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YY1 Represses Human Papillomavirus Type 16 Transcription by Quenching AP-1 Activity

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YY1 is a multifunctional transcription factor that has been shown to regulate the expression of a number of cellular and viral genes, including the human papillomavirus (HPV) oncogenes E6 and E7. In this study, we have analyzed the YY1-mediated repression of the HPV type 16 (HPV-16) E6-E7 promoter. A systematic analysis to identify YY1 sites present in the HPV-16 long control region showed that of 30 potential YY1 binding motifs, 24 bound purified recombinant YY1 protein, but only 10 of these were able to bind YY1 when nuclear extracts of HeLa cells were used. Of these, only a cluster of five sites, located in the vicinity of an AP-1 motif, were found to be responsible for repressing the HPV-16 P97 promoter. All five sites were required for repression, the mutation of any one site giving rise to a four- to sixfold increase in transcriptional activity. The target for YY1-mediated repression was identified as being a highly conserved AP-1 site, and we propose that AP-1 may represent a common target for YY1 repression. We also provide data demonstrating that YY1 can bind the transcriptional coactivator CREB-binding protein and propose a potentially novel mechanism by which YY1 represses AP-1 activity as a result of this YY1–CREB-binding protein interaction.

A small number of the human papillomaviruses (HPVs), most notably HPV type 16 (HPV-16) and HPV-18, are associated with the majority of instances of cervical carcinoma (25). Central to the role of these viruses in the etiology of cancer is the production of the oncoproteins E6 and E7 (13, 52, 76, 80). These oncogenes are transcribed at high levels in tumor tissue and in tumor-derived cell lines (35, 64) and are also required for the maintenance of the transformed phenotype (75). However, there is relatively little transcription of E6 and E7 in the basal epithelial cells of normal keratinocytes and low-grade neoplasias (9, 36). As the grade of neoplasia increases, however, there is a concomitant increase in the levels of E6 and E7 in basal cells and throughout the undifferentiated epithelium (9, 36). Thus, there is a correlation between the levels of E6 and E7 and the severity of the neoplastic phenotype.

The transcription of HPV-16 E6 and E7 oncogenes is controlled by a single promoter, termed P97 (19), which is itself modulated by regulatory elements within the long control region (LCR) (8, 18, 28, 70). The organization of the HPV-16 LCR (Fig. 1) is typical of all genital HPVs (56). The majority of transcription factor binding sites are present within the central segment of the LCR, between two E2 elements. The cellular factors that have previously been identified and shown to regulate a number of genital HPVs include NFI (1, 2), AP-1 (10), SP1 (27), Oct-1 (55), TEF-1 (38), and YY1 (7, 21, 47). The viral protein E2 is also an important modulator of E6-E7 promoter activity (48) and has been shown to repress transcription by displacing both Sp1 and TFIID (22, 71) from their cognate sites.

One current hypothesis is that circumstances which result in increased levels of E6 and E7 expression could represent cardinal events in the onset of tumorigenesis. One finding consistent with this idea is that most cervical carcinomas contain integrated copies of HPV (20, 64), and integration of the virus into the host genome nearly always disrupts the E2 open reading frame or E2 expression (61). However, approximately 30% of HPV-16-positive cervical carcinomas contain only extrachromosomal viral DNA with intact E2 open reading frames (16, 20, 24). Recently, a number of independent primary tumors or metastases were found to contain HPV-16 episomes with deletions or point mutations that affected one or more binding sites for the transcription factor YY1 (Yin-yang 1) (21, 47). These mutations resulted in an increase in activity of the E6-E7 promoter.

YY1 (67), also known as NF-E1 (58), UCRBP (23), CF1 (59), and δ (34), is a 414-amino-acid zinc finger protein that possesses multifarious properties. In the adeno-associated virus (AAV) P5 promoter, YY1 has been shown to interact with a regulatory element at −60 (the P5−60 site) and repress transcription of the P5 promoter (67). This negative regulation can be converted into positive stimulation by E1A in an interaction that is mediated by the E1A-associated protein p300 (39, 67). YY1 also binds to the AAV P5+1 site, where it acts to stimulate initiation of transcription (66, 67, 73), and there are numerous examples of cases in which YY1 has been shown to play an important role in the control of transcription of both viral (6, 12, 23, 43, 46, 47, 50, 51, 67) and cellular (34, 41, 44, 49, 54, 58, 59, 74, 79) genes. The role of YY1 as repressor, activator, or initiator appears to be context dependent (33).

YY1 was first implicated in the regulation of HPV transcription by Bauknecht et al. (6), who studied the regulation of the HPV-18 E6-E7 promoter. In these studies, a YY1 site was found to repress both constitutive and 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced enhancer activity. Later studies by the same group suggested this regulation was complex and could be influenced by sequences upstream of the YY1 site (7). The recent identification of a number of potential YY1 sites within the HPV-16 enhancer, which were found to be mutated in episomal copies of viral genomes obtained from tumor tissue and which resulted in elevated levels of E6-E7 transcription compared with the wild type, demonstrated the clinical importance of YY1 regulation in HPV-16 (21, 47). The details of YY1 regulation in HPV-16 are not, however, fully understood. For example, it is not known how many potential YY1 sites are involved in negatively regulating the E6-E7 pro-

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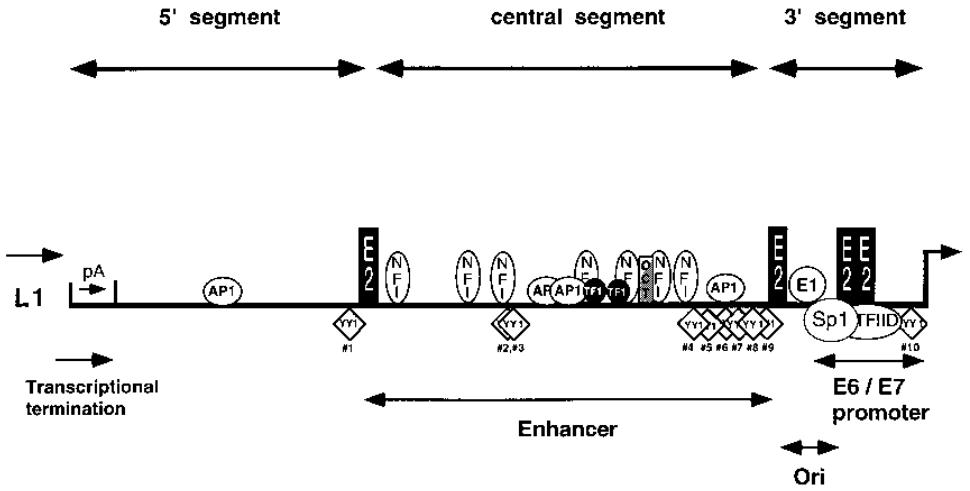

FIG. 1. Schematic representation of the HPV-16 LCR. Four E2 binding sites divide the LCR into functionally distinct segments labeled the 5′, central, and 3′ segments. The 5′ segment contains the transcription termination signal (pA), the central segment includes the epithelial cell-specific enhancer, which contains the majority of transcription factor binding sites, and the 3′ segment contains the origin of replication (Ori), the binding site for the HPV E1 protein, and the E6-E7 promoter. The positions of transcription factors NFI, Oct-1 (OCT), AP-1, TEF-1 (TF1), SP1, E2, and E1 are indicated. Not all binding sites shown have been functionally tested, and for clarity only those sites shown to be important for more than one genital HPV have been included. Also indicated are the positions of the 10 YY1 sites shown to bind nuclear YY1 protein in this study.

moter, nor has the target(s) for such repression been identified.

