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A Switch Region Determines the Cell Type-Specific Positive or Negative Action of YY1 on the Activity of the Human Papillomavirus Type 18 Promoter

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YY1 is a zinc finger transcription factor which acts as either a repressor or an activator dependent on the promoter context. YY1 is a potent activator of the genuine human papillomavirus type 18 (HPV-18) upstream regulatory region (URR) in HeLa cells, which are known for high-level expression of the HPV-18 early genes. The activating activity of YY1 is dependent on the presence of a newly identified switch region located upstream of the YY1 binding site. Deletion of this region causes YY1 to act as a repressor of HPV-18 promoter activity. In vivo footprinting of the HPV-18 URR and an in vitro electrophoretic mobility shift assay identified proteins binding to the switch region. Site-directed mutagenesis of the switch region and YY1 binding sites suggests that these two regions work in concert to yield high-level HPV-18 URR activity in HeLa cells but not in HepG2 cells, where HPV-18 is almost inactive. These data identified a novel mode of cell type-specific regulation of HPV-18 promoter activity by positive or negative action of YY1, determined by the switch region binding factor(s).

Only a subset of 73 distinct human papillomavirus (HPV) types identified to date, most commonly, high-risk HPV type 16 (HPV-16) and HPV-18, are found to be associated with the development of about 90% of cervical cancers (75). In cervical cancers and derived cell lines, the HPV DNA is usually integrated into the host genome, often disrupting the E1 and E2 genes, resulting in deregulated expression of the E6 and E7 genes (4, 57, 62). The E6 and E7 genes encode oncoproteins that act synergistically in the immortalization of primary human genital epithelial cells (24, 41). The immortalizing activities of both viral proteins appears to be partially accounted for by their ability to interfere with functions of the p53 protein and the retinoblastoma tumor suppressor gene product, pRB (15, 25, 42, 55, 71). Inactivation of p53 and pRB-mediated cell growth control by the E6 and E7 proteins, respectively, are believed to be important steps in cervical carcinogenesis (27, 70).

It has been postulated that because of loss or inactivation of negative cellular control mechanisms, activation of E6 and E7 gene expression is one essential event in the multistep process of HPV-linked cervical carcinogenesis (73, 74) and that some HPV-infected cells in vivo progress to malignancy over a period of many years because of the mutational activity of E6-E7 oncogene expression (75).

Active expression of HPV-18 E6 and E7 is regulated by the upstream regulatory region (URR). Transcription of HPV-18 E6 and E7 initiates at nucleotide 105 (P_{105}) immediately upstream of the E6 open reading frame (56, 64). In recent studies, it was shown that the HPV-18 P_{105} promoter exhibits stringent cell type specificity and functions only in certain human epithelial cells (6, 8, 18, 34, 45, 64). Binding sites for several nuclear proteins, e.g., AP-1, KRF-1, Oct1, and YY1, have been identified by in vitro DNase I footprinting analyses (6, 17, 34, 52). It has been shown that AP-1, KRF-1, and Oct1 contribute to the enhancer function of HPV-18 (26, 34, 45, 65) whereas YY1 acts as a repressor of HPV-18 promoter activity (6).

YY1 was recently cloned by Shi et al. (60) and described as a human Krüppel-related zinc finger protein that interacts with a negative regulatory element at −60 (the P5 −60 site) and at the transcription initiation region (the P5 +1 site) of the adeno-associated virus (AAV) P5 promoter. YY1 is widely expressed and highly conserved among mammalian species and thus can be categorized as a ubiquitous transcription factor (23, 53, 60). YY1 was also cloned and described as NF-E1, which binds to the immunoglobulin kappa 3′ enhancer and to the μE1 site in the immunoglobulin heavy-chain enhancer, where it may act as a repressor or an activator (46). Furthermore, YY1 was described as a positively acting factor, δ, that binds to downstream *cis* elements of the ribosomal protein L30 and L32 genes (23) and as the negative transcription factor UCRBP, which binds to the upstream conserved region core in the long terminal repeat of the Moloney murine leukemia virus (16). YY1 corresponds to the factor termed common factor 1, which binds to two sites in the c-*myc* promoter, to the μE1 site of the immunoglobulin H enhancer, and to the downstream CBAR site of the skeletal α-actin promoter (49). YY1 regulates expression of the human c-*fos* promoter by competition with serum response factor SRF for binding to the serum response element (19), to CArG boxes of skeletal α-actin promoters (19, 32), and to a CArG motif of the creatine kinase-M gene (69). Another function of YY1 may be represented by its ability to bend DNA of the mouse c-*fos* promoter (43). It also seems to regulate expression of human fetal globin (20, 21, 47), rabbit α-globin (72), and the β-casein genes (38, 48). On the other hand, it was recently shown that the c-*myc* promoter is activated by cotransfected YY1 (50) and that YY1 confers enhancer activity by binding to an intracisternal A-par-

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ticle upstream enhancer (54). YY1 also functions as an initiator of transcription, as shown by Seto et al. (59), upon binding to a transcriptional initiator element. An example is the cytochrome *c* oxidase subunit Vb promoter, where YY1 may act as an initiator protein (5). A direct protein-protein interaction of YY1 and Sp1 has recently been shown suggesting novel modes of regulation among sequence-specific DNA-binding transcription factors (31, 58). Previously it was shown that YY1 can function like a TATA-binding protein (TBP) (66). Association between YY1 and c-Myc inactivates both the repressor and activator functions of YY1 (61), and protein-protein interaction between YY1 and nucleolar phosphoprotein B23 has recently been shown to be responsible for relief of YY1-induced transcriptional repression (29). In addition, YY1 represses the BZLF1 gene of the Epstein-Barr virus (39) and human immunodeficiency virus type 1 transcription and virion production in vivo (35), emphasizing the important role of YY1 in regulating viral gene expression.

We have previously identified a YY1 binding site in the HPV-18 promoter (6). The 221-bp proximal promoter fragment of the HPV-18 URR is completely inactive despite the presence of an AP-1 binding site (6). YY1 binds to OL13, a negative regulatory domain in the HPV-18 promoter located between positions −125 and −82 (6). OL13 functions as a ubiquitous silencer which represses basal enhancer activity, as well as tetradecanoyl phorbol acetate (TPA) induction mediated by AP-1, when fused to the heterologous *tk* promoter (6). Recently, mutations of YY1 recognition sequences were found in extrachromosomal HPV-16 DNA (37). It has been suggested that mutations of YY1 recognition sequences permit the virus to escape cellular control.

In the present study, the functional role of YY1 was examined in the context of the authentic HPV-18 URR. Transfection studies of the HPV-18 URR with mutated YY1 binding sites revealed a cell type-specific and context-dependent activity of YY1. In HeLa cells, YY1 acts as an activator of the HPV-18 URR. This is in contrast to the results observed with the proximal promoter fragment (positions −221 to −2), where YY1 acts as a repressor (6; this study), suggesting that proteins binding to regions outside of the proximal promoter fragment may determine the mode of YY1 function. Indeed, we identified such a region, termed the switch region, that is responsible for the diverse function of YY1. In vivo genomic footprinting studies of the HPV-18 URR in HeLa and C4I cells demonstrate protein binding to this switch region and to YY1 binding site OL13. The specificity of this interaction was confirmed by electrophoretic mobility shift assay (EMSA) with HeLa and HepG2 nuclear extract. The ability of YY1 to repress the proximal promoter fragment of the HPV-18 URR is changed by its functional interaction with the switch region binding factor. As a result, YY1 and the switch region binding pro-
tein work in concert to activate the HPV-18 URR in HeLa cells. Mutations of both binding sites lead to complete loss of HPV-18 URR activity. YY1-switch region binding factor interactions may therefore be responsible for cell type-specific HPV-18 promoter activity and are likely to be key regulators of expression of tumor-promoting HPV oncoproteins E6 and E7.

MATERIALS AND METHODS

Plasmid constructions. Cloning of the HPV-18 URR fragment (nucleotides [nt] 6930 to 103) into chloramphenicol acetyltransferase (CAT) vectors pBLCAT2 and pBLCAT3 to construct plasmids p18URRtk and p18URR, respectively, has been previously described (6).

OL13, the YY1 binding and silencer element of the HPV-18 promoter (6), was mutated and introduced into the HPV-18 URR by PCR amplification as follows. To construct p18URR-M1tk and p18URR-M1 containing the mutated OL13-M1 sequences (see Fig. 1), plasmid p18URRtk or p18URR and internal primers $P_{13M1\text{-}A}$ (5′-CCGCGGTGAATTCAGCGAACAATTGTAGTATATAAAAAAGGG-3′; sense strand) and $P_{13M1\text{-}B}$ (5′-TTCGCTGAATTCACCGCGGGTATAGTATGTGCTGCCCAA-3′; antisense strand) were mixed with primers CAT_1 (5′-TTTTCTCCATTTTTAGCTTCCTTAGCTCCTG-3′; antisense strand), which binds in the CAT gene, and C_1 (5′-GTAACGCCAGGGTTTTCCCAGTCAC-3′; sense strand), which binds to vector sequences 5′ of the HPV-18 sequences, respectively. Amplification was performed at 94, 50, and 72°C for 2 min at each temperature for 30 cycles. The purified products were mixed, and amplification was repeated with primers C_1 and CAT_1, except that annealing of the primers was performed at 94°C for 1.5 min, 45°C for 2 min, and 72°C for 3 min. The resulting products were digested with *Hin*dIII and *Bam*HI and inserted into the corresponding sites of pBLCAT2 and pBLCAT3, respectively.

