JOURNAL OF VIROLOGY, July 1989, p. 2967–2976
0022-538X/89/072967-10$02.00/0
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Vol. 63, No. 7

Identification of a Novel Constitutive Enhancer Element and an Associated Binding Protein: Implications for Human Papillomavirus Type 11 Enhancer Regulation

MICHAEL T. CHIN, THOMAS R. BROKER, AND LOUISE T. CHOW*
Department of Biochemistry, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642

Received 21 November 1988/Accepted 27 March 1989

The human papillomavirus type 11 enhancer, when linked to the minimal simian virus 40 early promoter, has been dissected into two domains in monkey kidney CV-1 cells, one being constitutive (designated CEI) and the other inducible by *trans*-acting E2 proteins encoded by homologous and heterologous papillomaviruses (H. Hirochika, T. R. Broker, and L. T. Chow, J. Virol. 61:2599–2606, 1987; H. Hirochika, R. Hirochika, T. R. Broker, and L. T. Chow, Genes Dev. 2:54–67, 1988). We have demonstrated that the natural promoter regulated by this enhancer is located immediately upstream of the E6 open reading frame (the E6 promoter). We have mapped the cap site to nucleotide 99 by RNase protection. We further demonstrate a second constitutive enhancer element, CEII, which is required for transcription from the E6 promoter in the human cervical carcinoma cell lines C-33A and HeLa but not in CV-1 cells. By deletion mapping, we have localized this cell type-specific domain to 71 base pairs by using chloramphenicol acetyltransferase assays. Deletion of either CEI or CEII dramatically decreased the constitutive activity of the enhancer and the E6 promoter, whereas multimerization of either domain in the absence of the other could independently restore expression. Furthermore, when either of these elements was deleted, the full-length E2 protein of human papillomavirus type 11 abolished the remaining basal E6 promoter activity, demonstrating for the first time that the enhancer-activating E2 protein of human papillomaviruses can also function as a transcriptional repressor for the homologous E6 viral promoter. The presence of multiple copies of each element in tandem overcomes the repression by the E2 protein. The effects of CEII are at the level of transcription, without changing the cap site. By gel shift assay, we have shown that a protein present in nuclear extracts of C-33A and HeLa cervical carcinoma cells binds to the newly identified constitutive element II. This protein did not bind the simian virus 40 enhancer, nor did it bind to the enhancer region of many other papillomaviruses tested. UV cross-linking experiments revealed major 44-kilodalton and minor 34-kilodalton proteins that bound specifically to CEII. These two proteins are either related or bind to CEII with high cooperativity. We conclude that transcriptional activities directed by the enhancer and E6 promoter reflect an intricate balance among viral and cellular factors. We present a model on the regulation of the E6 promoter by host and viral transcription factors.

The papillomaviruses are a family of small, double-stranded DNA viruses that cause epithelial hyperplastic diseases, both benign and malignant (for a review, see reference 7). Their circular genomes of 7.9 kilobases are all similarly organized into early and late open reading frames (ORFs) encoded by the same DNA strand. Immediately upstream of the early ORFs is a region several hundred base pairs in length called the upstream regulatory region (URR) or the long control region. The URR contains a transcriptional enhancer which is *trans* activated by a protein encoded by the full-length viral E2 ORF (21, 22, 27, 41, 55, 59). One of the promoters modulated by this enhancer is located adjacent to the enhancer just upstream of the E6 ORF and is referred to as the E6 promoter (9, 15, 25, 54, 58). The URR-E6 promoter is important in regulating the expression of a number of viral genes, including the transformation genes (the E6 and E5 ORFs) of the bovine papillomavirus type 1 (BPV-1) (46, 61) and those of human papillomavirus type 16 (HPV-16) and HPV-18 (E6 and E7 ORFs) (3, 4, 30, 42, 56). In addition, the E6 promoter appears to direct the synthesis of a spliced transcript (12) which expresses functional E2 protein, as demonstrated by cDNA recovered via a retroviral vector (M. O. Rotenberg, M. Schweitz, T. R. Broker, and L. T. Chow, submitted for publication), suggesting that the E2 ORF might regulate its own expression.