In this study, we carried out a systematic approach to identify the YY1 binding sites present within the HPV-16 LCR and determined which of those sites were involved in transcriptional repression. We show that of 30 potential sites, 24 have the capacity to bind YY1, as determined by electrophoretic mobility shift assays (EMSAs) with recombinant YY1, but only 10 of these sites bind YY1 in EMSAs using nuclear extract. Of these 10 sites, 6 are clustered around an AP-1 site in the region shown to be deleted in the study of May et al. (47), and 5 of these 6 sites are responsible for repressing E6-E7 promoter activity. The other four sites present elsewhere in the LCR do not appear to be involved in transcriptional repression. Finally, we show that the cluster of YY1 sites represses transcription by quenching AP-1 activity, and we provide data demonstrating the interaction between YY1 and the transcriptional coactivator CREB-binding protein (CBP) (17). As a result of these findings, we propose a mechanism in which YY1 represses AP-1 activity as a consequence of the interaction between YY1 and CBP.

MATERIALS AND METHODS

Plasmid constructs. All constructs used in functional assays were based on the chloramphenicol acetyltransferase (CAT) construct pBLCAT3dH/N (55), a modified version of pBLCAT3 (63). The enhancerless HPV-16 promoter construct, p80 (previously designated p16 [55]), contains the HPV-16 promoter sequences from positions +16 to +80 cloned into the *Bgl*II and *Xho*I sites of pBLCAT3dH/N, while p103 contains the sequence from positions +16 to +103 in the same sites.

The HPV-16 enhancer fragment 0l/9e (nucleotides 7409 to 7852) was created by PCR and cloned into the *Hin*dIII and *Bam*HI sites of p103 to give the construct p103:0l/9e. To generate p103:0l (YY1m1)/9e, we used a 5′ primer which contained the YY1 mutation CAT to TGC, which abolishes YY1 binding (57). The same strategy was used to generate the construct p80:3e/9e (7572 to 7852) and the corresponding YY1 mutant constructs, p80:2e/9e (7524 to 7852) and p80:2e/8e (7524 to 7776). The construct p103 (YY1m10):0l/9e was generated from p103:0l/9e by replacing the wild-type promoter sequences from +16 to +103 with a double-stranded oligonucleotide containing the YY1m10 mutation. All PCR-generated fragments were sequenced by the method of Sanger et al. (62).

Constructs containing YY1, AP-1, or NFI mutations in the fp8e/9e region (see Results) were created by cloning a double-stranded oligonucleotide (99-mer) with the appropriate sequences and *Bam*HI and *Bgl*II complementary ends into the *Bam*HI site of the construct p80:2e/7e, the orientation of insertion being determined by a restriction digest with *Bam*HI and *Xho*I.

Plasmids used for in vitro translation included pBS:CTF-1 (a gift from Nick Mermod) and pGEM:YY1, which was constructed by cloning the *Bam*HI and *Eco*RI fragment of pGEX2T:YY1 (a gift Thomas Shenk and colleagues) into pGEM.

Cell culture and functional assay. Primary human keratinocytes were grown in serum-free medium 154 (both cells and media were obtained from Cascade Biologics, Inc.) according to the manufacturer's recommendations. HeLa cells were cultured in minimal essential medium supplemented with 10% fetal calf serum. Primary human keratinocytes were plated onto 10-cm-diameter culture dishes and transfected at 50 to 70% confluency, using Lipofectin reagent (GIBCO-BRL). For each transfection, 30 μl of Lipofectin was mixed with 5 μg of DNA in 1 ml of medium 154 and left at room temperature for 15 min before being added to the 9 ml of medium covering the cells. After 18 to 24 h, the medium containing Lipofectin was replaced with 10 ml of normal fresh medium 154, and the cells incubated for a further 24 h before harvesting. Transfection of HeLa cells was performed with either Lipofectamine (GIBCO-BRL) reagent in conditions identical to those described above or by electroporation as previously described (55).

To determine CAT activity (31), assays were performed essentially as described by Chan et al. (11), and CAT activities were determined as picomoles of chloramphenicol acetylated per minute per milligram of protein extract by quantification of radioactive spots on thin-layer chromatograms. Each value obtained represents between three and six independent transfections using two different DNA preparations.

Expression and purification of His-YY1 fusion protein. Forty milliliters of overnight culture of *Escherichia coli* RR containing plasmid pDS56-6 x HIS: YY1 (67), a gift from Thomas Shenk, was used to inoculate 800 ml of fresh LB broth containing 100 μg of ampicillin per ml and incubated at 37°C for 1 h until the optical density at 600 nm reached 0.4 to 0.5. YY1 expression was induced by the addition of isopropylthiogalactopyranoside (IPTG) to 0.5 mM, and the culture was incubated for a further 3 h at 30°C, after which time the cells were pelleted by centrifugation and lysed by sonication. Bacterial cell lysate was passed down a 1-ml nickel chelate column, washed with 400 ml of phosphate-buffered saline (PBS) followed by 20 ml of PBS plus 50 mM imidazole and then 10 ml of PBS plus 100 mM imidazole, and finally eluted with 6 ml of PBS supplemented with 200 mM imidazole, 1 mM dithiothreitol, and 20% glycerol. The purity of the eluate was checked by electrophoresis on a sodium dodecyl sulfate (SDS)–9% polyacrylamide gel, and the concentration of His-YY1 was adjusted to approximately 200 μg/ml.

EMSAs and DNase I protection assays. All double-stranded oligonucleotides used in the EMSAs described here were of the same length, containing a 15-nucleotide YY1 recognition sequence around the core 5′-CAT-3′ plus flanking *Xba*I sequences 5′-CTAGA-3′. Fifty-nanogram aliquots of annealed oligonucleotides were labeled with [^{32}P]dATP and [^{32}P]dCTP by using Klenow polymerase. Approximately 250 pg of purified labeled probe with an activity of approximately 20,000 cpm was used in a standard reaction as previously described (27). Samples were run on a 4% polyacrylamide gel containing 0.25× Tris-borate-EDTA at 200 V for 2 h. Quantification of EMSAs was carried out by densitometric analysis

TABLE 1. YY1 sites in the HPV-16 LCR[a]

Label	Sequence	Position of central A	Level of radioactive probe shifted by YY1[b]		Designation
			rYY1[c]	NE[d]	
	*				
AAV P5−60	TTG CGA CAT TTT GCG	AAV P5−60	+++	++	
a	CAG GGC CAT TTT GTA	7437	++++	++++	1
b1	CGG TTG CAT GCT TTT	7476	−	−	
b2	AAA AAG CAT GCA ACC (r[e])	7477	−	−	
c	AAA ACA CAT TTT GTG (r)	7484	−	−	
d	TGC TGA CAT AGA ACT (r)	7506	++	−	
e	TTA AAC CAT AGT TGC (r)	7518	++	−	
f	GCT TGC CAT GCG TGC	7548	+	−	
g	GCC AAC CAT TCC ATT	7594	++	+	2
h	CCA TTC CAT TGT TTT	7599	++	+	3
i	GTT GCA CAT AGT GCA (r)	7620	−	−	
j	AAT GTA CAT AGT GAT (r)	7641	++	−	
k	TAT GTA CAT TGT GTC	7646	+	−	
l	TTG TGT CAT ATA AAA	7654	+	−	
m	GCC TTA CAT ACC GCT	7691	+	−	
n	TAG GCA CAT ATT TTT	7705	++	−	
o	TAA TTG CAT ATT TGG	7738	−	−	
p	ATT TGG CAT AAG GTT	7747	−	−	
q	GGG TGA CAT TTA GTT (r)	7777	+++	+	4
r	CTA GTT CAT ACA TGA	7791	++	−	
s1	TTC ATA CAT GAA CTG	7795	+	+	5
s2	ACA GTT CAT GTA TGA (r)	7796	+	+	
t	GTT AGT CAT ACA TTG	7817	++	+	6
u	GTC ATA CAT TGT TCA	7821	++	+	7
v	ATT GTT CAT TTG TAA	7828	++	+	8
w1	ACT GCA CAT GGG TGT	7844	++	+	9
w2	CAC ACC CAT GTG CAG (r)	7845	++	+	
x	GTT ACA CAT TTA CAA	7875	+	−	
y1	ATA ATT CAT GTA TAA	14	+	−	
y2	TTT ATA CAT GAA TTA (r)	15	+	−	
z	AGC AGA CAT TTT ATG	78	++++	++++	10
Consensus sequences					
Binds YY1	NTN ATA CAT TTA TTA A T				
Does not bind YY1	ATA TNG CAT ANT GTN *				