5′ deletions of the HPV-18 URR and the corresponding HPV-18 URR mutation URR-M1 were constructed as follows. 5′ deletions p18URR-380.w (wild-type sequences) and p18URR-380.M1 (mutation OL13-M1) were generated by digestion of p18URRtk and p18URR-M1tk, respectively, with *Bgl*I, which cuts in the HPV-18 URR at position −380 relative to the transcription start site (the first proximal nucleotide of the URR [nt 104] was referred to as nt −1 for simplicity [see Fig. 1]), filled in with DNA polymerase I (Klenow fragment), and subsequently digested with *Bam*HI. These fragments were isolated and religated into pBLCAT3, which was digested with *Xba*I, filled in, and cut with *Bam*HI. 5′ deletions p18URR-335.w and p18URR-335.M1 were generated by digestion of p18URR-380.w and p18URR-380.M1, respectively, with *Hin*dIII and *Xho*I. The 380-bp 5′ deletion fragments of the HPV-18 URR (wild type, M1) were isolated, digested with *Bst*NI, which cut at nt −335 of the HPV-18 URR fragments, filled in with the Klenow enzyme, subsequently digested with *Bam*HI, and religated to *Xba*I-filled-in and *Bam*HI-digested pBLCAT3. 5′ deletions p18URR-275.w and p18URR-275.M1 were generated by PCR amplification. Plasmids p18URR-tk and p18URR-M1tk, respectively, were mixed with primers P_{-275} (5′-GTAATAAAACTGCAGTTAGGCACA-3′; sense strand) and CAT_1, and amplifications were performed as described above at 50°C. The resulting products were digested with *Pst*I and *Bam*HI, purified, and inserted into the corresponding sites of pBLCAT3. 5′ deletions p18URR-251.w and p18URR-251.M1 were generated as −275 deletions by using primer P_{-251} (5′-CATATTTTAGTCTGCAGTTACTTAAG-3′). 5′ deletions p18URR-243.w and p18URR-243.M1 were generated as −275/−251 deletions by using primer P_{-243} (5′-GTTTTTACTGCAGCTAATTGCA-3′). 5′ deletions −237, −232, and −228 will be described in detail elsewhere. Previously, we described cloning of the proximal fragment of the HPV-18 URR (nt 7740 to 103) into pBLCAT2 and pBLCAT3, resulting in plasmids p18-Itk and p18-I, respectively (6). The proximal promoter fragment of the HPV-18 URR is a 5′ −221 deletion. We recloned this proximal promoter fragment, referred as 5′ deletion −221, to ensure identical 3′ ends as obtained with the various 5′ deletions described above. 5′ deletions p18URR-221.w and p18URR-221.M1 were generated by digestion of p18URR-335.w and p18URR-335.M1, respectively, with *Hin*dIII and *Bam*HI. The 335-bp 5′ deletion fragments of the HPV-18 URR (wild type, M1) were isolated and digested with *Rsa*I, which cut at nucleotide position −221 of the HPV-18 URR fragments. After isolation, these fragments (5′ deletions −221.w [wild type] and −221.M1 [OL13-M1]) were religated to filled in *Xba*I- and *Bam*HI-digested pBLCAT3.

To introduce mutation OL22-M1 (see Figure 5; mutations are between positions −251 and −246) into the HPV-18 URR, PCR amplifications were performed. To construct p18URR-22M1-13w (mutated OL22-M1, wild-type OL13) and p18URR-22M1-13M1 (mutated OL22-M1, mutated OL13-M1), plasmids p18URR and p18URR-M1 (mutated OL13-M1), respectively, and internal primers $P_{22M1\text{-}A}$ (5′-TAGTTTGTTTCCGCGGAAGCTAATTGCATA-3′; sense strand) and $P_{22M1\text{-}B}$ (5′-GCAATTAGCTTCCGCGGAAACAAACTAAAAT-3′; antisense strand) were mixed with CAT_2 (5′-GCTCCTGAAAATCTCGCCAAGCTC-3′, antisense strand), which binds in the CAT gene, and with primer C_1 (described above), which binds to vector sequences 5′ of the HPV-18 sequences, respectively. Amplification was performed as described above for plasmid p18URR-M1. After the second round of amplification, the resulting products were digested with *Hin*dIII and *Bam*HI and inserted into the corresponding sites of pBLCAT3, respectively.

5′ deletions p18URR-251(22M1/13.w) (mutated OL22-M1, wild-type OL13) and p18URR-251(22M1/13.M1) (mutated OL22-M1, mutated OL13-M1) were constructed by PCR amplification. Plasmids p18URR-22M1-13w and p18URR-22M1-13M1, respectively, were mixed with primers $P_{-251(22M1)}$ (5′-CATATTTAGTCTGCAGCCGCGGAAGC-3′; sense strand) and CAT_2, and amplification was performed at 50°C. The resulting products were digested with *Pst*I and *Bam*HI, purified, and inserted into the corresponding sites of pBLCAT3.

To construct p18URR-31M1, containing the mutated OL31-M1 sequences (see Fig. 1), plasmid p18URRtk and internal primers $P_{31M1\text{-}A}$ (5′-CCTCCACCGCGGTGTGCAACCGATTTCGGTTGCCTTTGG-3′; sense strand) and $P_{31M1\text{-}B}$ (5′-TTGCACACCGCGGTGGAGGATTGTAGGATAAAATGGATG-3′; antisense strand) were mixed with primers CAT_1 and C_1, respectively, as described above for construct p18URR-M1. After two rounds of amplification, the resulting products were digested with *Hin*dIII and *Bam*HI and inserted into the corresponding sites of pBLCAT3.

To construct p18URR-21M1, containing the mutated OL21-M1 sequences (wild-type AP-1, mutated YY1) (see Fig. 1), plasmid p18URRtk and internal primers $P_{21M1\text{-}A}$ (5′-AGTCACCGCGGTGTCCAGGTGCGCTACAACAATT GCTTGC-3′; sense strand) and $P_{21M1\text{-}B}$ (5′-GCACCTGGACACCGCGGTG ACTAATACCAGGTGCGCCTT-3′; antisense strand) were mixed with primers CAT_1 and C_1, respectively, as described above for construct p18URR-31M1. After two rounds of amplification, the resulting products were digested with *Hin*dIII and *Bam*HI and inserted into the corresponding sites of pBLCAT3. All of the HPV-18 plasmids were sequenced across the internal mutations and 5′ deletions.

Cell culture, transfections, and CAT assays. HeLa, HepG2, and C4I cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. HeLa and HepG2 cells were transfected by the calcium phosphate coprecipitation method (9). Transfections were performed as previously described (6) and were done with 3 to 18 μg of the various CAT gene-containing reporter constructs and 1 to 2 μg of the RSV/L, a luciferase gene under control of the Rous sarcoma virus (RSV) long terminal repeat (12). The amount of DNA used is given in the figure legends. CAT assays were performed by using equal luciferase counts. CAT activities were quantified by cutting spots from thin-layer chromatography plates and determining the radioactivity by liquid scintillation. Results from individual transfections varied by less than 20%.

For generating the HeLa cell clone containing a single copy of the HPV-18 URR-CAT plasmid (clone 13), cells were transfected as described above by using 5 μg of the CAT plasmid and 0.5 μg of SV40-neo. At 48 h after transfection, selection of individual cell clones was started in culture medium containing G418 (final concentration, 600 μg/μl). The number of correctly integrated copies of the CAT construct was determined by Southern blot analysis.

Genomic footprinting. Cells were washed with phosphate-buffered saline and subsequently incubated in 0.5% dimethyl sulfate (DMS) in Dulbecco's modified Eagle's medium–10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.5) at room temperature for 2 min. After being washed twice with phosphate-buffered saline, genomic DNA was prepared (22). In parallel, protein-free genomic DNA was methylated in vitro for 30 s. Piperidine cleavage of both in vitro and in vivo methylated DNAs was performed as described elsewhere (36). Subsequently, selective amplification of the cleavage products by ligation-mediated PCR was performed (40). The oligonucleotide primers of the coding strand of the HPV-18 promoter were as follows: 1, 5′-CTCACATCTTTTATA TACACCG-3′; 2, 5′-TACACCGTTTTCGGTCCCGACC-3′; 3, 5′-CGGTCC CGACCGTTTTCGGTCACTCCC-3′. The primers for the noncoding strand were as follows: 1, 5′-CCTGGTATTAGTCATTTTCCTGTC-3′; 2, 5′-GTCAT TTTCCTGTCCAGGTGCGCTAC-3′; 3, 5′-CCTGTCCAGGTGCGCTACAA CAATTGCTTGC-3′. To determine protein binding to the HPV-18 URR-CAT construct (HeLa clone 13), primer 1 was exchanged against a primer with the sequence 5′-GCTCCTGAAAATCTCGCCAAGCTC-3′, which specifically binds to the CAT sequences. PCRs were performed in a Cetus-Perkin Elmer TempCycler with the following program: 1 min at 94°C, 2 min at 63°C, and 3 min at 72°C for 20 cycles. After addition of end-labelled primer 3, denaturation for 2 min at 95°C, and annealing for 2 min at 66°C, a single extension step of 10 min at 75°C was performed. Amplification products were resolved on a 6% standard sequencing gel (36).

EMSAs. EMSA reactions with bacterially expressed, purified protein YY1 or Jun were performed with a buffer containing 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 5 mM dithiothreitol, 4% glycerol, and an end-labelled oligonucleotide probe. The bacterial lysate protein preparations were mixed in 25-μl reaction mixtures and incubated on ice for 10 min. For depletion experiments, anti-YY1 monoclonal antibodies or heterologous monoclonal antibodies, recognizing unrelated HPV-11 protein L1 (kindly provided by Martin Müller), were added to the reaction mixture and incubated for a further 10 min at 4°C. Binding reactions with HeLa and HepG2 nuclear extracts, usually 10 μg, were performed with a reaction mixture containing 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 2 mM dithiothreitol, 4% glycerol, 0.5 μg of poly(dI-dC) as a nonspecific competitor, and an end-labelled oligonucleotide probe at 25°C for 10 min. For competition experiments, the reaction mixture with the proteins was incubated together with the unlabelled oligonucleotide at a 25- and a 50-fold molar excess with respect to the labelled probe to determine specific binding. Reactions were resolved on a 4% polyacrylamide gel in 0.25× TBE (1× TBE is 50 mM Tris-borate [pH 8.3] plus 1 mM EDTA) at 4°C.

