JOURNAL OF VIROLOGY, Nov. 1994, p. 7159–7168
0022-538X/94/$04.00+0
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Vol. 68, No. 11

The Transcriptional Regulator YY1 Binds to the 5′-Terminal Region of B19 Parvovirus and Regulates P6 Promoter Activity

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Received 25 May 1994/Accepted 4 August 1994

We performed a systematic study to identify cellular factors that bound to the terminal repeat region of B19 parvovirus. Using electrophoretic mobility shift assays, we detected one cellular factor which prominently bound to the repeat region. The factor was purified from K562 nuclear extract by specific DNA affinity column chromatography and identified as YY1, a multifunctional transcription factor. Of multiple possible YY1 binding sites in the upstream region of the P6 promoter, three showed specific strong binding. Transcription enhancement by YY1 was demonstrated in vitro by transient transfection assays. In studies using truncated and mutated versions of this region, YY1 activity was diminished by the alteration of any two of these three sites and abolished by the alteration of all three sites. Our results suggest that YY1 is a positive regulator of B19 parvovirus transcription.

B19 parvovirus is the only member of the *Parvoviridae* family that is pathogenic in humans (45). B19, like other parvoviruses, is a small nonenveloped icosahedral virus with a single-stranded linear DNA genome (4). The B19 parvovirus genome is composed of 5,596 nucleotides (nt), which encode a nonstructural protein, two structural proteins (VP1 and VP2), and several small polypeptides of unknown function (9, 22, 28, 38). At both ends of the genome are identical inverted repeat sequences of 383 nt; the distal 365 nt of the repeat are imperfect palindromes that form hairpin structures (1, 10).

Although B19 parvovirus shares several general features with other parvoviruses, some important aspects of its cellular and molecular biology are unusual. B19 parvovirus has extraordinary tropism for human erythroid progenitor cells (26); only semipermissive or abortive propagation has been achieved in a few cell lines (40). The transcription map of B19 parvovirus is more complex than those reported for other parvoviruses and is striking in the presence of only one promoter (28). Transcription must be largely regulated by splicing and termination mechanisms. Structurally, the terminal repeats of B19 are the longest of the parvoviruses. A small region (nt −249 to −157) of the terminal repeat has been implicated in the regulation of the P6 promoter (30).

All parvovirus termini contain palindromic sequences. For adeno-associated virus (AAV) and densoviruses as well as B19 parvovirus, identical sequences are present at both the 3′ and 5′ termini. For other parvoviruses, previous studies have shown that the inverted terminal repeats play important roles in the viral life cycle: they serve as primers for the synthesis of the complementary strand of virus DNA and are essential for the replication, transcription, and packaging of virus DNA (4). When applied to vector construction, the termini of AAV are adequate for gene expression and DNA integration (35). The length of the B19 parvovirus terminal repeat regions and their GC-rich character have prevented a similar use of them for gene transduction as well as the construction of practical infectious virus clones.

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The evident importance of terminal repeats in the life cycles of parvoviruses and their likely roles in the regulation of B19 parvovirus gene expression led us to systematically investigate proteins which bound to DNA in this region. It was discovered that the most prominent of these factors is YY1, and YY1 appears to increase B19 parvovirus transcription in cells in a transient transfection assay system.

MATERIALS AND METHODS

Cell culture. Human K562 erythroleukemia cells were grown in suspension in microcarrier flasks (Wheaton, Millville, N.J.) in improved minimal essential medium (Biofluids, Rockville, Md.). HeLa or $UT7_{EPO}$ (40) cells were grown in monolayer in improved minimal essential medium zink option (Gibco BRL, Grand Island, N.Y.) or Iscove's modified Dulbecco's medium (Gibco BRL), respectively. Medium was supplemented with 10% fetal bovine serum, 50 U of penicillin per ml, and 50 μg of streptomycin per ml, and for $UT7_{EPO}$ cells, 1 U of erythropoietin per ml (Amgen, Thousand Oaks, Calif.).

Nuclear extract. K562, HeLa, or $UT7_{EPO}$ cells were harvested in the late logarithmic phase of growth. Nuclear extracts were prepared in the presence of aprotinin, leupeptin, pepstatin, and phenylmethylsulfonyl fluoride (11). Extracts were dialyzed against 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (pH 7.8) containing 100 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 20% glycerol and stored in liquid nitrogen.

Plasmid construction. For probes F1, F2, F3, and F4 (see Fig. 1B) used in electrophoretic mobility shift assays (EMSA), four plasmids carrying parts of the B19 terminal repeat sequences, pUC18/F1, pUC18/F2, pUC18/F3, and pUC18/F4, were constructed. B19 parvovirus DNA was extracted from the serum of a patient with transient aplastic crisis (6). Different segments of the terminal repeat region were amplified by PCR using a modified protocol. In brief, reactions were performed in a total volume of 100 μl containing 2 U of Vent DNA polymerase, its reaction buffer (New England BioLabs, Beverley, Mass.), four deoxynucleoside triphosphates at 200 μM each, the primers described below, and B19 parvovirus DNA used as a template. Reaction mixture was incubated at 96°C for

5 min, immediately chilled on ice, and then subjected to 35 cycles of PCR (95°C for 45 s, 55°C for 15 s, and 72°C for 45 s). On the basis of the published B19 terminal repeat sequence (10), we constructed two primers flanked by *Sal*I and *Pst*I sites (5′-TTGTCGACCCAAATCAGATGCCGCCGGTC-3′, equivalent to nt −166 to −186, and 5′-TTCTGCAGCCGCTT GATCTTAGTGGCACG-3′, corresponding to nt −348 to −327) (see Fig. 1). (We indicate the numbered positions of nt in relation to the transcription start site [+1] of the published B19 sequence [5].) These amplified fragments were digested with the restriction enzymes *Sal*I and *Pst*I and subcloned into the appropriate sites of a pUC18 vector. Six clones were sequenced with a Sequenase version 2.0 DNA sequence kit (United States Biochemical, Cleveland, Ohio). Our amplified sequences were identical to the published sequence, except that 3 to 5 bp were deleted at around nt −190 to −195 (10). One construct carrying the nucleotide duplex corresponding to nt −166 to −347 (with three bases deleted at nt −195 to −193) was referred to as pUC18/F1, in which nt −348 to −351 at the turnaround region of the published terminal repeat sequence are missing. Some mismatching base pairs were observed in the published B19 sequence, while there was no mismatching in the terminal repeat of pUC18/F1 (see Fig. 1A). F2, F3, and F4 were prepared from pUC18/F1 by PCR amplification with appropriate primers flanked by *Sal*I and *Pst*I sites and by subcloning into the corresponding sites of pUC18. The resulting plasmids were designated pUC18/F2, pUC18/F3, and pUC18/F4, respectively.