[a] Thirty potential YY1 sites from HPV-16 are listed along with the P5−60 YY1 site from AAV. Each site has been given a label (a to z), and those also labeled 1 and 2 (for example, b1 and b2) represent two overlapping sites, one on each strand, staggered by a single nucleotide which results when a YY1 site contains the palindromic sequence 5′-CATG-3′. Also shown is the sequence of the putative YY1 site and the nucleotide position of the central A (below the asterisk) within the HPV-16 genome. Results obtained from EMSA experiments depicted in Fig. 2 are also shown. Those YY1 sites which were able to bind both recombinant YY1 and YY1 in HeLa nuclear extracts were given designations 1 to 10; the positions of these YY1 sites are shown in Fig. 1.

[b] Determined by densitometric analysis. −, less than or equal to 0.1% shifted probe; +, 0.2 to 1.0%; ++, 1.1 to 10%; +++, 11 to 50%; and ++++, more than 50%.

[c] Level of affinity of these sites for bacterially expressed and purified His-tagged recombinant YY1 (rYY1).

[d] Ability to bind YY1 in HeLa nuclear extracts (NE).

[e] r, reverse orientation on the lower strand.

using the integration volume function of the ImageQuant software provided with the Molecular Dynamics PhosphorImager. For EMSAs in which YY1 polyclonal antibody was used, 1 μl (1.5 μg) of antibody was added to the reaction prior to the addition of radiolabeled probe, and the mixture was incubated on ice for 10 min.

DNase I protection assays were performed according to standard methods as modified by Gloss and Bernard (27) and used approximately 200 ng of recombinant YY1 protein.

In vitro translation of radiolabeled proteins. Radiolabeled products of in vitro translation of YY1 (from pGEM:YY1) and NFI/CTF-1 (from pBS:CTF-1) were synthesized by using the TNT coupled transcription-translation system as recommended by the manufacturer (Promega).

GST fusion proteins and pull-down assay. Glutathione *S*-transferase (GST) fusion proteins were prepared by using the pGEX system (Pharmacia). Bacterial lysates were made as described above for His-YY1 lysates except that aliquots of the lysate were stored at −70°C until required for the pull-down assay. For the pull-down assay, bacterial lysates containing either GST, GST-CBP 1, or GST-CBP 2 were incubated with 25 μl of glutathione-Sepharose beads (Pharmacia), which existed as a 1:1 slurry with NENT buffer (100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 20 mM Tris [pH 8.0]) plus 0.5% nonfat dry milk, at 4°C for 30 min. The glutathione-Sepharose beads with bound GST protein were then spun down, the supernatant was removed, and the beads were washed twice with 1 ml of NENT buffer. After removal of the supernatant, the beads were resuspended in 300 μl of incubation buffer (50 mM KCl, 40 mM [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.5], 5 mM 2-mercaptoethanol, 0.1% Tween 20, 0.5% nonfat dry milk). After addition of either the radiolabeled translation product for YY1 or CTF-1, the mixture was subjected to 1 h of incubation and mixing at 4°C. The beads were then spun down, and the supernatant was removed before being washed twice with 1 ml of incubation buffer containing 100 mM KCl. Finally, the beads were heated to 95°C in 40 μl of sample buffer (125 mM Tris [pH 6.8], 1% SDS, 2% 2-mercaptoethanol, 25% glycerol, 0.05% bromophenol blue), subjected to electrophoresis on an SDS–9% polyacrylamide gel, and subsequently fixed, stained, dried, and autoradiographed.

RESULTS

Identification of YY1 binding sites within the HPV-16 LCR. Previous studies have demonstrated the importance of YY1 in

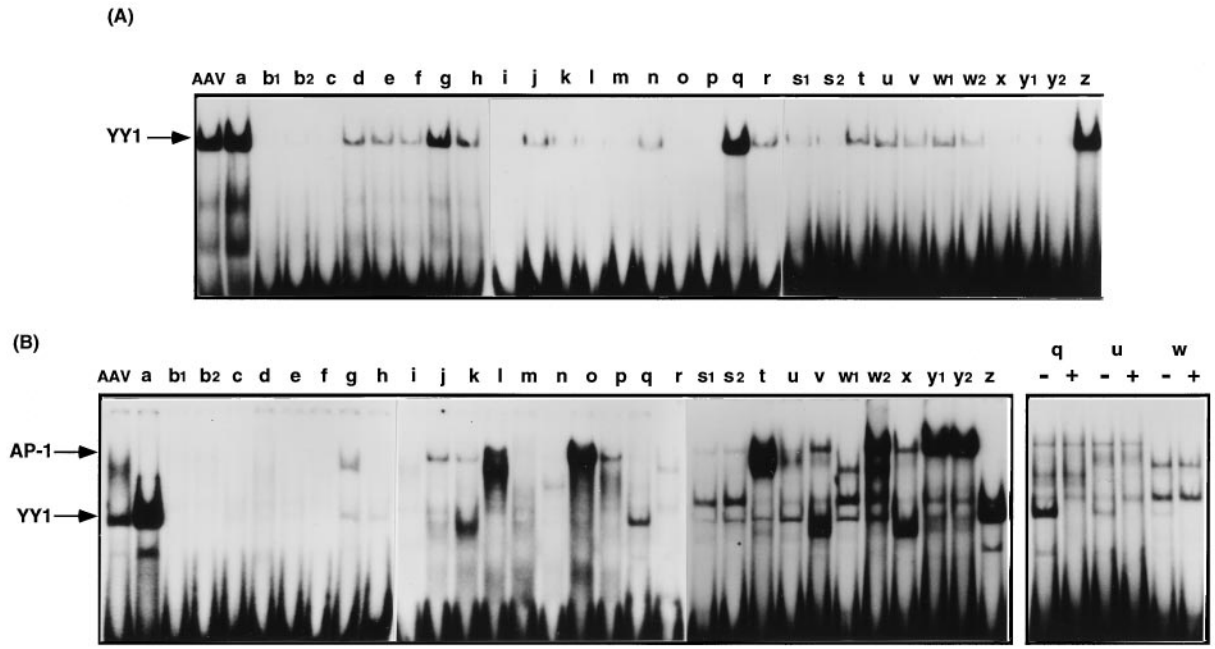

FIG. 2. EMSA of YY1 binding sites within the HPV-16 LCR. Thirty potential YY1 sites containing the sequence 5′-CAT-3′ were identified in the HPV-16 LCR (positions 7401 to 103). The sequences are listed in Table 1 and have been labeled a to z. Letters followed by the numbers 1 and 2 represent two overlapping sites on each DNA strand which result from the palindromic sequence 5′-CATG-3′. Also shown are results for the classical AAV P5−60 sequence. Double-stranded oligonucleotides containing these sequences were ^{32}P labeled and then used in EMSAs with either bacterially expressed and purified recombinant His-tagged YY1 protein (A) or HeLa nuclear extracts (B). Of the 30 potential sites, 24 bind recombinant YY1 to various degrees (Table 1). Of these 24, only 10 appear to bind nuclear YY1 in EMSAs using HeLa nuclear extract. Confirmation that the bands labeled YY1 do in fact represent YY1 binding is provided for sites q, u, and w by the use of a specific polyclonal anti-YY1 antibody (+ lanes) in which the complex representing YY1 binding is no longer present. Showing the highest affinity for YY1 are sites 1 and 10, followed by site 4 and then the remaining seven sites. The positions of these 10 YY1 sites are indicated in Fig. 1. All EMSAs were carried out with excess radiolabeled oligonucleotide and YY1 protein (see Materials and Methods).

the regulation of HPV oncogenes E6 and E7 (6, 21, 47). We wished to extend the studies on HPV-16 to determine more exactly the nature of YY1 repression and to identify other possible YY1 binding sites within the LCR that might represent potential targets for mutation and a means for the virus to escape cellular transcriptional repression.