Expression and purification of bacterial protein and preparation of nuclear extract. Bacterially synthesized protein HIS_6-YY1 was expressed, purified on a nickel chelate column, and renatured as previously described (6). The Jun protein was expressed in *Escherichia coli* BL21, purified by ammonium sulfate precipitation, and subjected to heparin-Sepharose chromatography (63) followed by DNA affinity chromatography using the AP-1 site of the human collagenase promoter (2). Eluted samples were dialyzed against EMSA buffer as previously described by Stein et al. (63). Crude nuclear extract was prepared essentially as described by Dignam et al. (14).

Oligonucleotides used in EMSAs. Some oligonucleotides were designed to contain *Hin*dIII sites at their 5′ ends and *Bam*HI sites at their 3′ ends, as indicated by lowercase letters. Mutations are underlined. The sequences of their double-stranded portions were as follows: P5 +1 (the initiator sequence of the AAV P5 promoter comprising the P5 +1 YY1 binding site [60]), 5′-AGG GTCTCCATTTTGAAGCGGG-3′; OL13 (the silencer and YY1 binding element of the HPV-18 URR proximal promoter fragment; positions −82 to −125 [6]), 5′-agcttGCAGCACATACTATACTTTTCATTAATACTTTTAACAATT GTAGg-3′; OL13-M1 (the mutated OL13-M1 element of the HPV-18 URR, showing no binding of YY1), 5′-agcttGCAGCACATACTATACCCGCGGTG AATTCAGCGAACAATTGTAGg-3′; OL13-M2 (the mutated OL13-M2 element of the HPV-18 URR, showing binding of YY1), 5′-agcttGCAGCAC ATATGATGCCCGCGGTTAATACTTTTAACAATTGTAGg-3′; OL21 (the AP-1 and YY1 binding element of the HPV-18 URR enhancer fragment; positions −337 to −359), 5′-agcttCTGGTATTAGTCATTTTCCTGTCg-3′; OL21-M1 (the mutated OL21-M1 element of the HPV-18 URR, showing binding of *jun* to AP-1 and no binding of YY1), 5′-agcttCTGGTATTAGTCACCGCGGT GTCg-3′; OL22 (the switch region of the HPV-18 URR enhancer fragment, showing binding of a specific switch region binding factor; positions −240 to −260), 5′-AGTTTGTTTTTACTTAAGCTA-3′; OL22mt (the mutated OL22mt element of the HPV-18 URR, showing no binding of the switch region binding factor), 5′-AGTTTGTTTTTATTTAAGCTA-3′; OL31 (the YY1 binding element of the HPV-18 URR distal fragment; positions −503 to −525), 5′-ACAA TCCTCCATTTTGCTGTGCA-3′; OL31-M1 (the mutated OL31-M1 element of the HPV-18 URR, showing no binding of YY1), 5′-agcttACAATCCTCCACC CGCGGTGTGCAg-3′; MRE (a 27-mer sequence from the human metallothionein gene II_A promoter, comprising one AP-1 binding site [51]), 5′-GAGCCG CAAGTGACTCAGCGCGGGGCG-3′.

RESULTS

YY1 binds to the distal, central, and proximal fragments of the HPV-18 URR. The HPV-18 URR (nt −824 to −1) can be digested with *Rsa*I to generate distal, central, and proximal fragments (6; Fig. 1). The 221-bp proximal promoter fragment of the HPV-18 URR is completely inactive, despite having an AP-1 binding site in OL12 (Fig. 1). We have previously shown that transcription factor YY1 binds silencer element OL13 (6). OL13 contains two TTTT stretches. Mutations were introduced either into both T motifs to generate OL13-M1 or into the 5′ T motif to create OL13-M2 (Fig. 1). OL13-M1 fails to bind YY1, and this mutation results in an increase in basal promoter activity (6; Fig. 2A). OL13-M2, on the other hand, was able to bind bacterially synthesized and purified YY1 in an EMSA (Fig. 2A). To ensure that the DNA-protein complex observed is due to YY1 binding, a monoclonal antibody against YY1 was included in the EMSA using labelled OL13 oligonucleotides as substrates. As a positive control, the P5 +1 YY1 binding oligonucleotides were used (60). As shown in Fig. 2B, the DNA-protein complexes formed on the OL13 or P5 +1 oligonucleotide were ablated by addition of the monoclonal antibody against YY1 but not by addition of an unrelated antibody. Taken together, these results demonstrate that YY1 can bind specifically to HPV-18 silencer OL13 and that OL13-M1, but not OL13-M2, can disrupt YY1 binding.

In addition to OL13, we detected at least two other putative YY1 binding sites located in the distal and central fragments of the HPV-18 URR (Fig. 1). Oligonucleotide OL31 of the HPV-18 distal fragment comprises YY1 binding motif 5′-CTC CATTTT-3′, which is identical to YY1 binding site P5 +1 of the AAV promoter (60). OL21 of the HPV-18 central fragment comprises a potential YY1 binding motif (5′-AGTC ATTTT-3′) homologous in seven of nine base pairs to the P5 −60 YY1 binding site (5′-CGACATTTT-3′) of the AAV P5 promoter. As shown in Fig. 2C (left panel), YY1 bound to OL31 and OL21 in an EMSA. Mutations of both YY1 binding sites, OL31-M1 and OL21M1 (Fig. 1), respectively, abrogated YY1 binding (Fig. 2C, left panel). However, YY1 bound to OL31 significantly more strongly than to the OL21 site (Fig. 2C). Obviously, the base change of the first nucleotide, C, in the P5 −60 motif, which is a main contact point for YY1 binding, as shown in a methylation interference analysis (60), versus an A in the YY1 binding motif of OL21 reduced YY1 binding affinity to OL21. Interestingly, the YY1 binding motif in OL21 overlaps the enhancer AP-1 site (34, 65) of the HPV-18 URR (Fig. 1). This mutation of the YY1 binding site

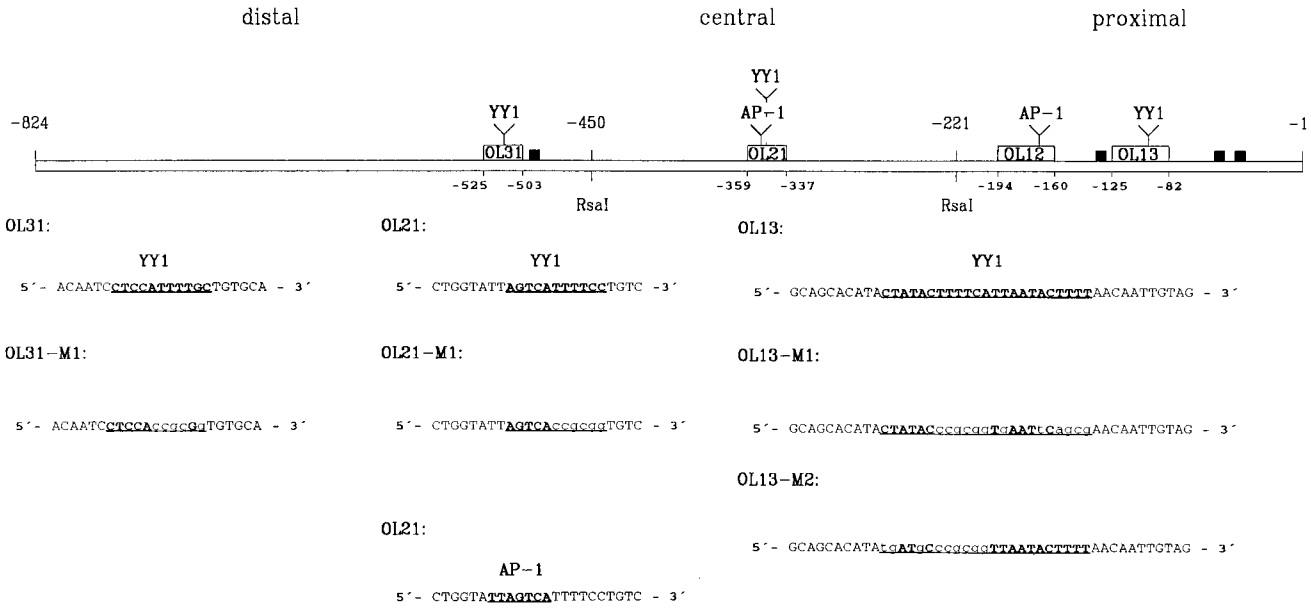


FIG. 1. Schematic representation of the HPV-18 URR. The 824-bp HPV-18 URR is located within the 1,049-bp *Bam*HI fragment of the HPV-18 genome (nt 6930 to 123). The first proximal nucleotide of the URR (nt 104) is referred to as nt −1 relative the transcription initiation site at position +1. The URR consists of 5′ distal (positions −824 to −450), central (positions −450 to −221), and 3′ proximal (positions −221 to −1) fragments. The positions of the distal YY1 site (OL31), the central overlapping AP-1 and YY1 sites (OL21), the proximal AP-1 (OL12) site, the proximal YY1 (OL13) site, and the E2 binding sites (■) are indicated. In the lower panel, wild-type and mutant sequences of three selected regions of the URR are indicated. The binding sites for YY1 and AP-1 are underlined and in boldface letters, and mutated sequences are shown by lowercase letters. OL31, comprising the distal YY1 binding element of the HPV-18 URR, is located at positions −503 to −525; OL21, the AP-1 and YY1 binding element of the central fragment is located at positions −337 to −359; OL12, the proximal AP-1 binding element, is located at positions −160 to −194; and OL13, the proximal YY1 binding element, is located at positions −82 to −125.