For luciferase assays, 12 luciferase reporter plasmids (see Fig. 8) and a YY1 expression plasmid were constructed. The wild-type luciferase reporter plasmid (pGL2/P6), which carried a fragment spanning the P6 promoter and its upstream region except for about one-half of the terminal repeat, was made by amplification of the nt −260 to +84 region from pYT103c (a nearly full-length B19 parvovirus clone; a gift of P. Tattersall). The 5′ end of the forward primer was flanked by a *Bgl*II site and an additional nine bases (CACAGGAAA) in order to create a putative YY1 sequence (lacking in pYT103c but predicted from the published sequence [10]), and the 5′ end of the reverse primer was flanked by a *Hin*dIII site. The amplified fragment was inserted into the corresponding sites of pGL2-Basic vector (Promega, Madison, Wis.) that carried the firefly luciferase gene downstream of multiple cloning sites but lacked eukaryotic promoter and enhancer sequences. This plasmid was also used to generate several mutant plasmids with truncated or base-substituted B19 parvovirus terminal repeat sequences. Substituted versions of pGL2/P6 were generated by PCR-mediated site-directed mutagenesis with pGL2/P6 as a template and adequate primers with or without mutated bases; in some cases, several steps using a PCR-based overlap-extension technique were required (17). Mutated bases were introduced into the terminal repeat sequences to produce seven substituted mutants, pGL2/−220M, pGL2/−110M, pGL2/−60M, pGL2/−220, pGL2/−110, pGL2/−60, and pGL2/P6M. To construct truncated versions of pGL2/P6, three fragments spanning nt −145 to +84, −86 to +84, and −50 to +84 were amplified with the pGL2/P6 plasmid as a template and appropriate primers with flanking *Bgl*II and *Hin*dIII sites. By insertion of the obtained fragments into the equivalent sites of pGL2-Basic vector, three 5′-end-truncated mutants were constructed, pGL2/−145D, pGL2/−86D, and pGL2/−50D. For construction of the YY1 expression plasmid, a fragment including the YY1 sequence was obtained from the pCMV-hYY1 plasmid (a gift of T. Shenk) with *Bam*HI and *Eco*RI sites and blunt ended with Klenow DNA polymerase, and the YY1 fragment was inserted into the blunt-ended *Not*I site of the pOPRSVICAT vector (Stratagene, La Jolla, Calif.) to replace the chloramphenicol acetyltransferase gene; this construct was called pOPRSVI/YY1. The relevant regions of all constructs were verified by sequencing. For control experiments, a luciferase reporter plasmid carrying the P5 promoter of AAV was constructed. By PCR, the AAV P5 promoter region, including two YY1 binding sites [P5(−60) and P5(+1) sequences], was amplified from the plasmid psub201(+) (a kind gift from R. Samulski) with appropriate primers, including template sequences, to produce a 5′ extension as a *Bgl*II site and a 3′ extension as a *Hin*dIII site. The obtained fragment, which corresponded to nt −88 to +64 (relative to the transcription start site [+1] of the AAV P5 promoter), was inserted into the respective restriction sites of pGL2-Basic vector. This construct was termed pGL2/AAV and used in luciferase assays to evaluate the effect of YY1 on transcriptional regulation.

Probe preparation. For EMSA, two series of probes spanning the B19 parvovirus terminal repeat region, longer probes generated from plasmid constructs and shorter probes created from synthetic double-stranded oligonucleotides, were prepared. The long probes F1, F2, F3, and F4 (see Fig. 1B) were prepared from pUC18/F1, pUC18/F2, pUC18/F3, and pUC18/F4 as described above. Plasmids were linearized with *Sal*I enzyme and labeled at the 3′ end with [α-^{32}P]CTP by using Klenow polymerase (a filling reaction) and standard techniques. The labeled F1, F2, F3 and F4 probes were released by digestion with *Pst*I enzyme, electrophoresed on a 1% agarose gel, and purified with a gel extraction kit (Qiagen, Chatsworth, Calif.). To obtain 12 short probes (see Fig. 6B), oligonucleotides with or without mutated bases were designed on the basis of the putative YY1 binding sites in the B19 parvovirus 5′-terminal region (see Table 2) and synthesized on an Applied Biosystems 308B DNA synthesizer (Foster, Calif.). Figure 6B shows the sense strands of these probes. The 5′ ends of the antisense strands (data not shown) contained GGG overhangs to facilitate the labeling of annealed probes with [α-^{32}P]CTP and Klenow DNA polymerase. Labeled probes were purified with a Select-D G25 spin column (5 Prime-3 Prime, Boulder, Colo.).

Protein purification and identification. Crude nuclear extract obtained from 500 liters of K562 cell culture was used to purify and identify protein(s) binding to the B19 parvovirus terminal repeat region. Extract was subjected to three kinds of column chromatography and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Binding activities were assessed by EMSA with ^{32}P-labeled P(−220) (see Fig. 6B). In brief, extract was applied to a heparin-Sepharose column equilibrated in buffer A (20 mM HEPES [pH 7.8], 0.2 mM EDTA, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 20% glycerol, 0.15 M KCl) and then was eluted in a linear KCl gradient from 0.15 to 1.0 M. Fractions with binding activities were pooled, the KCl concentration was adjusted to 0.15 M, and then pooled fractions were applied to a double-stranded salmon sperm DNA column, also equilibrated in buffer A. The flowthrough fraction from the column was incubated on ice for 15 min with 10 μg of poly (dI-dC) · poly(dI-dC) (Pharmacia LKB Biotechnology, Uppsala, Sweden) and then applied to a specific DNA affinity column in buffer A. This affinity column was constructed with concatemerized oligomers of sequence identical to the P(−220) probe coupled to CNBr-activated Sepharose 4B (Pharmacia LKB Biotechnology). Bound proteins were eluted with a linear KCl gradient from 0.15 to 0.5 M. After three cycles of specific DNA affinity column chromatography, active fractions were pooled. Samples were concentrated by precipi-