DNA sequences recognized by YY1 have suggested a requirement for the trinucleotide sequence 5′-CAT-3′ (33, 37, 68). We therefore carried out a search for this sequence in the HPV-16 LCR (from nucleotide positions 7401 to 103). There were 30 potential sites, which are listed in Table 1 along with the classical YY1 motif from the AAV P5−60 site.

To test the ability of each site to bind YY1, the corresponding double-stranded oligonucleotide was created and used in EMSAs with recombinant, bacterially expressed YY1 protein (Fig. 2A). Of the 30 HPV sites tested, 24 were able to bind purified bacterial YY1 to various degrees. The relative affinities of the binding sites for YY1 are given in Table 1 and were determined by measuring the percentage of radiolabeled oligonucleotide probe shifted under conditions in which neither probe nor YY1 protein was limiting. From the 24 sites that bound YY1, a consensus sequence (Table 1) similar to those obtained in previous studies (68) was obtained. One noticeable difference is that the core recognition sequence, usually cited as being 5′-CCAT-3′ (37), was found to be 5′-ACAT-3′ in the majority of HPV-16 recognition sequences. This is not too surprising, since an 5′-ACAT-3′ core was recently observed in a number of oligonucleotides selected for the ability to bind YY1 (77). In addition to core sequences with 5′-CCAT-3′ and 5′-ACAT-3′, 6 of the 24 sites in this study were also found to contain the core 5′-TCAT-3′, and these motifs were also able to bind YY1. However, no sequence that contained the core 5′-GCAT-3′ was found to bind YY1, suggesting that a guanine in this position may effectively prevent binding. To test this idea, we mutated one of the strongest HPV-16 sites (site a) so that it now contained the core 5′-GCAT-3′. The presence of this substitution virtually abolished YY1 binding (data not shown).

While the use of purified YY1 in EMSA experiments provides information about the relative affinity of the recognition sequence for YY1, it is still necessary to carry out similar experiments with nuclear extract, which reflects more accurately the conditions found in the nucleus, including the concentration of YY1 relative to those of other transcription factors and the effect of any posttranslational modification of the YY1 protein. Figure 2B shows that of the 24 sites that can bind purified YY1, only 10 bound YY1 when HeLa nuclear extract was used. Confirmation of the identity of YY1 bound to these 10 sites was obtained by using a specific polyclonal anti-YY1 antibody in EMSAs; in Fig. 2B, it can be seen that the complex representing YY1 for sites q, u, and w is absent in those lanes in which the reaction mixtures were pretreated with YY1 antibody. The relative strength of YY1 binding to the different sites is indicated in Table 1. The positions of the 10 sites in the HPV-16 LCR are also shown diagrammatically in Fig. 1. It can be seen that sites 1 and 10 bind YY1 most strongly, even more than the classical AAV P5−60 site, and followed in decreasing order by site 4 and then the remaining seven sites. Also indicated in Fig. 2B is the position of the AP-1 complex in lanes l and t. The sequences 5′-TGTGTCA-3′ and 5′-TTAGTCA-3′ present in these oligonucleotides have previously been shown to bind AP-1 (10), and it can be seen that AP-1 binding to the