(OL21-M1) was determined not to affect AP-1 binding activity, which allowed us to analyze its functional activity in the context of the complete HPV-18 URR (see below). To ascertain that this mutation does not affect AP-1 binding activity, OL21 and OL21-M1 were first tested for the ability to bind the bacterially expressed and purified Jun protein (63) in an EMSA. As shown in Fig. 2C (right panel), purified Jun was able to form a complex regardless of whether oligonucleotide OL21 (com-

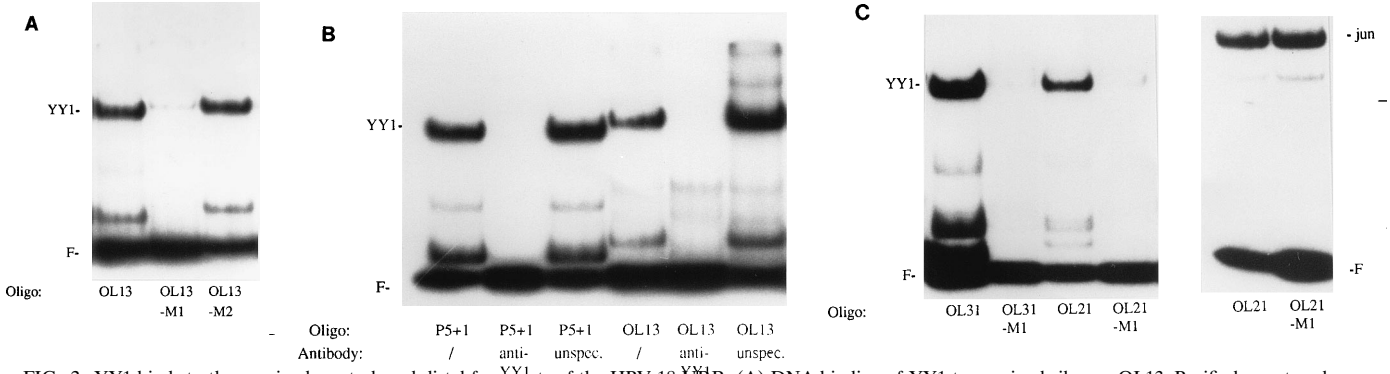


FIG. 2. YY1 binds to the proximal, central, and distal fragments of the HPV-18 URR. (A) DNA binding of YY1 to proximal silencer OL13. Purified, renatured HIS-YY1 fusion protein was incubated with ^{32}P-radiolabelled oligonucleotide (Oligo) OL13, mutated OL13-M1, and OL13-M2 (Fig. 1) of the HPV-18 promoter and used in an EMSA as indicated at the bottom. Retarded YY1-DNA complexes and the free DNA probe (F) are indicated at the left. (B) Ablation of complex formation by anti-YY1 monoclonal antibodies. The purified, renatured HIS-YY1 fusion protein was incubated with ^{32}P-radiolabelled oligonucleotide P5 +1 of the AAV P5 promoter (60) and with ^{32}P-radiolabelled YY1 binding site oligonucleotide OL13 corresponding to the sequence from positions −82 to −125 of the HPV-18 promoter. Either buffer alone or 1.5 μl of anti-YY1 or a heterologous monoclonal antibody was added to the binding reactions as indicated on the bottom, incubated at 4°C for 10 min, and subjected to EMSA analysis. The positions of the YY1 DNA-protein complex and the free DNA (F) are indicated at the left. (C) Left panel: localization of YY1 binding sites in the distal and central fragments of the HPV-18 URR. Purified, renatured HIS-YY1 fusion protein was incubated with ^{32}P-radiolabelled oligonucleotides OL31 and OL31-M1 (Fig. 1) of the HPV-18 distal fragment and with ^{32}P-radiolabelled oligonucleotides OL21 and OL21-M1 (Fig. 1) of the HPV-18 central fragment as indicated at the bottom. OL31, the distal fragment YY1 binding site (5′-CTCCATTTT-3′) of the HPV-18 URR, is identical to YY1 binding site P5 +1 of the AAV P5 promoter (60). OL21, the central fragment YY1 binding site (5′-AGTCATTTT-3′), is homologous at seven of nine base pairs to the P5 −60 YY1 binding site (5′-CGACATTTT-3′) of the AAV P5 promoter (60). Retarded YY1-DNA complexes and free DNA (F) are indicated at the left. Right panel: Jun binds to its cognate AP-1 binding site of the HPV-18 central fragment. The AP-1 binding motif overlaps the YY1 binding motif (Fig. 1). The bacterially expressed, purified Jun protein was incubated with ^{32}P-radiolabelled oligonucleotides OL21 and OL21-M1 (Fig. 1) of the HPV-18 central fragment as indicated below the lanes. Introduction of mutations into OL21-M1 was performed to inhibit YY1 binding but preserve an intact AP-1 binding site as shown in Fig. 1. Retarded Jun-DNA complexes and free DNA (F) are indicated at the right.

prising the YY1 binding site of the HPV-18 central fragment) or mutated oligonucleotide OL21-M1 (where YY1 no longer binds) was used as a probe (Fig. 2C, right panel).

HPV-18 transcriptional activity is not affected by the distal and central but by the proximal YY1 binding site in a cell type-specific manner. To determine the functional roles that each of the detected YY1 binding sites may play in the context of the authentic HPV-18 URR, mutant OL31-M1, OL21-M1, and OL13-M1 sequences were introduced by site-directed mutagenesis into the HPV-18 URR. Wild-type and mutated URR fragments were cloned into plasmid pBLCAT3 upstream of the CAT gene (constructs p18URR, p18URR-31M1, p18URR-21M1, and p18URR-M1, respectively). These constructs were transiently transfected into HeLa and HepG2 cells, and extracts of transfected cells were assayed for CAT activity. Our data showed that only mutations in OL13 affected the transcriptional activity of HPV-18 URR (Fig. 3), whereas mutations in OL31 and OL21 had no effect (data not shown). This suggests that YY1 binding sites at positions −515 (OL31) and −347 (OL21) do not play a major role in regulating HPV-18 URR activity. As shown in Fig. 3A, in the context of the complete URR, mutation of the proximal YY1 binding site (OL13-M1) resulted in an increase in the basal transcriptional activity of the promoter. This observation demonstrates that YY1, binding to OL13 in the context of the HPV-18 URR, acts as a repressor and contributes to the very low level of HPV-18 URR activity in HepG2 cells. Surprisingly, in HeLa cells, introduction of mutation OL13-M1 into the HPV-18 URR (construct p18URR-M1) led to a reduction of CAT activity by about three- to fourfold compared with that of wild-type p18URR (Fig. 3B). Similar results were observed with constructs in which the HPV-18 URR was cloned upstream of the *tk* promoter in pBLCAT2. Introduction of mutation OL13-M1 (construct p18URR-M1tk) repressed the heterologous *tk* promoter CAT activity about threefold in HeLa cells (data not shown). Previously, we showed that activation of the proximal promoter in HeLa cells can be achieved by mutating the YY1 binding site (OL13-M1) (6). This plasmid was referred to as p18-I(M) (6). In HepG2 cells, however, neither OL13-M1 [p18-I(M)] nor wild-type proximal promoter sequences exhibit any transcriptional activity.

This result demonstrated that YY1 functions as a repressor of the HPV-18 proximal promoter fragment in HeLa cells but leaves us with a paradoxical situation: YY1 binding site OL13 represses HPV-18 promoter activity in HeLa cells when analyzed in the context of the proximal promoter fragment (6; Fig. 4B), but the same YY1 binding site activates the HPV-18 promoter in HeLa cells in the context of the authentic HPV-18 URR (Fig. 3B). It is possible that sequences upstream of the OL13 YY1 binding site determine the positive or negative activity of YY1.

A switch region is responsible for diverse YY1 functions. As described above, a region upstream of the proximal promoter fragment of the HPV-18 URR seems to affect the transcriptional activity of YY1. To further define this region, which we term the switch region, we constructed a series of 5′ deletion mutants of the HPV-18 promoter. Each 5′ deletion mutant was made as a set of two different plasmids which contain either the wild-type OL13 or the mutated OL13-M1 sequence. HeLa cells were transfected with 10 to 18 μg of the various CAT plasmids. As shown in Fig. 4A, 5′ deletions −380 and −335 show the same pattern of expression as observed with the complete HPV-18 URR (Fig. 3B). Introduction of OL13-M1 resulted in reduced CAT activity, in contrast to plasmids harboring wild-type OL13 sequences (the proximal YY1 binding site) (Fig. 2A). 5′ deletion construct p18URR-380.w was