The E2 gene product binds directly to the motif $ACCN_6GGT$ (2, 28, 36, 38). Deletion of this motif precludes *trans* activation (24, 28, 54). This motif alone, but not mutant versions of it, confers E2 inducibility on the simian virus 40 (SV40) early promoter (26, 28). A protein derived from the 3′ end of the E2 ORF, called E2-tr, sE2, or E2-C by different investigators, acts as a repressor of E2-dependent *trans* activation (9, 15, 33) by competitive binding to the E2-responsive sequence (E2-RS) (9, 36).

Since viral transcriptional regulation occurs in vivo in a species-, tissue-, and differentiation-specific fashion (11), cellular factors are assumed also to play important roles. This is indeed the case as demonstrated in HPV-11, HPV-16, and HPV-18 in that the enhancers also contain constitutive domains functional in the absence of E2 stimulation (9, 15, 22, 23, 28, 57, 58). Proteins derived from the E2 ORF have been shown to repress such E2-independent activity. HPV-11 E2-C protein, for example, can act as a repressor of E2-independent activity, but only when the HPV-11 enhancer is linked to its natural promoter (9). In chloramphenicol acetyltransferase (CAT) assays, the full-length BPV-1 E2 protein has a similar suppression effect on both the HPV-18 and HPV-11 enhancers linked to their respective E6 promoters in the natural context, but not to the heterologous

* Corresponding author.

SV40 promoter (9, 59). Since binding sites for E2 or E2-C in HPV-18 and HPV-11 are adjacent to the E6 promoter TATA motif but are distant from the SV40 promoter in the CAT plasmids, these data suggest that the mechanism involves steric hindrance of the binding of a TATA recognition factor. One caveat to these studies, however, is that homologous E2 expression clones did not repress their natural enhancer-E6 promoter targets. On the contrary, the HPV-11 E2 protein has a slight but positive effect on the HPV-11 URR E6 promoter. This discrepancy could be due either to quantitative differences in E2 expression levels or to qualitative differences in the way that HPV and BPV E2 proteins interact with cellular factors to assemble transcriptional complexes (9) and is the subject of this investigation.

Clearly, the papillomavirus enhancers are analogous to those of SV40 and polyomavirus in that they consist of multiple functional domains (47). Some of the HPV enhancer domains appear to be cell type specific (15, 22, 23, 57), while others appear to be generally constitutive (28) or inducible by factors other than E2 (22, 23). The fine structure of many of these domains remains to be elucidated, and the identities of the cellular factors have yet to be determined. We present here further dissection of the HPV-11 enhancer and describe the identification of a novel, cell type-specific constitutive domain and an associated cellular DNA-binding protein. Our results suggest intricate interactions among cellular and viral transcription factors in association with their respective recognition sequences in the regulation of the viral promoter.