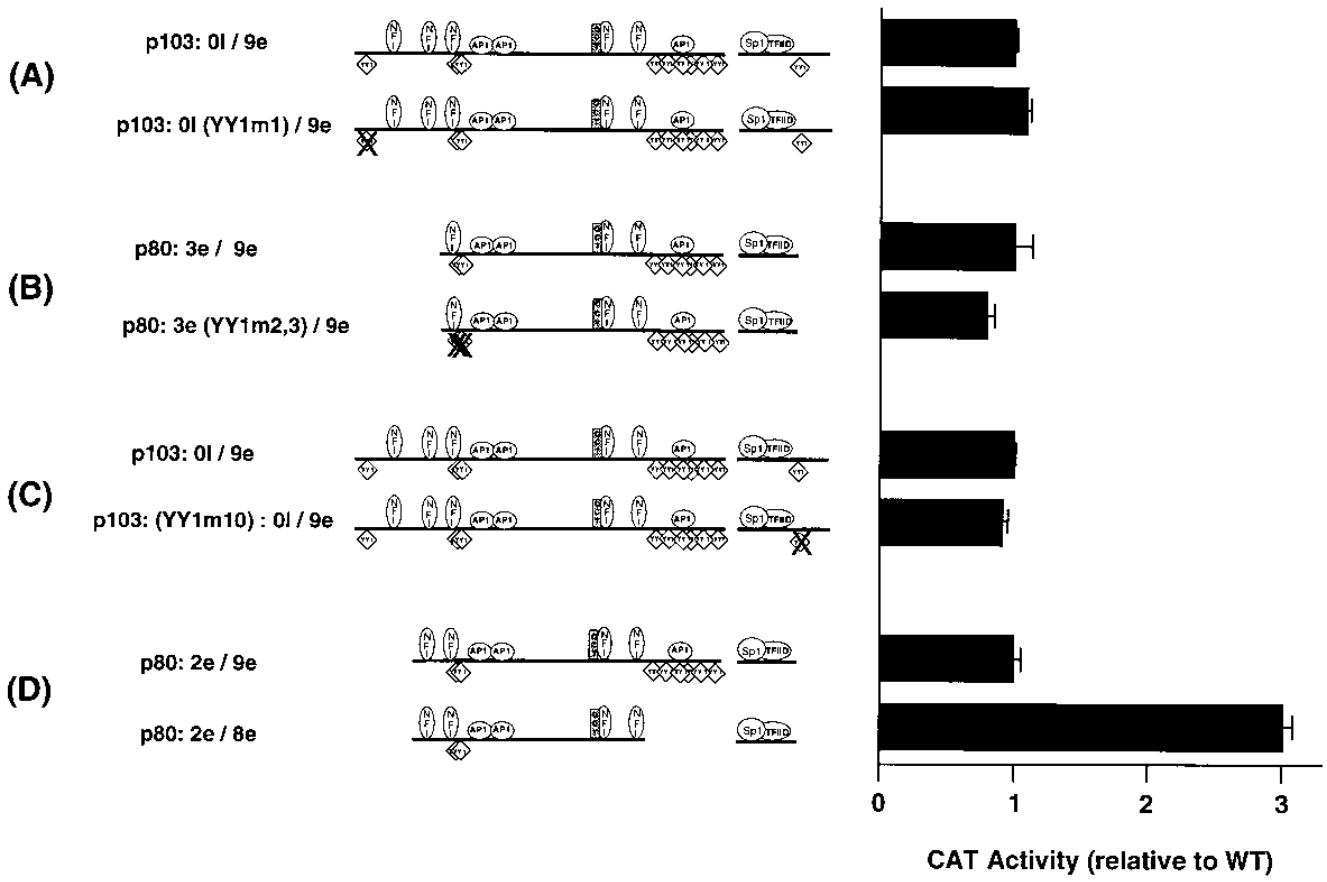

FIG. 3. The YY1 sites outside the fp9e region in the HPV-16 LCR are not involved in transcriptional repression. Each panel shows the pairwise comparison of CAT activities of two HPV-16 constructs differing only in the mutation of a single or cluster of YY1 sites. All constructs are based on pBLCAT3 (see Materials and Methods). Those constructs with the prefix p80 contain HPV-16 promoter sequences corresponding to the genomic positions +16 to +80, while the two constructs with the prefix p103 contain the HPV promoter sequence from genomic positions +16 to +103, which includes YY1 site 10. The various LCR fragments contained in each construct are also represented schematically, but for clarity not all binding sites are shown. Also indicated are the YY1 site point mutations (X). Construct p80:2e/9e contains the cluster of YY1 sites around the fp9e AP-1 site, while in p80:2e/8e, all of these YY1 sites (sites 4 to 9), as well as the AP-1 site, have been deleted. Results described here were obtained from the transient transfection of primary keratinocytes with 5 μg of the test construct. In each case, CAT activity was determined 48 h after transfection and calculated as picomoles of chloramphenicol acetylated per minute per milligram of protein extract. Each construct with intact YY1 sites was given an activity of 1, and the relative activity of the corresponding YY1 mutant construct was then determined. The activity for each construct represents the results from three to six independent transfection experiments and two different DNA preparations. Significance tests ($P < 0.05$) show that there is no significant difference in activity when YY1 sites 1, 2, 3, and 10 are mutated; however, there is a significant difference between the activities of constructs p80:2e/9e and p80:2e/8, in which YY1 sites 4 to 9 are deleted. WT, wild type.

sequence overlapping YY1 site 6 is particularly strong. We have previously designated this AP-1 site fp9e, the nomenclature being based on DNAse I protection patterns (29, 30).

Results of the EMSA experiments described by May et al. (47) suggested that there were four YY1 binding sites surrounding the fp9e AP-1 site which were located within the deleted region of episomal HPV-16 genomes from clinical samples. Two of these sites correspond to YY1 sites 5 and 9 of our study. The other two sites have the sequences 5′-CACCC TAGT-3′ and 5′-GTGCAGTTT-3′. Both of these sequences vary quite considerably from our observed core sequences, and the last site also possesses the core 5′-GCA-3′, which we have suggested may prevent YY1 binding. We have carried out EMSAs with both of these sites and find that neither can bind purified recombinant YY1 or YY1 in nuclear extracts (data not shown). The discrepancy between the two studies may have arisen from the fact that the first site was not directly tested in the EMSAs carried out by May et al., and the second site was on a DNA fragment larger than those used in our study, which, because of the proximity of many of these sites, would have led to complexes containing the additional YY1 binding sites identified here.

The YY1 sites outside the fp9e region in the HPV-16 LCR are not involved in transcriptional repression. EMSA studies presented here provide evidence for the binding of nuclear YY1 to 10 sites in the HPV-16 LCR (Fig. 1). One (site 1) is situated just upstream of the most 5′ E2 recognition site, two (sites 2 & 3) are overlapping sites present in the central segment, just downstream of a NFI binding site, one (site 10) is in the 3′ segment of the LCR, downstream of the TATA box, and the remaining six (sites 4 to 9) are clustered around the fp9e AP-1 site in the region of the enhancer previously shown to be deleted in episomal HPV-16 genomes that were associated with cervical carcinomas and metastatic tissue (21, 47).

One of our goals of this study was to determine whether any previously undescribed YY1 sites that might also be involved in the transcriptional repression of the E6-E7 promoter existed in the HPV-16 LCR. To this end, we cloned LCR fragments containing the HPV-16 E6-E7 promoter and enhancer sequences with either wild-type or mutated versions of YY1 sites

1, 2, 3, and 10 into a CAT expression vector and determined their transcriptional activities in primary human keratinocytes and in the cervical carcinoma cell line HeLa (see Materials and Methods). The results presented in Fig. 3 were obtained by transfection of primary human keratinocytes, but similar results were obtained with HeLa cells (data not shown).

Figure 3 shows pairwise comparisons of two HPV-16 LCR constructs that differ only in the presence of a mutation in a single YY1 site or a cluster of sites. The data show that the mutation of site 1, 2, 3, or 10 has no significant effect on activity compared with the corresponding constructs containing wild-type YY1 sequences. These data therefore suggest that YY1 sites 1, 2, 3, and 10 are not involved in transcriptional repression. This result is interesting in that both site 1 and site 10 have a very high affinity for the YY1 protein and both are conserved in a number of genital HPVs, suggesting that they may have a function in HPVs other than transcriptional repression. Deletion of the remaining six YY1 sites (sites 4 to 9), however, results in an increase in transcriptional activity, in agreement with previous observations (47).

DNase I protection experiments identify those binding sites that are simultaneously occupied by YY1 in the fp9e region. While we have identified six YY1 binding sites surrounding the fp9e AP-1 sequence, our EMSA experiments provide information only about the individual sites. The proximity of a number of these sites suggests that perhaps only some may be occupied simultaneously. Since it was our intention to create point mutations within those sites that bound YY1 (for use in functional studies), we decided to carry out DNase I protection analysis of the fp9e region to identify candidate YY1 sites for mutagenesis.

Figure 4 shows the results of these experiments, in which the lower strand of the HPV-16 fp9e region was labeled with ^{32}P and DNase I digestion was performed after incubation of the DNA with purified recombinant YY1 protein (see Materials and Methods). Four of the six YY1 sites in the fp9e region (sites 4, 5, 7, and 9) show protection over the core 5′-CAT-3′ sequence. The footprint covering YY1 site 4 is larger than the others, possibly because this site is of much higher affinity than the other YY1 sites in this region (Fig. 2A). The protected region of site 9 is also slightly larger than those of sites 5 and 7, probably because of the presence of staggered YY1 sites on both strands of the DNA. It was not possible from this experiment to determine whether YY1 site 8 was protected; although close examination suggests that there might be a footprint, there is not sufficient digestion by DNase I of the relevant sequences (in the lanes without YY1 protein) to be sure of this. This is not, however, the case for YY1 site 6: there is good evidence showing that this site is not protected from DNase I digestion. The close proximity of sites 6 and 7, along with the footprint data presented here, suggests that site 7 is bound by YY1 preferentially and to the exclusion of, site 6.

Five YY1 sites clustered around the fp9e AP-1 sequence contribute to transcriptional repression. We next wanted to determine which of the YY1 sites clustered around the fp9e AP-1 sequence were involved in transcriptional repression. To this end, we created point mutations within the four YY1 sites (sites 4, 5, 7, and 9) shown in Fig. 4 to bind YY1 as well as in YY1 site 8. Constructs were transfected into primary human keratinocytes, and their transcriptional activities were determined.