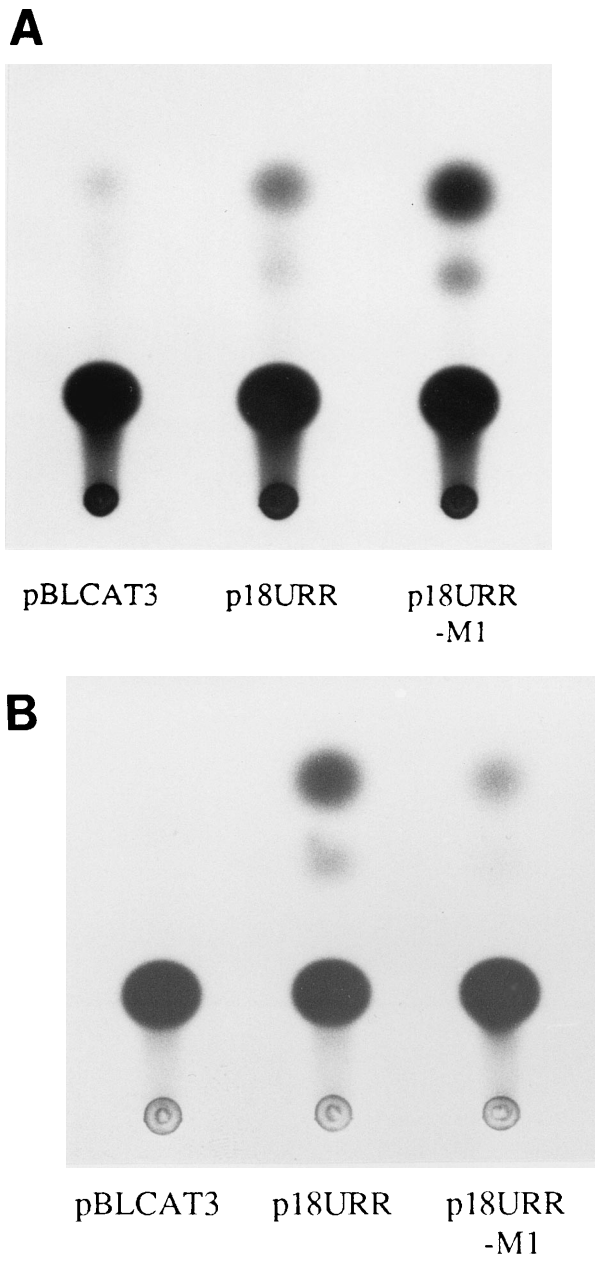


FIG. 3. YY1 functions in a cell type-specific manner, either as a repressor or as an activator of the HPV-18 promoter. (A) YY1 acts in HepG2 cells as a repressor in the context of the complete HPV-18 URR. CAT assays were carried out with extracts from HepG2 cells transfected with plasmid constructs derived from pBLCAT3, a promoterless CAT vector (33). Plasmid p18URR contains the complete HPV-18 URR fused to the CAT gene of pBLCAT3. In plasmid p18URR-M1, mutated OL13-M1 (Fig. 1) were introduced into the context of the HPV-18 URR. HepG2 cells were transfected with 18 μg of DNA from each plasmid together with 2 μg of RSV/L (12) as an internal control. CAT assays were performed for 60 min with equal luciferase counts. Relative CAT activities were determined by averaging four independent experiments quantified relative to the basal-level activity obtained with pBLCAT3, which was arbitrarily set at 1, and the result of a representative CAT assay is shown. Relative CAT activities: pBLCAT3, 1.0; p18URR, 3.0; p18URR-M1, 8.4. (B) In HeLa cells, YY1 acts as an activator in the context of the complete HPV-18 URR. CAT assays were carried out with extracts from HeLa cells transfected with plasmid constructs as described for panel A. HeLa cells were transfected with 3 μg of DNA from each plasmid together with 1 μg of RSV/L (12) as an internal control. CAT assays were performed for 15 min with equal luciferase counts. Relative CAT activities were determined as described for panel A, and the result of a representative CAT assay is shown. Relative CAT activities: pBLCAT3, 1.0; p18URR, 22.4; p18URR-M1, 7.0.

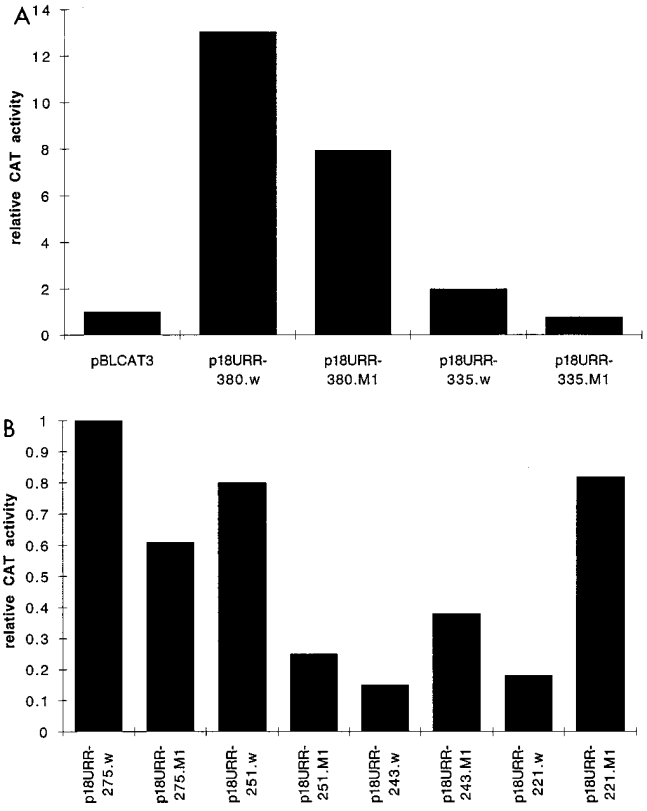


FIG. 4. 5′ deletion series of the HPV-18 URR revealing a switch element for YY1 function. CAT assays were carried out with extracts from HeLa cells transfected with the following 5′ deletion series of the HPV-18 URR containing the wild-type or mutated OL13 sequence: −380, −335, −275, −251, −243, and −221 of the HPV-18 URR (with the wild-type OL13 or mutated OL13-M1 sequence) are described in Materials and Methods. HeLa cells were transfected with 10 μg of DNA from each plasmid of the deletion −380 and −335 series together with 2 μg of RSV/L (12) as an internal control. CAT assays were performed for 60 min with luciferase calibration as described in Materials and Methods. CAT activities relative to the basal-level activity obtained with pBLCAT3 (A) are given, and they represent the means of four independent experiments. Transfections of HeLa cells with the various 5′ deletion constructs (−275, −251, −243, and −221) were carried out with 18 μg of DNA from each plasmid together with 2 μg of RSV/L (12) as an internal control. CAT assays were performed for 60 min with luciferase calibration as described in Materials and Methods. CAT activities (B) were determined by averaging five independent experiments and quantified relative to the activity obtained with p18URR-275.w, which was arbitrarily set at 1. (A) Relative CAT activities of 5′ deletion series −380 and −335: pBLCAT3, 1.00; p18URR-380.w, 13.04; p18URR-380.M1, 7.95; p18URR-335.w, 1.99; p18URR-335.M1, 0.78. (B) Relative CAT activities of 5′ deletion series −275, −251, −243, and −221: p18URR-275.w, 1.00; p18URR-275.M1, 0.61; p18URR-251.w, 0.80; p18URR-251.M1, 0.25; p18URR-243.w, 0.15; p18URR-243.M1, 0.36; p18URR-221.w, 0.18; p18URR-221.M1, 0.82.

about 35% less active than p18URR. Further deletion to nt −335 resulted in a drastic loss of activity to only about 10% of the level observed with the complete HPV-18 URR (Fig. 3B). This is likely due to removal of the AP-1 site, which was previously shown to be required for full URR activity (65). However, introduction of OL13-M1 further reduced the activity of the wild-type HPV-18 sequences about two- to threefold. These data imply that the region that is responsible for YY1's activating function is located within nt −1 to −335. Because the CAT activity of deletion mutants containing less than 335 bp (−1 to −335) of the upstream sequences declined rapidly, the following 5′ deletions were analyzed by transfection of HeLa cells with 18 μg of CAT plasmid. The result obtained with 5′ deletion −275, −251, −243, and −221 plasmid constructs harboring the wild-type OL13 or the mutated OL13-M1 sequence is shown in Fig. 4B. The 5′ deletion −275 and −251 plasmids gave a pattern of activity similar to that of 5′ deletions −335 and −380 or the complete HPV-18 URR: the presence of a functional YY1 binding site in OL13 resulted in enhanced CAT activity, which was reduced by the mutation that abolishes YY1 binding (OL13-M1). Further deletion to −243 altered the function of YY1. p18URR-243.w is the largest 5′ deletion which exhibits the same regulatory pattern as the −221 bp 5′ deletion mutant, the proximal promoter fragment of the HPV-18 URR, where YY1 acts as a repressor (6; Fig. 4B).

These results suggest the existence of a *cis*-acting element (switch region) which may serve as a binding site for a factor that determines the positive or negative action of YY1 on HPV-18 URR activity in HeLa cells. The switch region identified by this deletion mutant analysis comprises the HPV-18 URR sequence from positions −251 to −243 (5′-TTACTTA AG-3′) (Fig. 5A).

Synergistic activation of the HPV-18 URR in HeLa cells by the switch region and the proximal promoter YY1 binding site. To analyze further the function of this switch sequence in the context of the HPV-18 URR, OL22-M1 mutations (Fig. 5B) that are predicted to disrupt proteins binding to the switch region (on the basis of in vivo footprinting results described below) were introduced by site-directed mutagenesis into HPV-18 URR plasmids containing either wild-type (OL13) or mutated (OL13-M1) YY1 binding sites (p18URR-22M1-13w, mutated OL22-M1 and wild-type OL13; p18URR-22M1-13M1, mutated OL22-M1 and mutated OL13-M1). These constructs were transfected into HeLa cells for analysis of the functional involvement of YY1 and the switch region (Fig. 5C). Compared with wild-type HPV-18 URR, plasmid p18URR-22M1-13w (mutated 22-M1, wild-type OL13) (Fig. 5C) had decreased CAT activity (approximately 2.5-fold) similar to that of p18URR-M1 (wild-type OL22, mutated OL13-M1) (Fig. 3B). This result suggests that both regions of the HPV-18 URR, OL22 and OL13, contribute to HPV-18 URR activity. Alternatively, through mutation of the switch region, the switch region binding factor can no longer communicate with YY1. Consequently, YY1 acts as a repressor similar to the situation observed with the proximal promoter fragment of the HPV-18 URR (6; Fig. 4B). Introduction of mutations of both sites (OL22-M1 and OL13-M1) into HPV-18 URR resulted in complete loss of HPV-18 URR activity in HeLa cells (Fig. 5C). Similar results were observed with −251 5′ deletion plasmid p18URR-251(22M1/13.M1) (data not shown). In HepG2 cells, compared with the wild-type HPV-18 URR, plasmid p18URR-22M1-13w had approximately twofold decreased CAT activity and no change in promoter activity was observed with p18URR-22M1-13M1 (data not shown), suggesting that the switch region binding factor acts as an activator in HepG2 cells.

These results demonstrate that in HeLa cells, HPV-URR activity is completely dependent on both the switch region and YY1 binding site OL13. Furthermore, the loss of activity in construct p18URR-22M1-13M1 suggests that YY1 not only functions as an activator-repressor of the HPV-18 URR but may also act as a general factor that mediates an important contact between transcription factors such as AP-1 and TBP.