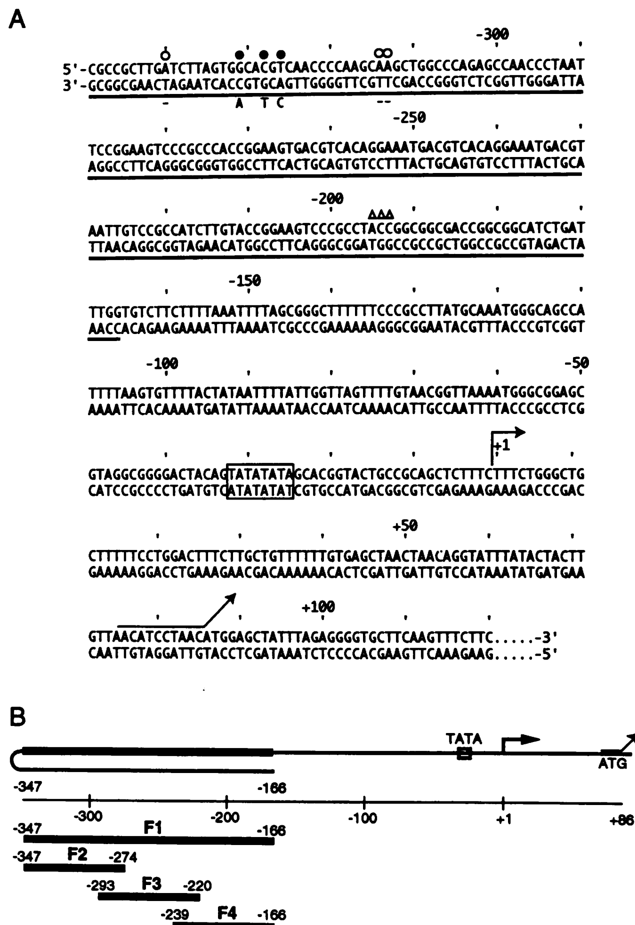


FIG. 1. Schema of the B19 parvovirus terminal hairpin region, showing the locations of the long probes for EMSA. (A) Nucleotide sequence of the B19 parvovirus terminal region used in this study. The terminal repeat region (underlined) was amplified by PCR from virus DNA extracted from a patient's serum and subcloned into the pUC18 vector. The pUC18/F1 construct was used to make probe F1, which was in turn used as a template to prepare probes F2, F3, and F4. The sequence of the terminal repeat region varied slightly from the published sequence. Base pairs shown by open triangles were absent in pUC18/F1. The original sequence contained some mismatched base pairs (open and closed circles), but there was no mismatching in pUC18/F1 (published bases appear beneath the underline, and dashes indicate bases not present in the original sequence). Any sequence other than that present in the terminal repeat region is that of pYT103c. Numbers above the sequence represent nt positions in relation to the transcription start site (+1) (5). (B) Locations of probes F1, F2, F3, and F4. Probes spanning the terminal repeat region were prepared from the corresponding plasmid constructs by 3′ end labeling. These probes are indicated by bold solid lines, with numbers showing the positions of nucleotides at both ends of the sequences. Above the probes is a diagram of the terminal repeat region, including the P6 promoter TATA box, transcription start site (↱), and translation initiation site (_↗) of the nonstructural protein gene.

tation with trichloroacetic acid and electrophoresed by SDS–7.5% PAGE for further purification and analyses of proteins. To reconstitute purified protein, the procedure of Hager and Burgess was used (14). Proteins on the SDS-polyacrylamide gel were electrotransferred onto a nitrocellulose membrane, part of which was silver stained with Protogold (BioCell Research Laboratories, Cardiff, United Kingdom). The membrane corresponding to the stained bands in an adjacent lane was excised. Bound proteins were eluted from the excised membrane in 50 mM Tris-HCl (pH 9.0) with 1% Triton X-100 for 1 h at room temperature and reconstituted by dialysis against the reaction buffer used for EMSA.

For analysis of protein sequences after SDS–7.5% PAGE, proteins were electrotransferred onto a polyvinylidene difluoride protein sequencing membrane (Bio-Rad Laboratories, Richmond, Calif.) and visualized by staining with Ponceau S (Sigma, St. Louis, Mo.). The band corresponding to the active protein in EMSA was excised within a 0.24-cm^2 membrane and subjected to microsequencing (W. Lane, Harvard University Microsequencing Facility). After digestion of the protein in situ with trypsin, peptides were separated by a reverse-phase high-performance liquid chromatography column and the sequence was analyzed by an ABI 477A protein sequencer with a 120-A on-line PTH-AA analyzer and subjected to a computer search of the GenBank database by TFASTA (31). The protein concentration was quantified by the Bradford method (7) with a commercial kit (Bio-Rad Laboratories).

Immunoblot analysis was performed by boiling the sample in Laemmli buffer, SDS–7.5% PAGE, electrotransfer to nitrocellulose, development of the membrane with an anti-YY1 monoclonal antibody (a kind gift of T. Shenk), and detection by a commercial alkaline phosphatase system (Promega). Supershift EMSA was performed with partially purified K562 cell extract and ^{32}P-labeled P(−220) probe in the presence and absence of monoclonal antibody to YY1.

EMSA. EMSA was performed with nuclear extract obtained from K562, $UT7_{EPO}$, and HeLa cells and various probes derived from plasmid constructs and synthetic oligonucleotides as described above. Reaction mixtures consisted of a binding buffer (20 mM HEPES [pH 7.8], 5 mM $MgCl_2$, 30 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol), 2 μg of poly(dI-dC) · poly(dI-dC) (or 0.1 μg for purified protein) (Pharmacia LKB), 10 μg of bovine serum albumin, ^{32}P-labeled probes (about 50,000 cpm), and various amounts of protein samples in a total volume of 20 μl. For competition binding assays, the same conditions were used but an excess amount of unlabeled competitor DNA was added before the labeled DNA probes. Mixtures were incubated for 15 min on ice and then 15 min at room temperature and electrophoresed on a 4% polyacrylamide gel in 0.5× Tris-borate-EDTA buffer (45 mM Tris-borate, 1 mM EDTA) at room temperature. The gel was dried and autoradiographed.

Methylation interference assay. The F1 fragment (Fig. 1) was labeled with ^{32}P at the 3′ end of either the sense or antisense strand with Klenow polymerase. The labeled fragment was partially methylated with dimethyl sulfate (24). Methylated ^{32}P-labeled fragments were used as probes. After the incubation of probes with K562 nuclear extracts, EMSA were performed as described above, except at 10-fold-greater volumes. Free and protein-bound DNAs were located by wet-gel autoradiography, and bands were excised from the gel. Gel strips were embedded on a 1% agarose gel. A piece of NA45 membrane (Schleicher and Schuell, Keene, N.H.), slightly wider than the acrylamide strip, was inserted into a slit that had been made with a razor blade 5 mm below the gel strip on the agarose gel in order to perform electrotransfer. To cleave DNA at methylated guanine residues, electrotransferred membranes were treated with 100 μl of 1 M piperidine at 90°C for 30 min. The unused methylated probes were also cleaved with piperidine to provide a guanine ladder. DNA samples were dried and dissolved in deionized formamide and then analyzed on a 12% sequencing gel (42).

In situ copper-phenanthroline protection assay. F1 frag-

ments (Fig. 1) were labeled with ^{32}P at the 3′ ends of antisense strands with Klenow polymerase and used as probes. Following incubation of probes with K562 nuclear extracts in 10-fold-higher volumes, samples were analyzed by EMSA. The entire polyacrylamide gel was subjected to in situ copper-phenanthroline protection (18). The gel was autoradiographed at 4°C. The bands of interest, representing DNA-protein binding complexes and free probe, were excised from the gel and analyzed on a 12% sequencing gel.

Transfection and luciferase assay. HeLa cells were plated on 60-mm-diameter dishes (3×10^5 cells per 4 ml) and cotransfected by calcium phosphate precipitation according to the Chen-Okayama procedure (8) with the following amounts of plasmids: 1 μg of each luciferase reporter plasmid with wild-type or mutated B19 parvovirus terminal sequence (see Fig. 8), 10 μg of YY1 expression plasmid (pOPRSVI/YY1) or nonexpression plasmid (pOPRSVICAT), and 1 μg of β-galactosidase expression plasmid (pCH110; Pharmacia LKB Biotechnology) used as an internal standard of transfection efficiency. Forty-eight hours after transfection, cells were harvested and cell extracts were prepared by the luciferase assay system (Promega) according to the manufacturer's instructions. Luciferase assays were performed with samples diluted to produce signals within the linear range of light detection. Twenty microliters of diluted lysate was added to 100 μl of luciferase assay reagent (Promega) containing luciferase assay substrate (Promega) and immediately mixed. Activity was measured with a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, Calif.) β-Galactosidase activity was measured by using the β-galactosidase enzyme assay system (Promega). Two lots of each luciferase plasmid were used for transfection experiments and produced indistinguishable results.