Figure 5 shows the activities of the various constructs relative to that of the wild-type plasmid (p80:2e/9e) in which all YY1 sites are intact. Mutation of all five sites results in a sixfold increase in CAT activity relative to the wild-type construct. Somewhat surprisingly, mutation of any of the five sites

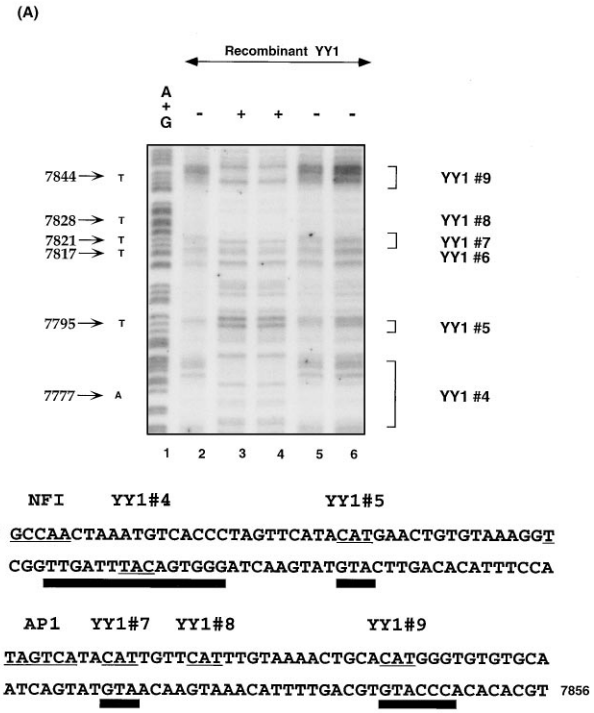

FIG. 4. DNase I protection analysis of bacterially expressed YY1 protein in the fp9e region. (A) Lane 1 contains the chemically cleaved DNA fragment, which includes the fp9e region and which has been labeled on the lower strand with ^{32}P to produce a sequence ladder. Lanes 2, 5, and 6 contained no YY1 protein, while lanes 3 and 4 contained 200 ng of bacterially expressed and purified YY1 protein. The numbers and arrows at the left designate the central A (T on the lower strand) of the YY1 recognition sequences 4 to 9, while on the right is indicated the extent of the DNase I protection (footprint). Protected areas include YY1 sites 4, 5, 7, and 9. YY1 site 8 could also be protected; however, there is insufficient DNase I digestion of the relevant sequences to be sure. YY1 site 6 does not appear to be protected. (B) Nucleotide sequence of the fp9e region showing the positions of YY1 sites 4 to 9, the fp8e NFI site, and the fp9e AP-1 site, all underlined. The thick black bar delineates the area of DNase I protection on the lower strand indicated by the autoradiogram in panel A.

individually also results in an increase in activity of between four to sixfold, suggesting that all five sites are required for transcriptional repression of the HPV-16 E6-E7 promoter. Similar results were also obtained from transfections in HeLa cells (data not shown). The mutation of YY1 site 5 in this study, which results in a fivefold increase in activity, can be compared with the naturally occurring point mutation found in this site, which was shown to result in a fourfold enhancement of promoter activity (47).

It could be argued that factors other than YY1 bind to these five sites and are responsible for this repression. This would seem unlikely, however, since no single factor (other than YY1) is common to all five sites. Also, mutating all of these sites, or each of the sites individually, results in the same range of effect, i.e., four to sixfold. Lastly, the ability of YY1 to repress transcriptional activity is well documented.

The target of YY1 transcriptional repression in HPV-16 is AP-1. Previously published work has not identified the target for YY1 repression in HPV-16. One possibility is that YY1, once bound to the cluster of five sites, could repress transcription by interacting directly with components of the basal transcription machinery (15, 73). Alternatively, YY1 might repress transcription by interfering with transcriptional activators present within the LCR. Since the cluster of YY1 sites surround an AP-1 recognition sequence, previously shown to en-

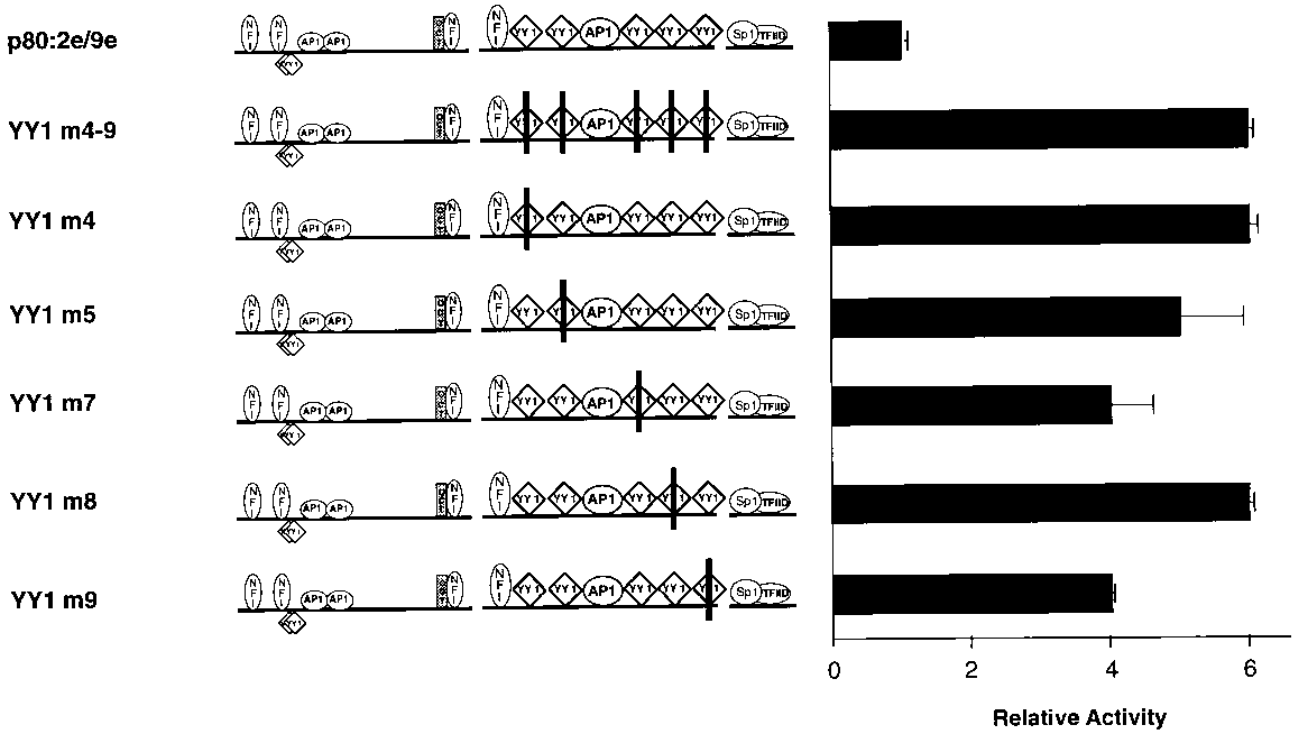

FIG. 5. Five YY1 sites contribute to transcriptional repression of the HPV-16 E6-E7 promoter. Shown are the CAT activities of the various YY1 mutant p80:2e/9e constructs relative to that of the wild-type p80:2e/9e construct. The constructs are presented schematically, and a vertical black bar indicates that the YY1 site has been mutated to prevent YY1 binding. Construct YY1m4-9 contains mutations in all five sites and results in a sixfold increase in transcriptional activity. Mutations in any of the five sites individually also results in an increase in CAT activity of between four- to sixfold. The experiments were carried out in primary human keratinocytes as described in Materials and Methods.

hance activity in response to phorbol esters (10), we decided to determine whether this was the target for YY1. As there is also an NFI binding site (fp8e) just upstream of YY1 sites 4 and 5, we also wished to investigate this as a possible target for repression. The site at fp8e has been shown to bind NFI with lower affinity than the other six NFI sites in HPV-16 (30) but has not been functionally tested.

We reasoned that if the activity of either NFI or AP-1 were being repressed, then we should detect a decrease in transcriptional activity in constructs containing mutations in these sites when all five YY1 sites were also mutated. However, we would not predict a change in activity when these sites were mutated in the presence of functional YY1 sites, as they should already have been inactivated by YY1.