MATERIALS AND METHODS

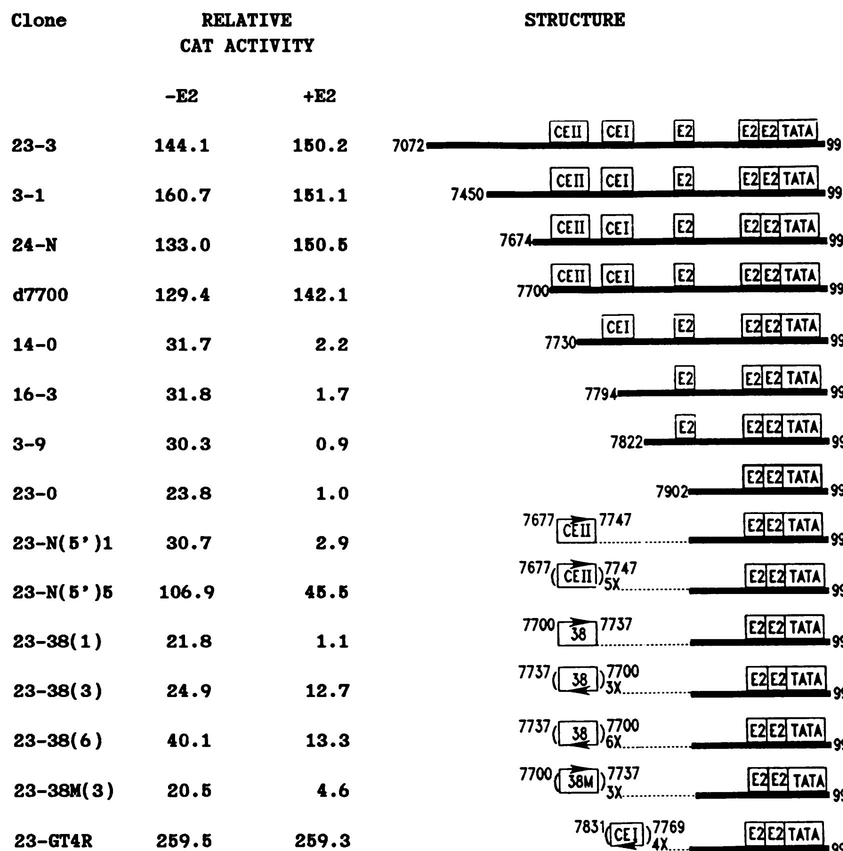
Tissue culture, nuclear extracts, transfections, and CAT assays. C-33A cells were cultured and transfected as described elsewhere (9). Nuclear extracts were made by the method of Dignam et al. (17). Protein concentration was determined by the method of Bradford (6). CAT assays were performed several times, each in duplicate, and were quantitated by liquid scintillation counting after thin-layer chromatography, as described previously (28). All assays were performed within the linear range.

Construction of recombinant clones. The CAT expression clones containing 5′ deletion mutations of the HPV-11 URR E6 promoter were generated by isolating the *Sph*I-*Oxa*NI fragments containing HPV-11 sequences from existing deletion clones (28) and cloning them in place of the *Sph*I-*Oxa*NI fragment of pUR23-3 (9). pUR23-0 (nucleotides [nt] 7902 to 99) was generated by digesting pUR23-3 (nt 7072 to 99) with *Bam*HI and *Bst*EII, treating the DNA with the Klenow fragment of DNA polymerase I, and ligating at low DNA concentration. pUR23-N(5′)1, pUR23-N(5′)5, and pUR23-GT4R were generated by inserting the *Pst*I-to-*Xba*I fragments of pCAT-N(5′)1, pCAT-N(5′)5, and pCAT-GT4R, respectively, (28) between the *Pst*I and *Xba*I sites of pUR23-0. pUR23-38(1) and pUR23-38(6) were prepared by cloning one and six copies, respectively, of the 38-base-pair (bp) synthetic oligonucleotide (5′ TGCCAACAACACACCTGG CGCCAGGGCGCGGTATTGCA 3′) into the Klenow-treated *Sal*I site of pUR23-0. The clone containing a single-base deletion, pUR23-38M, was discovered during sequencing of several pUR23-38 isolates. Complementary synthetic oligonucleotides 5′ TCGACTGCCAAGC 3′ and 5′ TCGAC GAAGCCAAAGC 3′, homologous to distal element II (DEII) and the cytokeratin gene octamer, respectively, were cloned as one and two copies in pCAT-A, the enhancer assay CAT expression plasmid (27). CAT plasmids containing the URRs of HPV-1a, -6b, -7, -16, and -18 and BPV-1 have been described previously (27).