Binding of nuclear proteins to the HPV-18 URR in vivo. To determine whether the switch region of the HPV-18 URR was occupied by DNA-binding proteins, in vivo footprinting analysis using HeLa and C4I cells was performed. HeLa cells contain multiple copies of integrated HPV-18, whereas C4I, another cervical carcinoma cell line, contains one copy of

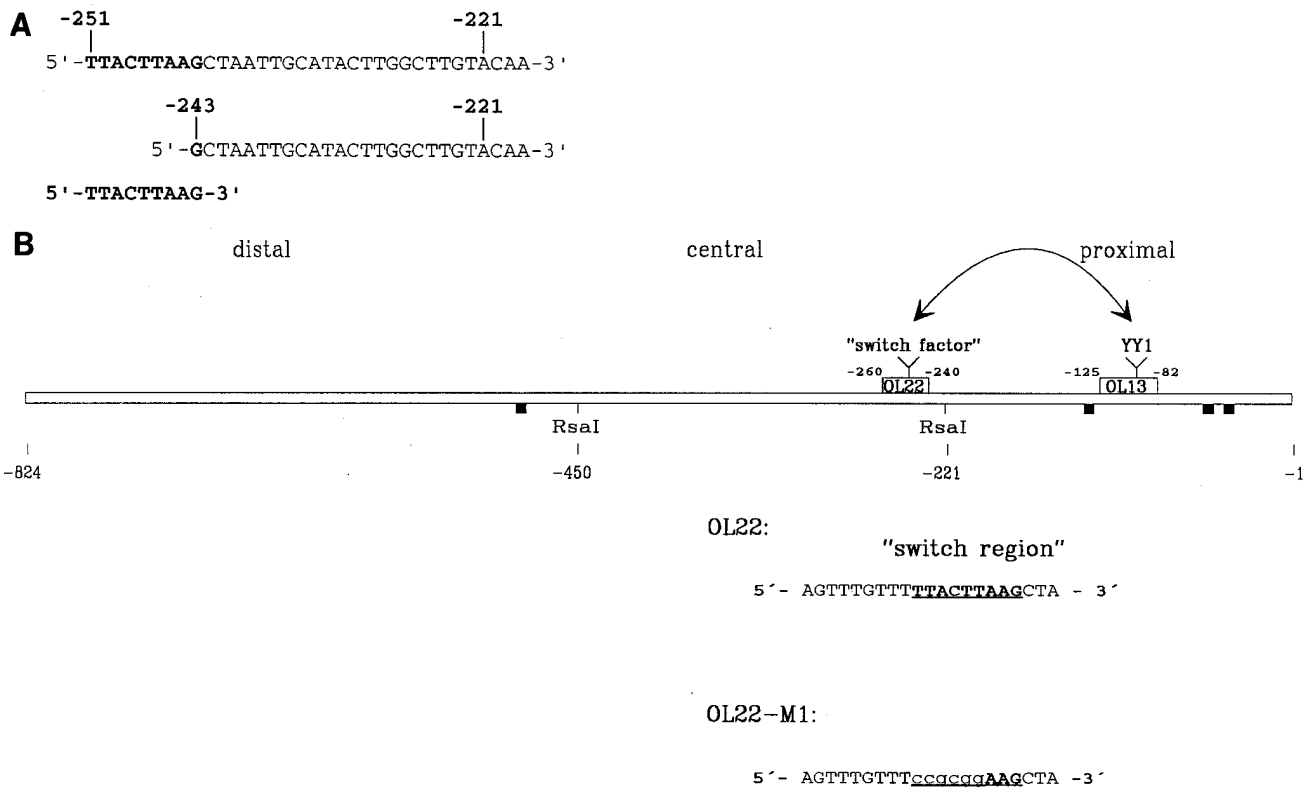
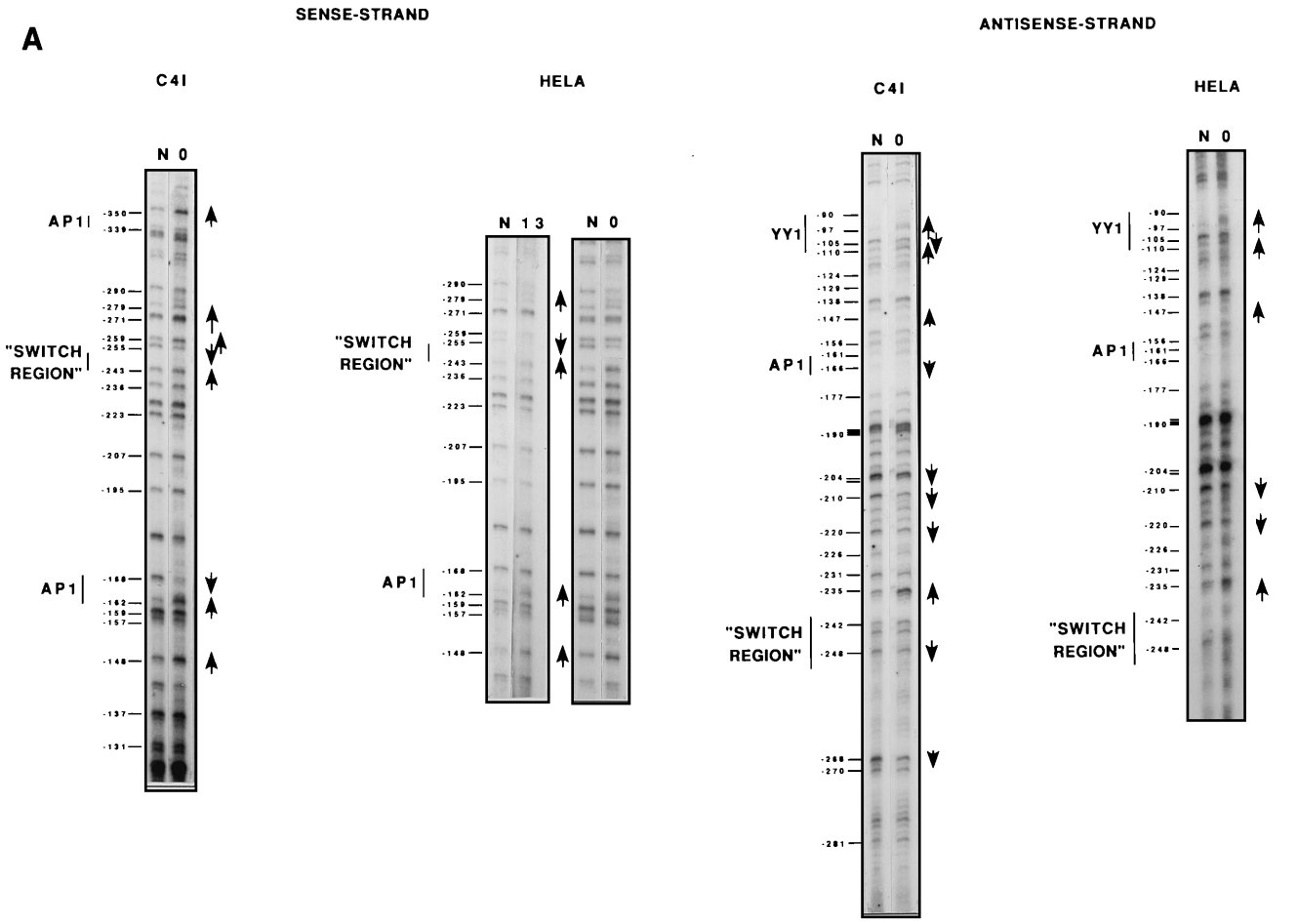


FIG. 5. Synergistic activation of the HPV-18 URR in HeLa cells by the switch region and YY1. (A) Switch region of the HPV-18 URR, determining positive or negative YY1 activity. The HPV-18 switch sequence was detected by analyzing 5′ −251 and −243 deletion constructs containing wild-type OL13 and mutated OL13-M1 sequences as shown in Fig. 4. (B) Schematic representation of the HPV-18 URR. The positions of OL22, comprising the switch sequence, and OL13, comprising the proximal YY1 binding site, are indicated. The arrow points to a functional interplay between a switch region binding factor and YY1, as deduced from the results obtained in experiments whose results are shown in Fig. 4. Black boxes indicate the relative positions of E2 binding sites. In the lower panel, wild-type OL22 and mutated OL22-M1 sequences are depicted. The switch region is underlined by bold letters, and mutated sequences are shown by lowercase letters. (C) HPV-18 URR activity in HeLa cells is dependent on a functional synergism between the switch region and YY1. CAT assays were carried out with extracts from HeLa cells transfected with plasmids derived from pBLCAT3. Plasmids p18URR and p18URR-M1 are described in Fig. 3A and were used to introduce mutation OL22-M1 by PCR amplification, as described in Materials and Methods, to construct plasmids p18URR-22M1-13w (mutated OL22-M1 and wild-type OL13) and p18URR-22M1-13M1 (mutated OL22-M1 and mutated OL13-M1). HeLa cells were transfected with 5 μg of DNA from each plasmid together with 1 μg of RSV/L (12) as an internal control. CAT assays were performed for 20 min with equal luciferase counts. Relative CAT activities were determined by averaging four independent experiments and quantified relative to the activity obtained with p18URR, which was arbitrarily set at 1. The result of a representative CAT assay is shown. Relative CAT activities: p18URR, 1.00; p18URR-22M1-13w, 0.41; p18URR-22M1-13M1, 0.02.