In Fig. 6 it can be seen that the mutation of the fp8e NFI site in constructs with all five YY1 sites mutated (YY1m4-9/NFIm) does not lead to any significant decrease in activity, suggesting that NFI does not contribute to enhancer activity from this site. In contrast, mutation of the AP-1 site under identical conditions (YY1m4-9/AP-1m) leads to a decrease in CAT activity of about sixfold, thereby demonstrating the potential importance of the fp9e AP-1 site for transcriptional activation of the E6-E7 promoter. However, in the presence of functional YY1 sites, the mutation of the AP-1 site does not lead to any decrease in CAT activity (compare YY1WT with YY1WT/AP-1m), suggesting that AP-1 function is already quenched in the wild-type context as a consequence of YY1 repression. Moreover, the levels of AP-1 contribution and YY1 repression are the same, i.e., approximately sixfold, which suggests that fp9e AP-1 is the sole target for repression by the five YY1 sites.

If this is the case, then deletions that remove the AP-1 site at fp9e along with the YY1 sites should not show any net change in transcriptional activity. However, in both clinical samples (21, 47) and Fig. 3, deletion of the fp9e region results in an increase in the activity of the E6-E7 promoter. We propose that this increase in activity is not due to the removal of YY1 sites but rather may result from a distance effect, that is, the shifting of upstream regulatory sequences closer to the HPV-16 promoter. It is also possible that the deletions remove some other unidentified repressor whose function does not depend on the quenching of the fp9e AP-1 activity. However, this seems less likely, since there is no obvious candidate sequence in this region.

YY1 interacts in vitro with the AP-1 transcriptional coactivator CBP. The experiments described above suggest that YY1 represses the HPV-16 E6/E7 promoter by inactivating AP-1 function. As this was the first time that YY1 had been directly shown to interfere with AP-1 function, we were curious as to the mechanism involved. Previous studies involving YY1-mediated repression of transcription have led to proposals of steric interference with target factors (32, 41, 74), DNA bending (54), or direct protein-protein interaction (15, 40, 42, 65, 69, 79). It seemed unlikely to us that YY1 repressed AP-1 function by preventing its binding to the fp9e site because of the high affinity that this site has for AP-1 (compared with the relatively weak YY1 sites). Also, only one of the five sites (site 7) appears close enough to interfere directly with AP-1 binding. Lastly, EMSAs investigating the binding of AP-1 and YY1 to the fp9e region of HPV-16 have provided no evidence for increased AP-1 binding upon the addition of competitor YY1 oligonucleotides, or vice versa (57). However, one potential mechanism did occur to us as a consequence of recent work in

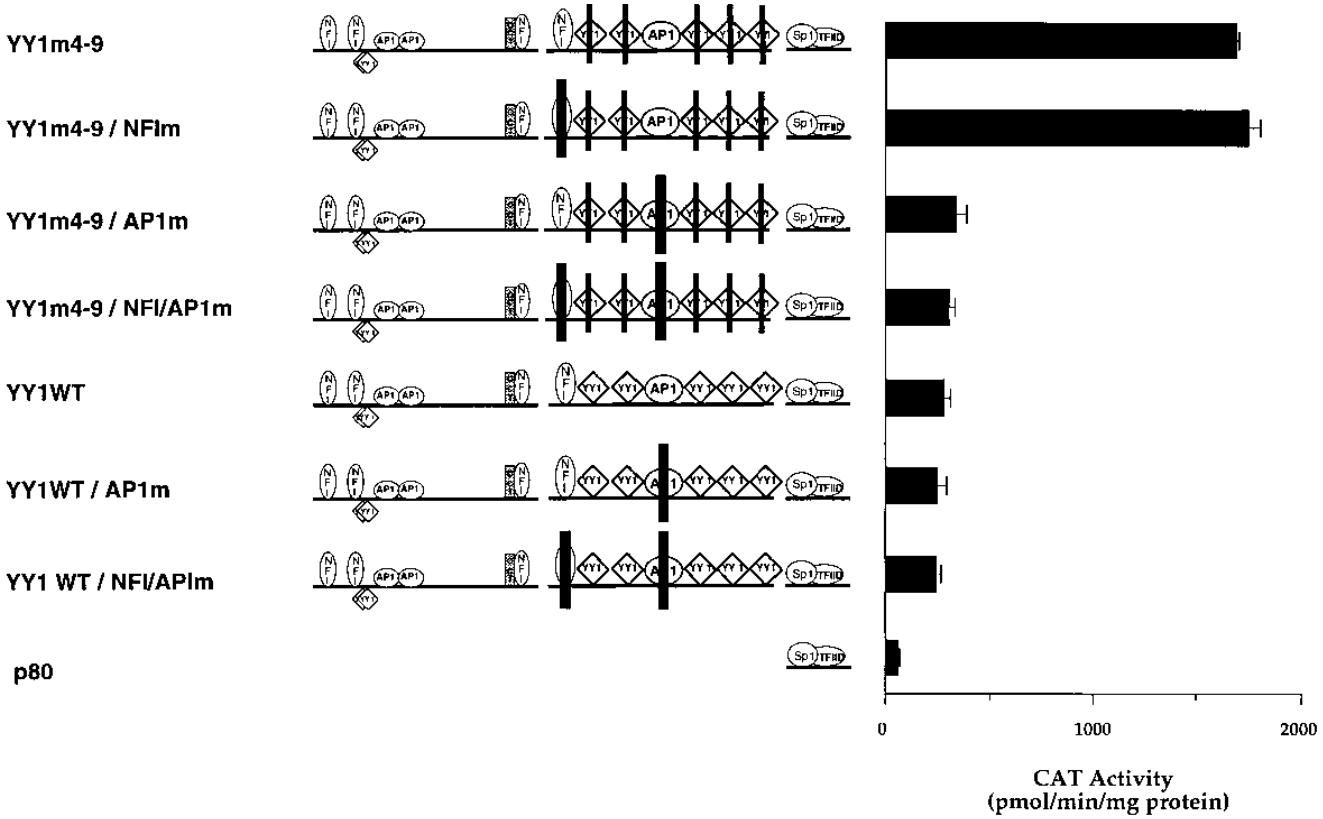

FIG. 6. The target of YY1 transcriptional repression is AP-1. The diagram shows the levels of CAT activity (given in picomoles of acetylated chloramphenicol per minute per milligram of protein extract) of wild-type (wt) p80:2e/9e and various YY1, NFI, and AP-1 mutant constructs. The high level of CAT activity, driven by the construct YY1m4-9, is not reduced when an additional mutation is introduced into the fp8e NFI site (YY1m4-9/NFIm), suggesting that the NFI site at fp8e does not contribute to transcriptional activation. In contrast, mutation of the AP-1 site in the presence of the YY1 mutations (YY1m4-9/AP-1m) results in a sixfold decrease in activity, providing evidence that this AP-1 site has the potential to activate the E6-E7 promoter. However, when the same AP-1 site is mutated in the presence of functional YY1 sites (YY1WT/AP-1m), there is no reduction in activity compared with the p80:2e/9e (YY1WT) construct, suggesting that the activity of this AP-1 site has already been quenched by YY1.

which the transcriptional coactivator CBP was shown to mediate the activity of the AP-1 components c-Fos and c-Jun (4, 5). This, observation, coupled with the recent finding that YY1 can interact with the adapter protein p300 (39), which shares many regions of homology with CBP (3, 45), suggested to us that YY1 might also be able to interact with CBP and in doing so quench AP-1 function.