RESULTS

Binding of proteins to DNA within the B19 parvovirus terminal repeat region. To investigate the cellular proteins that bind to the terminal repeat region of B19 parvovirus, we attempted to construct a full-length version of this region by cloning from virus or by gene amplification. These attempts were unsuccessful, probably because of the high GC content and length of the B19 parvovirus terminal repeat region. We were able to produce a nearly complete terminal repeat, called F1, by PCR from virus DNA by modifying the conditions (see Materials and Methods). The F1 probe lacked four bases (GCGC) located at the hairpin turn, and some mismatched base pairs present in the original sequence were missing from the amplified sequence (Fig. 1A shows the sequence of the subcloned terminal repeat and compares it with the published sequence of pYT103c). Amplified fragments were inserted into the pUC18 vector to produce the plasmid pUC18/F1, which was in turn used either to prepare the F1 probe or as a template for gene amplification and construction of plasmids pUC18/F2, pUC18/F3, and pUC18/F4 (Fig. 1B).

The F1 probe was used in EMSA to screen crude nuclear extract from $UT7_{EPO}$, K562, and HeLa cells for proteins binding to the B19 termini. A similar major retarded band was observed with extracts from all three cell types; multiple minor retarded bands were also seen (Fig. 2A). To better determine the DNA-binding sites, three shorter probes, F2, F3, and F4, that spanned the longer F1 region were used in EMSA (Fig. 1B). The F4 probe gave rise to a strong retarded band on incubation with K562 nuclear extract (Fig. 2B). Competition with cold F4 probe showed that the binding of F4 to cell extract was specific, and cold F4 also completely competed with the

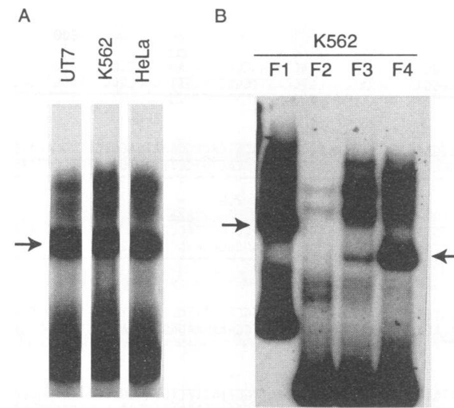


FIG. 2. EMSA with F1, F2, F3, and F4 probes. Each probe was incubated with nuclear extract from $UT7_{EPO}$, K562, or HeLa cells in 20 μl of reaction mixture for 15 min on ice and then 15 min at room temperature before being subjected to 4% PAGE. (A) EMSA with nuclear extracts from different cell lines and the F1 probe. (B) EMSA with K562 nuclear extract and probes F1, F2, F3, and F4. Arrows indicate the positions of the major DNA-protein complexes.

labeled F1 probe (data not shown). A faint retarded band, whose migration was retarded similarly to that with F4, was also produced with the F3 probe; no similar retarded band was observed with the F2 probe. These results suggested that specific binding sites for nuclear proteins were located in discrete regions of F4 and F3 or that a single site was situated in the overlapping sequence between F4 and F3.

To determine the precise protein-binding site within the hairpin region, we performed methylation interference assays. For these experiments, either the sense or antisense strand of the F4 probe was radioactively labeled at its 3′ end, partially methylated, and then incubated with K562 nuclear extract. After the subjection of DNA-protein complexes to EMSA, DNA probe bound to protein as well as free DNA probe was eluted from gels and exposed to piperidine, which cleaves DNA at methylated guanine sites. Comparison of the chemical cleavage patterns allows more precise determination of the contact point of the DNA probe with protein. The methylation of guanine residues at nt −221 on the sense strand and at nt −216, −219, and −220 on the antisense strand strongly interfered with the binding of protein (Fig. 3). Partial interference was observed at nt −222 and −223 on the antisense strand.

To confirm these DNA-protein contact sites, in situ copper-phenanthroline protection assays were performed. For this technique, the antisense strand of F4 probe was labeled at the 3′ end and reacted with K562 nuclear extract for EMSA. The gel was treated with copper-phenanthroline, which cleaves all nucleotides except those protected by complexing with protein. The region protected was then analyzed by footprinting and shown to be identical to the specific site implicated by the methylation interference assay (Fig. 3A). From both analyses, the sequence containing guanine residues at nt −221 on the sense strand and at nt −216, −219, and −220 on the antisense strand appeared to be essential for protein binding (Fig. 3B). A mutant probe of 40 bp, which spanned the predicted protein-binding site, was prepared from synthetic oligonucleotides; mutated base pairs were introduced from nt −224 to −215 to exchange purine for pyrimidine residues and vice versa. When EMSA were performed with mutant probes and K562 nuclear extract, no retarded complexes were observed (data not

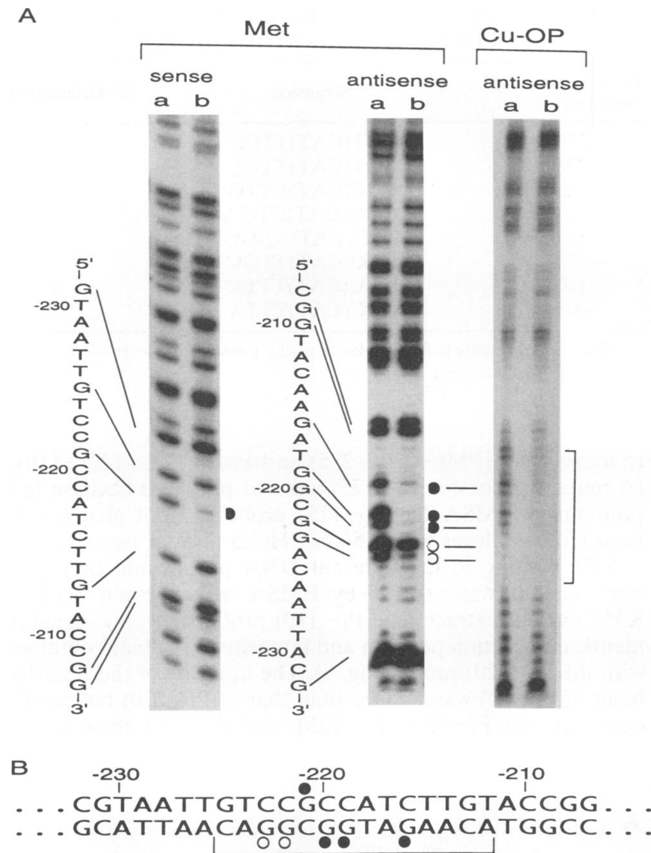


FIG. 3. Methylation (Met) interference and copper-phenanthroline (Cu-OP) protection assays. (A) For methylation interference assay, the F1 probe labeled at the 3′ end of either the sense or antisense strand was lightly methylated and incubated with K562 nuclear extract. Following EMSA, DNA was isolated from the retarded complex and analyzed on a 12% sequencing gel. For the in situ copper-phenanthroline protection assay, F1 probe labeled at the 3′ end of the antisense strand was used for EMSA with K562 nuclear extract. The whole gel was subjected to copper-phenanthroline cleavage. After DNA-protein complexes were excised and eluted from the gel, DNA samples were analyzed on a 12% sequencing gel. The protected region on the antisense strand is indicated by the bracket. (B) Shown in the summary of the footprinting data from these two assays are the locations of the deduced protected regions on the sense and antisense strands. Lanes a and b, the piperidine cleavage patterns for free and bound probes, respectively. Partial sequences of the probes are shown adjacent to the lanes, and the guanine contact points are indicated by solid adjacent lines. Numbers to the left of the sequences indicate nt positions in relation to the transcription start site (+1) of the B19 virus gene. Closed and open circles represent strong and weak sites of interference, respectively.