HPV-18 DNA integrated into the genome (57). Figure 6A shows the pattern of DMS reactivity of guanines of both the coding and noncoding strands of the URR analyzed by the ligation-mediated PCR technique (40). The cleavage pattern of DNA methylated in vitro (N) and that of DNA methylated in vivo (O) showed specific differences representing areas of protein-DNA interaction. On the sense strand, DNA-protein interaction across the proximal AP-1 site is reflected by an increase in DMS protection of guanine −168 (visible only in C4I cells) and by a strong hyperreactivity of guanine −162 (in both cell lines), which is located just 3′ of the AP-1 core sequence. On the antisense strand, a weak increase in DMS protection of guanine −166 was visible in C4I cells. Binding of factors to the distal AP-1 site was reflected by strong hyperreactivity of guanine −350 on the sense strand. Binding of YY1 to OL13 in the URR was seen on the antisense strand, as indicated by the hyperreactivity of guanines −110 and −97 in both cell lines and protection of guanine −105 in C4I cells. These changes are summarized in Fig. 6B. Binding of other, as-yet-unidentified factors was visible by DMS protection and the hyperreactivity of guanines −279, −272, −271, and −148 of the sense strand and −268, −235, −220, −210, and −204 of the antisense strand. Most importantly, binding to the switch region on the sense strand was clearly visible by the hyperreactivity of guanine −243 in both cell lines and −259 in C4I cells, located at the 3′ border and 7 nt upstream of the switch region, respectively. In addition, an increase in DMS protection at guanine −255 was visible in both cell lines. On the noncoding strand, DMS protection was visible in C4I cells at guanine −248 located in the center of the switch region. To determine whether similar protein binding patterns can be observed for the URR promoter constructs which were used for identification of the switch region by transfection analysis (Fig. 4), in vivo footprinting analysis was performed with a


A
SENSE-STRAND
ANTISENSE-STRAND
C4I
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C4I
HELA
N 0
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AP1
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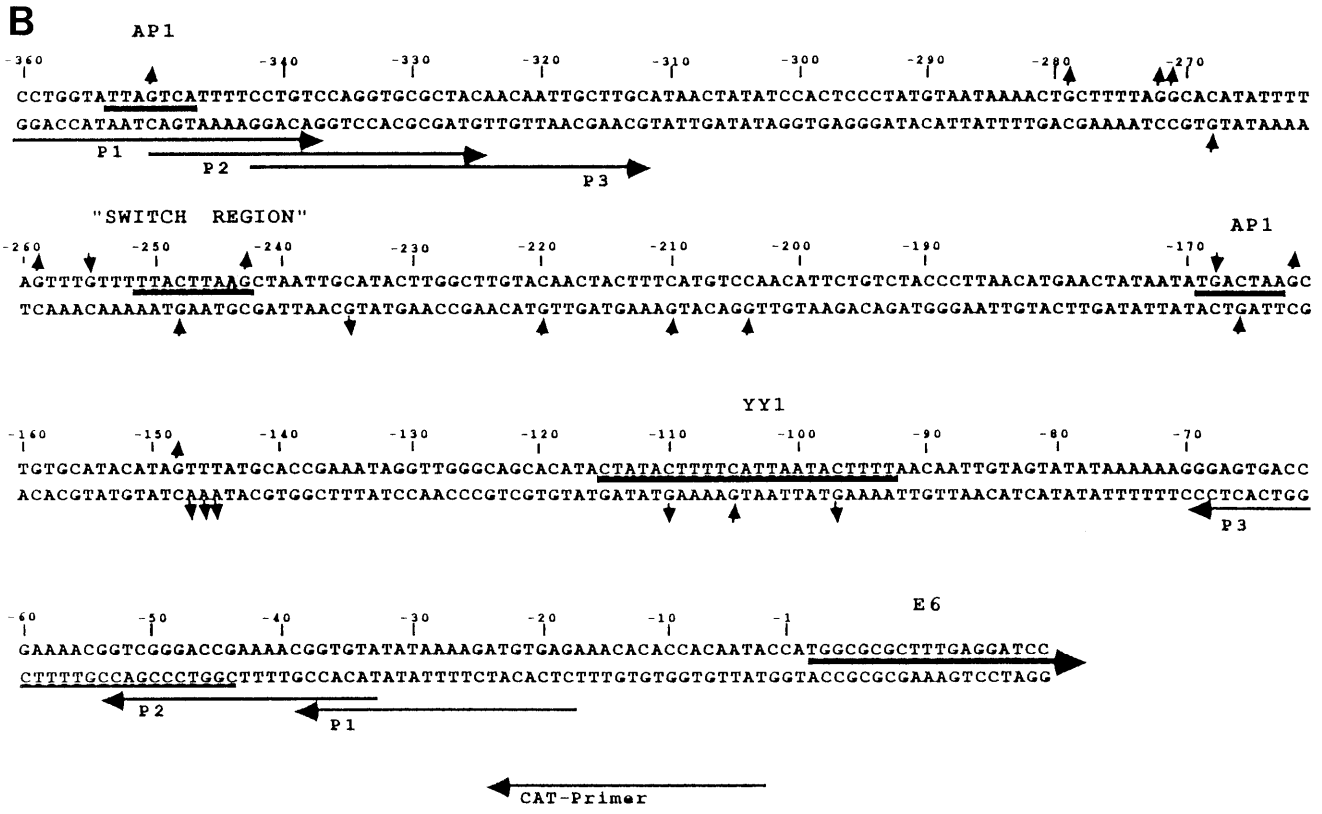
B
AP1
CCTGGTATTAGTCATTTTCCTGTCCAGGTGCGCTACAACAATTGCTTGCATAACTATATCCACTCCCTATGTAATAAAACTGCTTTTAGGCACATATTTT
GGACCATAATCAGTAAAAGGACAGGTCCACGCGATGTTGTTAACGAACGTATTGATATAGGTGAGGGATACATTATTTTGACGAAAATCCGTGTATAAAA
P1
P2
P3
"SWITCH REGION"
AP1
AGTTTGTTTTTACTTAAGCTAATTGCATACTTGGCTTGTACAACTACTTTCATGTCCAACATTCTGTCTACCCTTAACATGAACTATAATATGACTAAGC
TCAAACAAAAATGAATGCGATTAACGTATGAACCGAACATGTTGATGAAAGTACAGGTTGTAAGACAGATGGGAATTGTACTTGATATTATACTGATTCG
YY1
TGTGCATACATAGTTTATGCACCGAAATAGGTTGGGCAGCACATACTATACTTTTCATTAATACTTTTAACAATTGTAGTATATAAAAAAGGGAGTGACC
ACACGTATGTATCAAATACGTGGCTTTATCCAACCCGTCGTGTATGATATGAAAAGTAATTATGAAAATTGTTAACATCATATATTTTTTCCCTCACTGG
P3
E6
GAAAACGGTCGGGACCGAAAACGGTGTATATAAAAGATGTGAGAAACACACCACAATACCATGGCGCGCTTTGAGGATCC
CTTTTGCCAGCCCTGGCTTTTGCCACATATATTTTCTACACTCTTTGTGTGGTGTTATGGTACCGCGCGAAAGTCCTAGG
P2
P1
CAT-Primer

FIG. 6. In vivo protein binding to the HPV-18 promoter. (A) In vivo protein binding to the endogenous HPV-18 promoter in C4I and HeLa cells (lane O) or in HeLa cell clone 13 containing one copy of CAT plasmid p18URR (lane 13) was analyzed by genomic footprinting as described in Materials and Methods. Guanine ladders for the sense and antisense strands are shown. O, in vivo methylated DNA from HeLa or C4I cells; 13, in vivo methylated DNA from HeLa clone 13. N, in vitro methylated, protein-free (naked) DNA from the appropriate cells. The marked regions represent the distal and proximal AP-1 sites (−353 to −347; −169 to −163), the YY1 binding site (−115 to −93), and the switch region (−251 to −243) identified by 5′ deletion mutants. The arrowheads pointing upward and downward indicate hyperreactivity and protection from DMS modification, respectively. (B) Summary of the binding of proteins to the HPV-18 URR. On the sense strand, DMS-hyperreactive sites are shown by arrows pointing upward and single protected nucleotides are shown by arrows pointing downward (vice versa on the antisense strand). Unmarked nucleotide positions indicate that no difference was found between purified DNA and treated cells. Some of the DMS protections and hyperreactivities are visible only in C4I cells and are possibly due to the lower abundance of the factors interacting with these regions in HeLa cells. Core recognition sequences for bound transcription factors are underlined. The positions of the primers (P1, P2, and P3) used for in vivo footprinting are indicated. Note that the CAT primer hybridized only to the CAT sequences of the stably transfected p18URR construct in HeLa clone 13.

clonal line of HeLa cells (clone 13) which contains one copy of the HPV-18 URR-CAT construct (p18URR) in the genome. By using a CAT-specific primer, the pattern of DNA-protein interactions of the transfected construct was found to be essentially the same as that of the endogenous copies of HPV-18 DNA (Fig. 6A, left panel).

Specific binding of nuclear proteins to the switch region. As demonstrated by in vivo footprinting experiments, the switch region (OL22) and YY1 binding site OL13 serve as protein binding recognition sites (Fig. 6). To identify DNA-protein interactions at the switch region, we analyzed HeLa and HepG2 cell extracts by EMSAs for protein binding to OL22. Specific DNA-protein complexes were formed with OL22 (Fig. 7, lanes 1 and 8) which were abolished in the presence of 25-fold and 50-fold molar excesses of unlabelled OL22 (Fig. 7, lanes 2 and 3 and lanes 9 and 10), whereas competition with either an unrelated oligonucleotide (MRE) from the human metallothionein gene II_A promoter (Fig. 7, lanes 4 and 5 and lanes 11 and 12) or an oligonucleotide containing a single point mutation in the switch region (OL22mt; C to T) (Fig. 7, lanes 6 and 7 and lanes 13 and 14) did not affect the formation of this DNA-protein complex. To control for the quality of the HeLa and HepG2 nuclear extracts used for the experiment, AP-1 activity was detected by using the proximal AP-1 site of the HPV-18 URR as the substrate (data not shown).

In summary, these data demonstrate that, in addition to the sites previously identified by transient transfection studies (AP-1 and YY1 [6, 34, 45, 65]), the newly identified switch region of the HPV-18 URR serves as a recognition site for DNA binding proteins in vivo.

DISCUSSION

YY1 is a multifunctional transcription factor which can act as a repressor (60), an activator (50), or an initiator (59). In the present study, YY1 was identified as a key activator of HPV-18 early gene transcription in HeLa cells. The novel aspect of this finding is that the activating activity of YY1 is dependent on a sequence termed the switch region, located upstream of the proximal promoter YY1 binding site (OL13) in the HPV-18 URR (Fig. 5A and B). In the absence of an intact switch region, YY1 acts as a repressor rather than an activator of the HPV-18 URR (Fig. 4B and 5C). This strongly suggests a functional interplay between the switch region and the YY1 binding site (OL13). Mutations of the switch region (OL22) and YY1 binding site OL13 together completely abolished HPV-18 URR activity in HeLa cells (Fig. 5C), suggesting that these two *cis* elements play an essential role in determining the activity of the HPV-18 URR. Two other YY1 binding sites were detected in the distal and central fragments (OL31 and OL21) of the HPV-18 URR (Fig. 1 and 2C). In contrast to OL13, introduction of mutations in OL31 or OL21 had no effect on the transcriptional activity of the HPV-18 URR. The functional role of these two YY1 sites has to be determined in further experiments by introduction of double mutations at these sites into the HPV-18 URR.