Figure 7 provides evidence that YY1 can indeed interact with CBP. To date, two separate regions of CBP have been shown to interact with CREB (17) and c-Jun (5) or with E1A, E2F (72), and c-Fos (4). These are the regions spanning amino acids 461 to 662 (termed CBP 1) and 1621 to 1877 (termed CBP 2). In the experiment shown in Fig. 7B, YY1 was translated in vitro and radiolabeled with [^{35}S]methionine. The labeled YY1 was then incubated with GST, GST-CBP 1, or GST-CBP 2 and subjected to a GST pull-down experiment (see Materials and Methods). GST-CBP 2, but not GST-CBP 1 or GST alone, resulted in the rescue of radiolabeled YY1. As a negative control, NFI/CTF-1 was also translated and labeled in vitro; as shown in Fig. 7C, it was not rescued by GST-CBP 2, in agreement with previously published data (4).

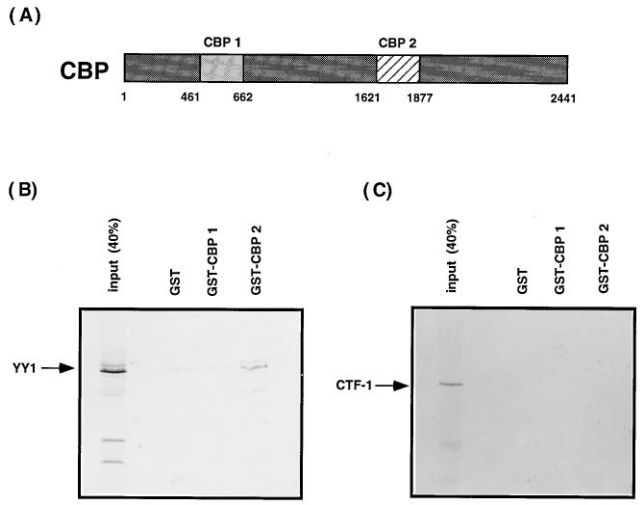

FIG. 7. YY1 binds CBP in vitro. (A) Diagrammatic representation of CBP depicting two regions previously shown to interact with CREB or c-Jun (CBP 1, amino acids 461 to 662) or with E1A, E2F, and c-Fos (CBP 2, amino acids 1621 to 1877). (B) In vitro-translated YY1 interacts with GST-CBP 2. Incubation of GST, GST-CBP 1, and GST-CBP 2 with in vitro-translated and radiolabeled YY1 was subjected to GST pull-down. GST-CBP 2, but not GST-CBP 1 or GST alone, rescues the radiolabeled YY1 protein. (C) In vitro-translated and radiolabeled NFI/CTF 1 does not interact with GST-CBP 1 or GST-CBP 2.

DISCUSSION

The multifunctional transcription factor YY1 has been shown to be an important regulator of both cellular and viral gene expression. One suggested role for YY1-mediated repres-

sion is in viruses that are involved in persistent or latent infection, including AAV (67), Epstein-Barr virus (50), herpes simplex virus type 1 (12), human cytomegalovirus (43), and HPVs (6, 47). In many of these cases, YY1 serves to keep viral gene expression at low levels until a specific trigger or phase in the viral life cycle when relief of YY1 repression occurs, often mediated by other transcription factors. For example, the production of E1A in AAV leads to both the relief of YY1 repression and transactivation of the P5 promoter (67). Another mechanism has been proposed for the regulation of the BZLF1 gene of Epstein-Barr virus (50) in which serum-responsive factor (SRF) may displace YY1 from its binding site, leading to a switch from repression to activation and a concomitant switch from latency to the lytic pathway (14). The antagonism between SRF and YY1 binding has also been identified in a number of cellular genes (32, 41, 74), including c-*fos*, and may arise from the similarities in their DNA binding requirements (SRF binds to the sequence 5′-CCATATTAGG-3′) (60).

The role of YY1 in the regulation of HPV is less clear. YY1 has previously been reported to down-regulate the expression of the HPV oncogenes E6 and E7 in the two viruses most commonly associated with cervical cancer, HPV-16 and HPV-18. Such repression may be important to maintain low levels of E6 and E7 in cells in which the oncoproteins are not required, i.e., in mitotically active basal epithelial cells. However, at present, the biological role of YY1-mediated regulation in HPV cannot be stated with any certainty, nor is it known if any mechanism exists that can alleviate YY1 repression in a way analogous to those described above. What is clear, however, is that mutations in YY1 sites that result in elevated levels of E6 and E7 expression may have important clinical ramifications.

In this study, we have analyzed the YY1-mediated repression of the HPV-16 E6-E7 promoter. We have shown that of all the sites demonstrated to bind YY1 protein, only a cluster of five sites, located in the vicinity of an AP-1 site, are responsible for repressing the HPV-16 P97 promoter. We have also provided evidence that all five sites are required for repression, the mutation of any one site giving rise to a four- to sixfold increase in transcriptional activity. Finally, we have demonstrated that the target for YY1-mediated repression is AP-1 and that YY1 can bind the transcriptional coactivator CBP, which is required for AP-1 function.

Three of the five YY1 sites in the fp9e region have not previously been described. Of the two sites identified by May et al. (47), one (site 5) has been found to be mutated in naturally occurring HPV-16 episomes associated with cervical carcinoma. At present it is not clear why all five YY1 sites should be required for the repression of AP-1. We can think of two possible explanations for this observation. The first is that all five sites may need to be bound by YY1 before the repression of AP-1 activity takes place. The second possibility is that the cluster of low-affinity sites surrounding AP-1 could lead to an effective decrease in the dissociation of YY1 from this region. If this were the case, not all sites would necessarily need to be bound by YY1 simultaneously in order to repress AP-1 activity, as it might be sufficient, for example, to have a single YY1 protein bound on either side of the AP-1 factor. According to this model, the loss of any one of these sites would be sufficient to decrease the local affinity for YY1 below a certain threshold level. We are currently investigating these two possibilities. Whatever the reason, the consequence is a number of potential target sequences which, if mutated to prevent YY1 binding, will result in the loss of repression and elevated levels of E6-E7 expression.

AP-1 also appears to be the target of YY1-mediated repression in HPV-18 (6). Bauknecht et al. (6) identified a single YY1 site downstream of an AP-1 motif, which was present on a 221-bp fragment that contained no obvious transcriptional activity. The AP-1 motif had previously been shown to activate a thymidine kinase promoter in a TPA-inducible manner once removed from the HPV-18 context (26). This led to the proposal that YY1 probably inactivated AP-1 (especially since the AP-1 motif was the only obvious activator on the 221-bp fragment) (6). Significantly, it is the homologous AP-1 motif that is repressed in both HPV-16 and HPV-18 and whose position at the 3′ end of the enhancer is conserved in virtually all genital HPVs (56). Moreover, surrounding the HPV-18 AP-1 site are not one but six potential YY1 sites. Clusters of four or more potential YY1 sites are also found surrounding the equivalent AP-1 motif in HPV-31 and HPV-35 (57), two more high-risk viruses that are also associated with cervical cancer, suggesting that the same mechanism could occur in a number of HPVs. The association of YY1 sites with AP-1 motifs is not limited to HPVs, however. For instance, recent data provide a link between AP-1 activation and YY1 repression in the granulocyte-macrophage colony-stimulating factor gene promoter (78). Another example is the c-*fos* promoter, which not only is regulated by an overlapping YY1 and serum response element (53) but also has a nearby AP-1 recognition motif. While there are at present no published data describing the interaction of YY1 and AP-1 in either the granulocyte-macrophage colony-stimulating factor gene promoter or c-*fos*, the evidence provided in this report raises the possibility that AP-1 is a common target for YY1 repression.

There are a number of examples, mostly involving SRF, in which the mechanism of YY1 transcriptional repression involves the steric inhibition of activator binding to its recognition sequence (32, 41, 74). The results presented in this study provide a novel and alternative model for the mechanism of YY1-mediated repression of AP-1 activity, in which YY1 represses AP-1 function by binding and inactivating the cofactor CBP.

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