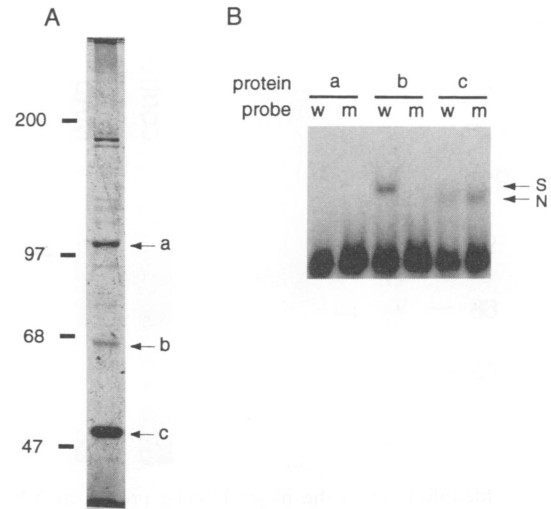


FIG. 4. Purification of proteins binding to the terminal repeat region. K562 nuclear extract was used to purify protein binding to the hairpin sequence. Nuclear extracts were applied to a heparin-Sepharose column, and active fractions were subjected to a double-stranded salmon sperm DNA column, three rounds of a specific DNA affinity column, and SDS-PAGE. Binding activities were assayed by EMSA with P(−220) probe. Proteins on the gel were electrotransferred onto nitrocellulose for further analysis. (A) Silver staining of proteins on the nitrocellulose membrane showed 15 bands; a, b, and c, three major bands. The molecular masses of protein standards (in kilodaltons) are on the left. (B) EMSA with purified and reconstituted proteins. The 15 proteins isolated from the membrane were reconstituted and subjected to EMSA with wild-type P(−220) (w) and mutant PM(−220) (m) probes. The results for three proteins, a, b, and c, are shown. Arrows indicate specific (S) and nonspecific (N) DNA-protein complexes.

shown). This experiment localized the protein-binding site to between nt −224 and −215 of the hairpin region.

Identification of the binding protein for the B19 parvovirus terminal repeat region. We used nuclear extract from 500 liters of K562 cell culture to purify and identify the binding protein; activity was assessed by EMSA with a 40-bp DNA probe, P(−220), containing the binding site (Fig. 4). When nuclear extract was applied on a heparin-Sepharose column, binding activity was detected in fractions that eluted between 0.35 and 0.7 M KCl. Active fractions were then applied to a double-stranded salmon sperm DNA column and eluted in flowthrough fractions. As a third step, a specific DNA affinity column was constructed with concatemerized oligomers of a sequence identical to P(−220). Protein that bound specifically to P(−220) eluted between 0.3 and 0.5 M KCl, but this fraction was contaminated with nonspecifically binding proteins, even after three cycles of DNA affinity chromatography. PAGE under denaturing conditions was required to sufficiently purify the protein for amino acid sequencing.

Following SDS-PAGE, proteins were transferred onto a nitrocellulose membrane. By silver staining the membrane, 15 bands were visible (Fig. 4A). All were isolated by excision and elution from the membrane. By EMSA with wild-type and mutant P(−220) probes, only one protein produced a specifically retarded complex (Fig. 4B).

To identify this probe-specific binding protein, an internal peptide derived from the protein was analyzed for amino acid sequence. This analysis showed the sequence LEGEFSVTM WSSDEK. By computer search of the GenBank database by the TFASTA program, this peptide was identical to amino acid residues 215 to 229 (numbered from the amino-terminal methionine) of the ubiquitous mammalian transcription factor YY1. The identity of the protein was confirmed by immunoblot analysis and an antibody supershift experiment, both with an anti-YY1 monoclonal antibody (Fig. 5).

YY1 binding upstream of the B19 parvovirus P6 promoter. YY1 recognition sequences contain conserved CAT bases (Table 1). We searched for CAT sequences in the B19 parvovirus promoter region. CAT sequences were found at eight sites on the sense and antisense strands (Table 2). Three CAT sequences, in addition to the original site within probe

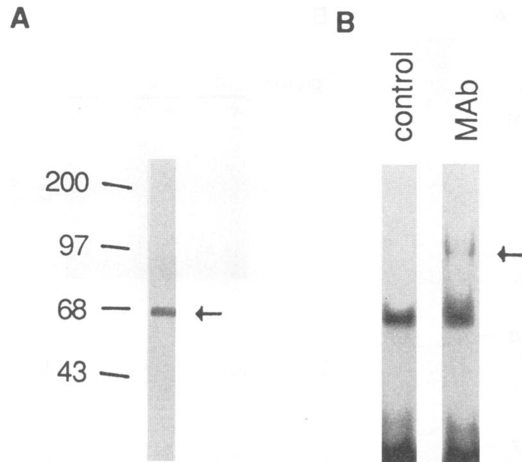


FIG. 5. Identification of the major binding protein as YY1. (A) Immunoblot analysis. Partially purified K562 cell nuclear extract was electrophoresed, electrotransferred, and exposed to a monoclonal antibody to YY1, which was detected with anti-mouse immunoglobulin G. Arrow, expected size (in kilodaltons) of YY1 by SDS–7.5% PAGE. (B) Supershift antibody assay. Partially purified K562 nuclear extract and ^{32}P-labeled P(−220) probe were incubated in the absence and presence of anti-YY1 monoclonal antibody (MAb) before undergoing EMSA. Arrow, supershifted band of complexed DNA.

P(−220), were present within the terminal repeat, and another four CAT sequences were located outside of the terminal repeat region.

To examine whether these CAT sequences represented authentic YY1 binding sites, eight probes composed of the CAT bases and their adjacent sequences were prepared from synthetic double-stranded oligonucleotides; additionally, a mutated version of each of the eight probes was constructed to evaluate binding specificity. The sequences, locations, and orientations of these eight probes are shown in Fig. 6. P(−220) probe was used as a control. Some CAT sequences were in such close proximity that single-mutant probes were employed to assay them [PM(−250/−235) and PM(−125/−120/−110)]. [A mutant probe for P(−175) was not prepared because in a preliminary EMSA the P(−175) probe did not give rise to retarded complexes with K562 or HeLa nuclear extracts.]

