

YY1/NF-D is an activator of Py late transcription. Since both the NF-D1 and NF-D2 sequences lie in a region of the Py regulatory region necessary for efficient late transcription (2), we tested their relevance in the late promoter function. To this end, we used a reporter construct, pPB-CAT, in which the CAT gene was placed under the control of the Py regulatory region sequence spanning from the *Bam*HI site at nt 4634 to the *Pvu*II site at nt 5132 (Fig. 3). The *Pvu*II-*Bam*HI fragment encompasses the A and D enhancer domains (36) and most of the 5′ heterogeneous initiation sites of late mRNAs (9, 35). It also contains late-transcribed sequences, including the VP3 intron and the VP3 and VP2 initiation codons (36). The construct was made in such a way that these two initiation codons, in the same frame, were also in frame with the initiation codon for the CAT protein.

In an almost identical construct, Bourachot et al. (2) determined that a major set of closely spaced start sites of the late mRNAs transcribed was centered on nt 5126 ± 2 and that the VP3 intron was spliced out in most of the transcripts.

To determine whether YY1/NF-D plays a role in the regulation of Py late transcription, we introduced nucleotide substitutions into the NF-D1 and NF-D2 DNA sequences carried by plasmid pPB-CAT. These mutations, which prevented YY1/NF-D binding as verified by mobility shift assays (data not shown), are unlikely to interfere with mRNA splicing, since the 5′ splice consensus sequence was not involved (16). The function of the NF-D3 sequence was not tested because of its very low affinity for YY1/NF-D. The plasmids mutagenized in the NF-D1, NF-D2, and NF-D1 plus NF-D2 sites were named pPBxD1-CAT, pPBxD2-CAT, and pPBxD1/xD2-CAT, respectively (Fig. 3).

Wild-type and mutant plasmids were transiently transfected into 3T3 fibroblasts, and extracts of transfected cells were assayed for CAT activity. Figure 3 shows that the mutations in either the NF-D1 or the NF-D2 site or both resulted in a threefold decrease of CAT activity, thus indicating that YY1/NF-D may function as an activator of the Py late promoter.

We also tested the wild-type and mutant CAT constructs in cell lines nonpermissive for Py replication, namely, human HeLa cells and F9 mouse teratocarcinoma cells. Figure 3 shows that the results obtained for HeLa cells were similar to those obtained for 3T3 cells. Mutations in either or both of the NF-D binding sites led to a twofold decrease in promoter strength. In F9 cells, both the single and double mutations similarly impaired the promoter activity; however, in this case CAT activity decreased almost to nil, indicating that in F9 cells, YY1/NF-D is strictly required for the late promoter activity.

DISCUSSION

In this paper, we report evidence indicating that NF-D and YY1 are either the same or highly related factors. By means of mobility shift assays, we have shown that NF-D and YY1 recognize the same DNA sequences and that an YY1-specific antibody can specifically block the binding of NF-D to its DNA recognition sequence.

We have also shown that the NF-D factor binds with high affinity to two sequences in the Py regulatory region. One of them, NF-D1, centered at nt 5040, lies in the D enhancer domain and has previously been described by us (6); the other is centered at nt 5003 and is described here for the first time. We have also identified a third sequence, NF-D3, centered at nt 5093; the binding of NF-D to this site, however, is barely detectable. The transcription factor YY1 has been implicated in the regulation of a wide variety of genes, although the location of the binding site and the regulatory role of the factor vary considerably among genes. In the upstream YY1/CF1 sites of the c-*myc* promoter (29), in the downstream YY1/δ sites of the ribosomal protein L32 (7), and in the initiator element of the promoter of the adeno-associated virus P5 (31), the factor has a positive effect on transcription; conversely, in other locations, such as upstream elements in the Moloney murine leukemia virus (10) and the 3′ enhancer of immunoglobulin κ genes (26), it has a repressive function. In the skeletal α-actin gene, its repressive effect is modulated by competitive binding of the serum response factor (12).

In the Py late promoter, both YY1/NF-D binding sites are located downstream of the late transcriptional starting points. The NF-D1 site lies in the region of the D enhancer corre-

sponding to the late leader exon; the NF-D2 site overlaps the initiation codon of the VP2 mRNA and lies in the intronic region of the VP1 and VP3 mRNAs.

The results of transfection studies with mutagenized NF-D binding sites indicate that both NF-D elements contribute to the activity of the Py late promoter. Moreover, the activities of promoters mutated in either of the two sites or both are similar, suggesting that the two YY1/NF-D binding sites may work cooperatively. It is worth noting that the two sites lie in opposite directions and are separated by exactly 40 nt; therefore, the bound NF-D molecules are on the same side of the DNA helix. Our data are in accord with those of Bourachot et al. (2), who described a *cis* element stimulating late transcription in Py, located between the *Bam*HI (nt 4634) and *Bcl*I (nt 5023) sites.

YY1 might activate transcription by two mechanisms (i) as an initiator element of the basal transcription machinery, binding in correspondence of the transcriptional start point, or (ii) as a classical transcription factor that modulates the basal transcription binding upstream or downstream of the +1 region.

The Py late promoter lacks a consensus TATA or a CAAT box, and late mRNAs are initiated at multiple sites. In a construct almost identical to our pPB-CAT, Bourachot et al. (2) determined that a major set of closely spaced start sites was centered on nt 5126 ± 2. Thus, considering that both YY1/NF-D binding sequences are located downstream of the major initiation sites, it seems more likely that YY1/NF-D activates the late promoter by stimulating the basal transcription and not by acting as an initiator.

In view of the multiple mechanisms by which Py LT activates Py late transcription (5, 20, 22) and of the ability of YY1 to mediate E1A activation of the adeno-associated virus P5 promoter (32), the possibility existed that YY1 not only activates the basal transcription but also mediates at least in part the late transcription activation by Py LT itself. This possibility, however, was essentially ruled out by the results of the following experiment. A Py LT expression plasmid was cotransfected with the pPB-CAT reporter construct (which contains the two YY1/NF-D binding sites but no LT binding site) in either 3T3 or HeLa cells. In both cases, no stimulation by LT on the reporter activity could be detected (data not shown).

The cell type dependence observed when the levels of transcriptional stimulation by YY1/NF-D were compared in different cell types deserves some comments. Although YY1/NF-D behaved as an activator in all cell lines tested, the importance of this factor appeared to be much greater in undifferentiated F9 cells than in other cells. In F9 cells, unlike in 3T3 and HeLa cells, promoters bearing mutations in either one of the two YY1/NF-D binding sites were in fact essentially unable to stimulate transcription. The simplest way to explain this difference is to consider that in undifferentiated F9 cells, some transcription factors are present at only minimal levels; a well-known example of this is PEA1 (which binds the Py enhancer and stimulates late transcription [21]). In contrast, in 3T3 and HeLa cells, the redundancy of transcription factors binding to the late promoter can probably offset the loss of NF-D binding. This situation bears some analogy to that observed for the YY1/δ element of the ribosomal protein L30 promoter (13): the importance of the YY1/δ element became manifest when the promoter activity was substantially decreased by mutations in two other elements located upstream of the start point (7).

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