Previously, we identified proximal YY1 binding site OL13 as a negative regulatory *cis* element of the HPV-18 proximal promoter fragment responsible for undetectable promoter activity in HeLa cells (6). However, YY1 acts as an activator of HPV-18 early gene transcription in the context of the complete HPV-18 URR in HeLa cells (Fig. 3B). This opposite action of YY1 in HeLa cells (repressor of the proximal promoter and activator of the HPV-18 URR) enabled us to identify the switch region that determines transcriptional activities of YY1. The functional interplay between YY1 and the switch region seems to be cell type specific, since the complete HPV-18 URR is virtually inactive in HepG2 cells (Fig. 3A) and human fibroblasts (data not shown), where YY1 acts as a repressor of HPV-18 URR activity. In this regard, it will be interesting to determine the mode of regulation of the HPV-18 URR in nonmalignant HeLa cell-fibroblast hybrids. Recently it was shown that both AP-1 binding sites are essential for HPV-18 transcriptional activity (65), but no activity of the HPV-18 URR could be detected in Jun-Fos-cotransfected (65) or Jun-overexpressing (1) mouse embryonic F9 cells. This may suggest the existence of cell type-specific negative regulators of the HPV-18 URR, since in control experiments, the collage-

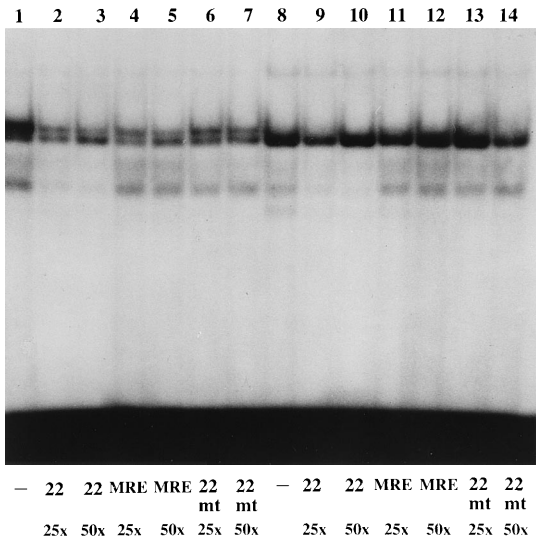

FIG. 7. Characterization of nuclear proteins from HeLa and HepG2 cells binding to the switch region of the HPV-18 URR. HeLa and HepG2 nuclear extracts contain a specific switch region binding activity. A ^{32}P-labelled OL22 oligonucleotide, corresponding to the sequence from −240 to −260 (Fig. 5B) of the HPV-18 enhancer, was used in EMSAs with HeLa (lanes 1 to 7) and HepG2 (lanes 8 to 14) crude nuclear extracts. Unlabelled competitor OL22, MRE, and OL22mt were used in 25- or 50-fold molar excess, as indicated at the bottom. Comp, competitors.

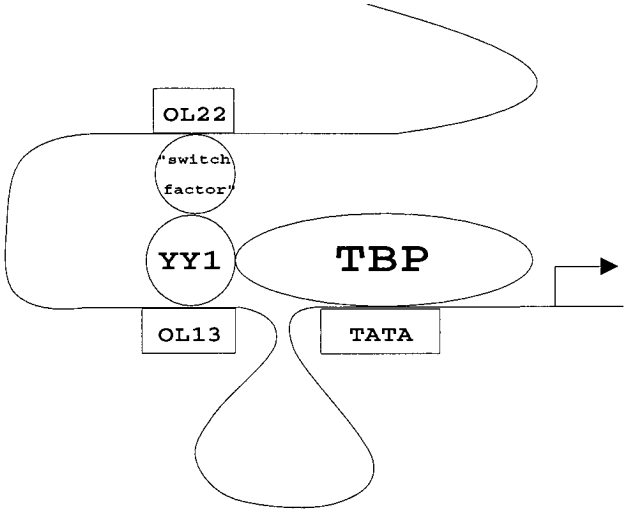

FIG. 8. Schematic model of synergistic interaction among the switch factor, YY1, and TBP. YY1 is a transcriptional regulator which can stimulate or repress transcription of different cellular or viral promoters. The functional role of YY1 can be modulated by interaction with other transcription factors. YY1 also interacts with several general transcription factors, such as TBP (31, 66). OL22 is the newly identified switch region and specifically binds a still unknown factor present in HeLa nuclear extracts. YY1 binds to proximal element OL13, as shown in Fig. 1 and 2A and B. Both factors together are important regulators of HPV-18 URR activity, as observed with mutants of both binding sites, OL22-M1 and OL13-M1, respectively (Fig. 5). A switch factor-YY1 complex may contact TBP and stimulate HPV-18 E6-E7 transcription in HeLa cells, as indicated by the crooked arrow.

nase gene promoter was strongly activated by cotransfected Jun in F9 cells (1, 3, 44).

Mutation of either YY1 (OL13) or the switch region (OL22) resulted in loss of about 50% of HPV-18 URR activity, analyzed in HeLa cells, as shown in Fig. 3B and 5C. This suggests that in the context of the HPV-18 URR, YY1 acts as an activator, possibly through functional interactions with the switch region binding factor(s). When the switch region is mutated, YY1 was likely acting as a repressor, which reduces HPV-18 URR activation by activators such as AP-1. In the context of the HPV-18 URR, YY1 may act not only as an activator or repressor, depending on the switch factor binding site. From its ability to communicate with the transcriptional machinery by interacting with TBP (31, 66), it seems that YY1, together with the switch region binding factors, may also function as a mediator to direct other transcription factors to the components of the RNA polymerase II initiation complex (Fig. 8). This assumption is supported by our observation that mutation of both the switch region and the YY1 site resulted in complete loss of HPV-18 URR activity even though both AP-1 sites of the HPV-18 URR, which are considered to be necessary for specific HPV-18 promoter activity (65), are present.

YY1 binding site OL13 and the switch region are separated by approximately 130 bp. To enable direct interactions between a factor binding to the switch region and YY1 to assemble a promoter-bound complex as schematically summarized in Fig. 8, DNA bending or looping may be involved. In fact, YY1 has been shown to bend DNA (43). Therefore, it is possible that, depending on the specific complex binding to the switch region, interactions between the switch region binding factor and YY1 (and possibly other factors binding to the URR) may differ, depending on specific DNA bending.

The DNA-bending property of YY1 resembles that of yeast ARS binding factor 1 (ABF1) and repressor-activator protein 1 (RAP1) (43). ABF1 and RAP1 have been implicated in transcriptional activation, transcriptional silencing, and DNA replication (13). Interestingly, the origin of replication for HPV-18, which corresponds to the origin of replication in bovine papillomavirus type 1 DNA (67, 68), was recently shown to map to the HPV-18 URR (10, 11) and overlaps YY1 binding site OL13. On the basis of these results, we suggest that YY1 may be involved in HPV-18 replication. In transient replication experiments with bovine papillomavirus type 1 E1 and E2 expression plasmids (kindly provided by A. Stenlund), we observed replication with plasmid constructs of the HPV-18 URR proximal promoter fragment, p18URR-221.w (OL13), and plasmid p18URR-221.M2 (OL13-M2), respectively, but not with p18URR-221.M1 (OL13-M1) (unpublished results). Our observation that YY1 binds in vitro to OL13 and OL13-M2 but not to OL13-M1 (Fig. 2A) may point to a direct involvement of YY1 in the replication process of HPV-18. Comparison of HPV sequences revealed that YY1 binding sites also exist in anogenital HPV-11, HPV-16, HPV-31, and HPV-33 (6). However, no sequence homologies could be found for the switch region in these HPV types. Recently, it was reported that extrachromosomal HPV-16 DNA from a lymph node metastasis of a cervical cancer contained a deletion of YY1 binding sites in the HPV-16 URR and that this deletion resulted in increased HPV-16 transcriptional activity (37). This may point to different modes of regulation of HPV-16 versus HPV-18 in certain cell types.

How does the switch region determine the transcriptional activities of YY1? It is possible that physical interactions between proteins that bind to the switch region and YY1 are the basis for the observed functional interplay between these factors. Indeed, the DNA-bound switch region binding factor may physically interact with the YY1 protein (7). Physical interactions between YY1 and other proteins that affect YY1 transcriptional activities have been documented. For example, YY1 has been found to interact with Sp1, c-Myc, and nucleolin (29, 31, 58, 61). These interactions affected YY1 activities to various degrees. However, the HPV-18 URR represents the first example of the presence or absence of a binding site for a yet to be identified DNA-binding protein (switch region binding factor) determining the positive or negative action of the second transcription factor (YY1). Work is in progress to characterize proteins that bind specifically to the switch region.

As demonstrated in this study, the functional interplay between the switch region and the proximal promoter YY1 binding site is highly cell type specific (Fig. 3). The mechanisms underlying this phenomenon are still unclear. Different possibilities can be considered. First, cell type-specific differences in the expression of the switch region binding factor or YY1 may result in enhanced or reduced association between the two proteins. Since YY1 expression seems to be comparable in HeLa and HepG2 cells (30), it will be interesting to determine whether the switch region binding factor(s) is differentially expressed. So far, no obvious difference between HeLa and HepG2 nuclear extracts were detected by EMSAs for proteins binding to switch region OL22 (Fig. 7). It is possible that cell type-specific interactions between the switch region binding factor and YY1 are regulated by posttranslational modifications such as phosphorylation. Phosphorylation is considered to be an important mechanism in the regulation of transcription factors with respect to their intracellular localization, DNA-binding activity (positive or negative), and transactivation activities (for a review, see reference 28). Differences in phosphorylation from one cell line to another may alter physical interactions between the switch region binding factor

and YY1 and possibly other factors binding to the URR of HPV-18.

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