YY1 binding to the different DNA probes and their mutant versions was assayed by EMSA with partially purified K562 nuclear extract. The P(−110) probe showed a signal of identical migration position and intensity to the band obtained with the P(−220) probe (Fig. 7). The intensity of the retarded band of P(−60) was weaker than that of P(−220) but significant, whereas P(−250), P(−125), and P(−120) showed only

TABLE 1. Documented binding sequences for YY1

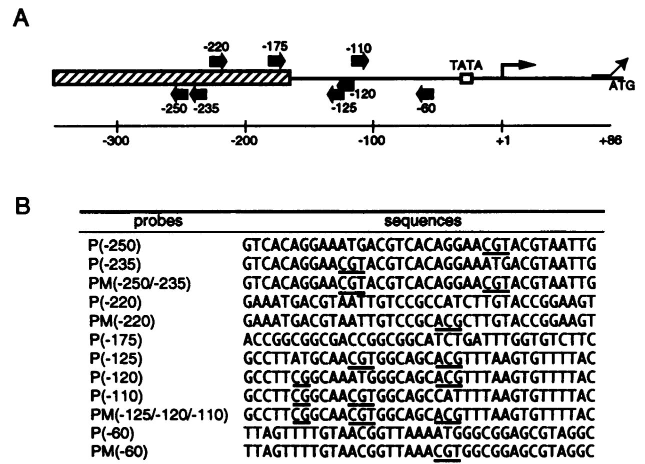
Site[a]	Sequence	Reference
AAV P5(+1)	CTC(CAT)TTTG	39
AAV P5(−60)	CGA(CAT)TTTG	39
MuLV-LTR UCR	CGC(CAT)TTTG	12
HSV-1	GGC(CAT)CTTG	25
ε-Globin	TAT(CAT)TTTG	32
Ig κE3′	CTC(CAT)CTTG	29
Ig μE1	GGC(CAT)CTTG	29
c-*myc* (−260)	GAC(CAT)TTTC	33
c-*myc* (−390)	CGC(CAT)GTAC	33
c-*fos* SRE	GTC(CAT)ATTA	13
α-Actin MRE	CGC(CAT)ATTT	20
MCK-CArG	GCC(CAT)ACAA	44
COX Vb	GCC(CAT)CTTG	2
rpL30	GGC(CAT)CTTG	15
rpL32-1	TGC(CAT)CTGT	15
rpL32-2	GGC(CAT)CCGC	15

[a] MuLV-LTR UCR, Moloney murine leukemia virus long terminal repeat upstream conserved region; HSV-1, herpes simplex virus type 1; Ig κE3′, immunoglobulin κ 3′ enhancer; Ig μE1, immunoglobulin heavy-chain μ E1; SRE, serum response element; MRE, muscle regulatory element; MCK-CArG, M isozyme of creatine kinase; COX Vb, cytochrome *c* oxidase subunit Vb promoter; rp, mouse ribosomal protein promoter.

TABLE 2. Nucleotide sequences containing CAT bases in the B19 parvovirus terminal region

B19 parvovirus terminal site[a]	Sequence	Orientation[b]
−250	CGT(CAT)TTCC	−
−235	CGT(CAT)TTCC	−
−220	CGC(CAT)CTTG	+
−175	CGG(CAT)CTGA	+
−125	TTG(CAT)AAGG	−
−120	GCC(CAT)TTGC	−
−110	AGC(CAT)TTTA	+
−60	GCC(CAT)TTTA	−

[a] Number represents position relative to the transcription start site (+1).
[b] +, sense; −, antisense.



probes	sequences
P(-250)	GTCACAGGAAATGACGTCACAGGAACGTACGTAATTG
P(-235)	GTCACAGGAACGTACGTCACAGGAAATGACGTAATTG
PM(-250/-235)	GTCACAGGAACGTACGTCACAGGAACGTACGTAATTG
P(-220)	GAAATGACGTAATTGTCCGCCATCTTGTACCGGAAGT
PM(-220)	GAAATGACGTAATTGTCCGCACGCTTGTACCGGAAGT
P(-175)	ACCGGCGGCGACCGGCGGCATCTGATTTGGTGTCTTC
P(-125)	GCCTTATGCAACGTGGCAGCACGTTTAAGTGTTTTAC
P(-120)	GCCTTCGGCAAATGGGCAGCACGTTTAAGTGTTTTAC
P(-110)	GCCTTCGGCAACGTGGCAGCCATTTTAAGTGTTTTAC
PM(-125/-120/-110)	GCCTTCGGCAACGTGGCAGCACGTTTAAGTGTTTTAC
P(-60)	TTAGTTTTGTAACGGTTAAAATGGGCGGAGCGTAGGC
PM(-60)	TTAGTTTTGTAACGGTTAAACGTGGCGGAGCGTAGGC

FIG. 6. Synthetic oligonucleotide probes. Oligonucleotides spanning a portion of the terminal repeat region containing CAT sequences were designed on the basis of the data shown in Table 2 and synthesized. The 5′ end of the antisense strands (not shown) contained GGG overhangs to facilitate the labeling of annealed probes with Klenow DNA polymerase. After the annealing of complementary strands, duplexes were 3′ end labeled with ^{32}P. (A) Relative locations of oligonucleotide probes and the orientation of the CAT sequence in each probe are schematically indicated by bold arrows. Numbers above and below arrows show the approximate positions of the probes with respect to the transcription start site (+1). ▨, terminal hairpin region; □, P6 promoter, including a TATA box; ↱, transcription start site; _↗, translation initiation site of B19 parvovirus nonstructural protein. (B) The names of the probes consist of uppercase letters, P (wild type) and PM (mutant), and parenthetical numbers of their approximate positions in the terminal repeat region. Sequences of the sense strands are shown, and mutated bases are underlined. In probes P(−250), P(−235), P(−125), P(−120), and P(−60), the antisense strands contain YY1 CAT motifs.

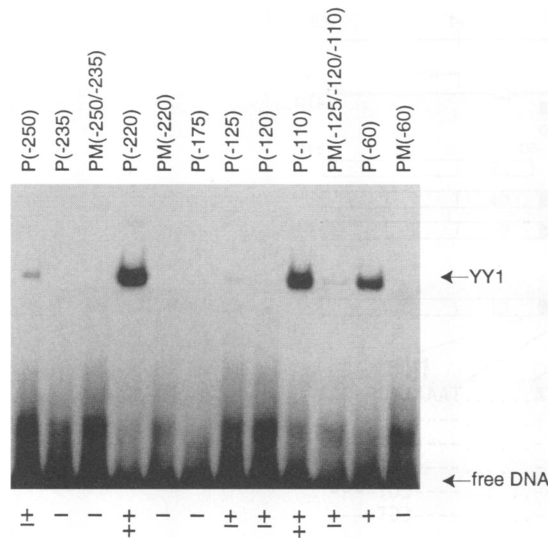


FIG. 7. EMSA with synthetic oligonucleotide probes. Wild-type and mutant probes (Fig. 6) were examined for binding activity to purified YY1 by EMSA. Purification of YY1 was performed by using K562 nuclear extract and sequential column chromatography steps followed by SDS-PAGE. The intensities of the binding activities are indicated as follows: ++, strongly positive; +, positive; ±, weakly positive; −, not detected.

trace amounts of specific DNA-protein complexes. P(−235), P(−175), and the mutant probes did not show specifically retarded complexes by EMSA (Fig. 7). The binding affinities of the probes are also summarized in this figure.

Functional effects of YY1 on B19 parvovirus promoter in transfection assays. To assess the functional role of YY1 binding sites in transcriptional regulation of B19 parvovirus, a series of luciferase reporter plasmid constructs containing point mutated or truncated versions of the B19 parvovirus terminal region were generated by PCR (Fig. 8A). In control experiments, a luciferase reporter plasmid that carried the AAV P5 promoter region, which includes two YY1 binding sites [P5(−60) and P5(+1) sequences], was constructed (Fig. 8B). Each plasmid was transfected into HeLa cells together with a YY1 expression plasmid (pOPRSVI/YY1) or a nonexpression plasmid (pOPRSVICAT) and a β-galactosidase expression plasmid (pCH110) and then luciferase activity in cell extracts was measured (Fig. 9). In the wild-type terminal region (pGL2/P6), YY1 activated transcription 1.7-fold; in contrast, YY1 downregulated transcription from the AAV promoter (pGL2/AAV) to 15% in the absence of E1A protein, as previously reported (39). In pGL2/−145D, pGL2/−86D, and pGL2/−50D, all of which carried 5′-truncated regulatory regions, the B19 P6 promoter activities decreased to 25, 11, and 1%, respectively, without YY1 overexpression. The mutation of any single YY1 binding site (pGL2/−220M, pGL2/−110M, or pGL2/−60M) did not affect basal or YY1-stimulated transcriptional activities. The mutation of two sites (pGL2/−220, pGL2/−110, or pGL2/−60) reduced basal levels of transcription to about 40%. However, a mutation within all three sites (pGL2/P6M) showed the most dramatic reduction of basal level of transcription and no stimulation by YY1 overexpression.

DISCUSSION

Parvoviruses are dependent on cellular factors for their replication because of the limited size of their genome. Our study began as an investigation of possible regulatory proteins of cellular origin within the terminal repeat region of B19 parvovirus. This region has important roles in the viral life cycles of parvoviruses, including DNA replication, transcription, packaging, and integration. By EMSA, we identified YY1 as the major hairpin binding protein for B19. Not only were YY1 binding sites within the hairpin identified but YY1 binding sites were also found upstream of the single strong P6 promoter of B19 parvovirus in an area previously shown to be important in transcription regulation (21). The presence of multiple YY1 binding sites in these key areas suggested that YY1 might play an important role in transcription from the P6 promoter. For B19 parvovirus, this is the first cellular protein shown to bind to the virus genome and to affect virus transcription. However, other cellular proteins might be identified by methods other than EMSA or by altering the specific conditions of this assay. Unfortunately, functional data could only be obtained with transfected HeLa cells, because a cell line that satisfactorily mimics the behavior of the virus in erythroid progenitor cells has not been developed and a practical infectious clone of the virus has not been constructed.

YY1 is a DNA-binding protein containing a zinc finger domain, related to the Krüppel family of transcriptional regulators of *Drosophila melanogaster*. YY1 is multifunctional. Depending upon the experimental system and cellular context, YY1 has been shown to be a transcriptional activator or repressor and to function as a transcriptional initiator for a variety of genes, including a number of oncogenes and virus genes. The multiple functions of YY1 in various cellular and virus genes have been demonstrated. YY1 activates the promoter of the c-*myc* gene (33) (and it has complex interactions with the c-*myc* gene product, as described below) as well as the genes for ribosomal proteins L30 and L32 (16), cytochrome *c* oxidase (2), and the leaky-late binding site of herpes simplex virus type 1 (25). YY1 represses skeletal α-actin (20), c-*fos* (13), immunoglobulin (29), human immunodeficiency virus type 1 (23), the major late promoter of adenovirus type 12 (46), the upstream regulatory region of human papillomavirus type 18 (3), and the upstream conserved region of Moloney murine leukemia virus (12). For the parvovirus AAV, YY1 functions as a repressor of gene transcription from the P5 promoter, but repression is relieved by the adenovirus gene product E1A (39). YY1 also serves as an initiator of transcription from the P5 promoter, and for this function it does not require a TATA-binding site or the TATA-binding protein (37, 43). In contrast, in the experiments reported here, YY1 functioned as a *trans* activator of the B19 parvovirus P6 promoter, even in the absence of adenovirus E1A protein. The difference between YY1 function for the B19 parvovirus P6 promoter and that for the AAV P5 promoter might be due to the context of *cis* elements, including the presence of a TATA box, as the cellular factors were the same in our experiments. The difference in YY1 function might be related to the autonomous behavior of B19 parvovirus compared with AAV. Although YY1's positive effect on transcription was weak (1.3- to 1.9-fold above basal transcription), it appeared to be specific because mutant reporters lacking the YY1 binding site were not activated in the presence of YY1. The relatively weak apparent YY1 activity might be due to the masking effect of endogenous YY1 or YY1-associated factors in HeLa cells.

YY1 binding sites are widely distributed in many cellular and virus promoters since YY1 has relatively degenerate DNA

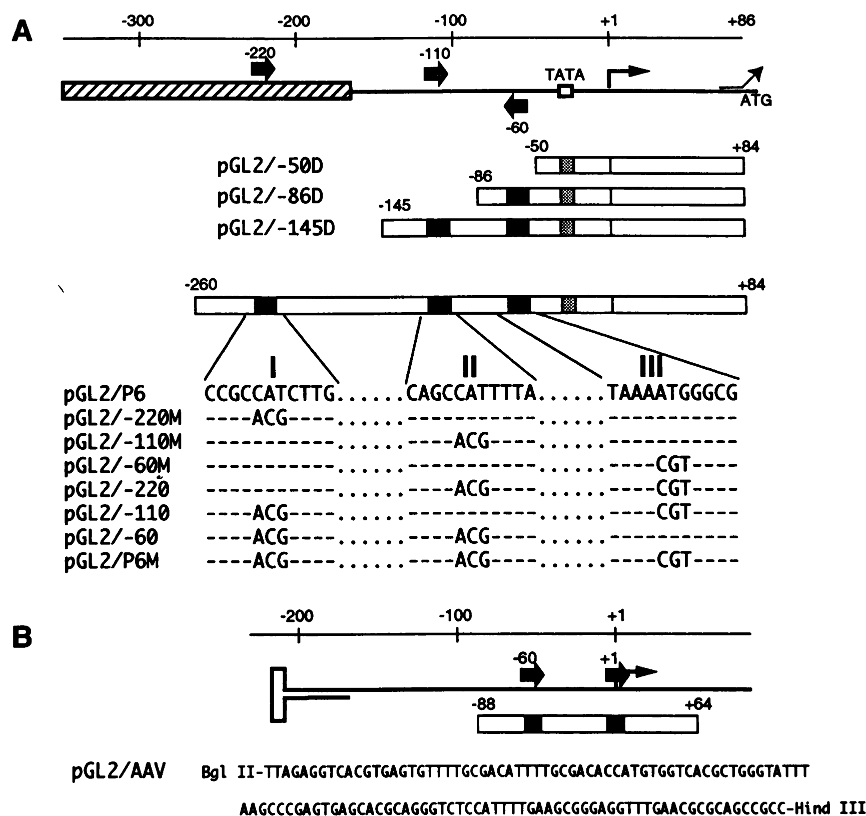


FIG. 8. Schema of luciferase reporter plasmids carrying truncated or base-substituted versions of the B19 parvovirus terminal repeat region. (A) The locations and orientations of YY1 binding sites are shown by hatched arrows. DNA fragment nt −260 to +84 spanned the terminal region (except for the 5′ half which contains no YY1 CAT motifs). The wild-type luciferase reporter plasmid, pGL2/P6, was constructed by inserting the fragment into the pGL2-Basic vector and was used to generate truncated or mutated sequences. The deleted terminal repeats carried in truncated mutants are schematically represented at the same scale as the wild-type repeat region, with their names and nt numbers to show the beginning and end of each fragment. The sense strand sequence of each base-substituted mutant is shown at the bottom. Only residues different from those in the wild-type sequence are indicated; identical nt are represented by dashes. The names of constructs are indicated on the left. ➧, YY1 binding site; □, P6 promoter, including a TATA box. (B) Luciferase reporter plasmid with sequence spanning the P5 promoter of AAV. A fragment (nt −88 to +64) containing the promoter region was amplified from psub201(+) (a molecular clone of AAV [34]). Plasmid pGL2/AAV was generated by inserting this fragment into the pGL2-Basic vector. The resulting plasmid and its relationship to the AAV terminal region is diagrammed above the sequence of the fragment. Positions and orientations of the YY1 binding sites [P5(−60) and P5(+1)] in the AAV promoter are indicated by hatched arrows; solid boxes show the relative positions of the P5(−60) and P5(+1) sites in the inserted fragment. Numbers represent nt positions with respect to the transcription start site (+1).

binding specificity. In the region upstream of the P6 promoter of B19 parvovirus, eight sequences contained CAT bases, but only three showed evidence of specific YY1 binding. In our functional cotransfection studies with truncated and mutated plasmids of this region, the removal or alteration of any two of the three major YY1 binding sites markedly decreased YY1 transcriptional upregulation, and transactivation was abolished if all three regions were mutated, indicating that any two binding sites were sufficient for YY1 activation. The transcription level of pGL2/−145D was lower than that of pGL2/−220M by 27%, although neither contained YY1 binding sites upstream of nt −145. In addition, pGL2/−86D showed 24% less activity than pGL2/−60, but both lacked YY1 binding sites upstream of nt −86. These results suggest that other factors associated with these upstream regions might affect transcriptional activation. In contrast to pGL2/−110 and pGL2/−60, pGL2/P6M did not show transactivation by YY1. This finding is consistent with a proposed effect of YY1 on transcription by bending DNA, so altering promoter topology as to facilitate the interaction of other regulatory proteins (27). For example, in studies of YY1 function in the murine c-*fos* promoter, the repression of transcription did not appear to be an intrinsic property of YY1, since it was altered by changing the orientation of YY1 binding or the phasing of other factors to the promoter (27).

The semipermissible cell line $UT7_{EPO}$ and several nonpermissible cell lines (K562, HeLa, TF1, MEL, and COS) showed similar binding activities with the YY1 probe, suggesting that YY1 alone probably does not play a role in the cell-specific propagation of B19 parvovirus. YY1 exists ubiquitously in mammalian cells, but its regulatory effects on gene expression are exerted by its association with other proteins. For example, YY1 interacts with Sp1 (19, 36) and c-*myc* (41). In several genes, YY1 binding sites overlap with other regulatory regions, including binding sites for serum response elements (13), which also can reverse YY1 repression. These interactions with other factors might be involved in the cell specificity of B19 parvovirus propagation. The interaction of YY1 with intranuclear factors would be best assessed in in vitro transcription systems in which the presence of purified factors can be fully

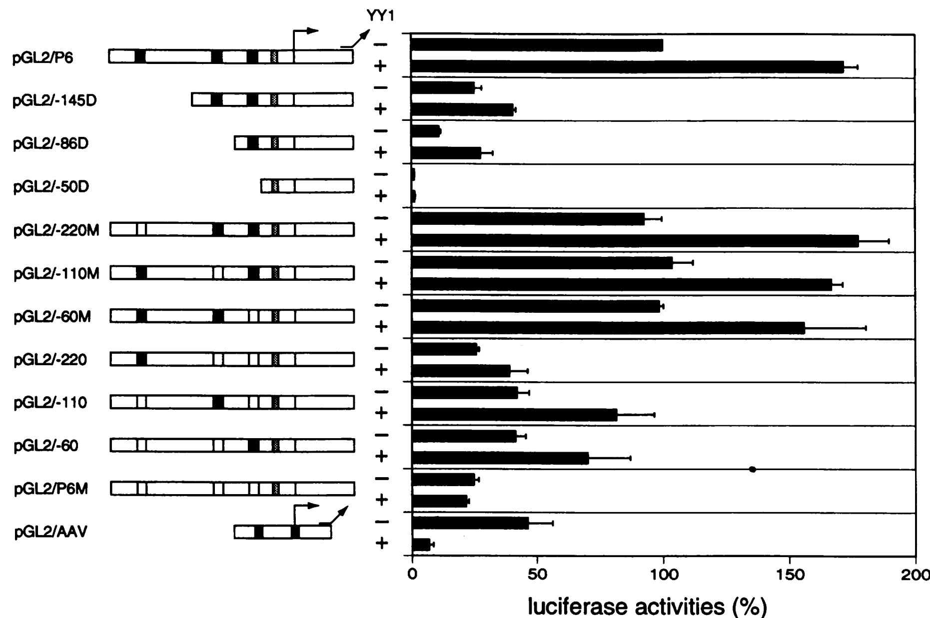


FIG. 9. Activation of B19 parvovirus P6 promoter in truncated or base-substituted mutants by YY1. Each luciferase reporter plasmid (Fig. 8) was cotransfected into HeLa cells with either a YY1 expression plasmid (pOPRSVI/YY1) or nonexpression plasmid (pOPRSVICAT) and a β-galactosidase expression plasmid (pCH110). At 48 h, cells were harvested and supernatants were assayed for luciferase activity. Plasmids are represented schematically on the left. Solid and open boxes indicate wild-type and mutated YY1 binding sequences, respectively. ▥, P6 promoter; ↗, translation initiation site. Levels of luciferase activity are expressed relative to the luciferase activity of cells in which pGL2/P6 was cotransfected with pOPRSVICAT and normalized by comparison with β-galactosidase activity. The results are averages of at least three experiments, each performed in duplicate.

controlled. We have recently produced highly purified and functionally active YY1 by using the baculovirus expression system (unpublished data), an important step towards this goal. Other efforts in our laboratory are directed to determining the structural changes in the B19 parvovirus promoter and hairpin regions induced by YY1 binding and other transcriptional regulatory proteins that interact with YY1.

ACKNOWLEDGMENTS

We are grateful to Johnson M. Liu and John M. Cunningham for helpful discussions. We thank Thomas Shenk for providing anti-YY1 monoclonal antibody and pCMV-hYY1, Peter Tattersall for providing pYT103C, and Richard J. Samulski for providing psub201(+).

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