RNase protection. The RNase protection assay was performed as described elsewhere (37), with several modifications. Briefly, an 864-nt antisense riboprobe containing HPV-11 URR sequences from nt 7450 to 99 and CAT sequences from nt 1 to 251 was synthesized in the presence of [^{32}P]UTP with T7 polymerase from pBS3-1, a pBS+ plasmid (Stratagene) containing the 833-bp *Pst*I-to-*Eco*RI fragment of deletion clone 3-1 (Fig. 1). The ^{32}P-labeled riboprobe was then hybridized with 50 μg of total RNA from transfected cells in 80% formamide–40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES, pH 6.4)–400 mM NaCl–1 mM EDTA overnight at 55°C. The reaction mixtures were diluted 13-fold and digested with 40 μg of RNase A per ml–2.2 μg of RNase T_1 per ml for 60 min at room temperature in 0.3 M NaCl–10 mM Tris hydrochloride (pH 7.5)–5 mM EDTA. Samples were then phenol extracted twice, ethanol precipitated twice, and suspended in 5 μl of 80% formamide–0.1% xylene cyanol–0.1% bromphenol blue. The samples were then heated to 68°C for 3 min and loaded onto 6% polyacrylamide sequencing gels.

Gel mobility shift DNA-binding assays. The restriction fragment containing a single copy of constitutive enhancer element CEII from 23-N(5′)1 was excised with appropriate flanking restriction enzymes and was 3′ end labeled with the Klenow fragment of *Escherichia coli* DNA polymerase I in the presence of [α-^{32}P]dCTP. Alternatively, the 71-bp CEII was excised from 23-N(5′)1 with *Bam*HI and *Hin*dIII and cloned into the multiple-cloning site of the vector pBS+ and labeled by annealing the M13-sequencing primer (New England BioLabs, Inc.) at 50°C and extending it with the Klenow fragment in the presence of 50 μM each dATP, dGTP, and dTTP and 40 μCi of [α-^{32}P]dCTP. The labeled CEII was then excised with appropriate enzymes and purified. The low-ionic-strength gel shift assay was performed by using a modification of published procedures of Fried and Crothers (18) and Garner and Revzin (19). Specifically, 5 to 10 μg of nuclear protein was preincubated in 25 μl of binding buffer [25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (K+) (pH 7.8), 10 mM $MgCl_2$, 225 mM KCl, 1 mM dithiothreitol, 1 mM EDTA, 50 μg of poly(dI-dC) per ml] for 45 min at 0°C. Labeled DNA (10,000 cpm, 4 fmol [250 pg]) and unlabeled competitor DNA were then added simultaneously, and the reaction was incubated for another 45 min at 0°C. The samples were then loaded onto 4% polyacrylamide gels equilibrated with 0.25× TBE (1× is 0.089 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA) and electrophoresed for 2 h at 300 V and 4°C. Gels were then dried and autoradiographed. Experiments using labeled DNA containing the 38-bp segment in clone 23-38 were performed similarly.

UV-induced cross-linking. The restriction fragment containing CEII (nt 7677 to 7747) was labeled in one strand with [^{32}P]dCTP along its entire sequence by the primer annealing method as described above, except that 5-bromodeoxyuridine was substituted for dTTP. Binding reactions were also performed identically. Samples were then exposed to a medium-wave UV (301 nm) transilluminator (Ultra-violet Products) for 40 min on ice at a distance of 3 cm. DNase I (15 U) was then added, and the samples were incubated for 30 min at 37°C. Samples were then mixed with 30 μl of 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer and boiled for 5 min, and electrophoresis was performed on 10% polyacrylamide-sodium dodecyl sulfate gels. Gels were then dried and autoradiographed.



Clone	RELATIVE CAT ACTIVITY		STRUCTURE
	-E2	+E2	
23-3	144.1	150.2	
3-1	160.7	151.1	
24-N	133.0	150.5	
d7700	129.4	142.1	
14-0	31.7	2.2	
16-3	31.8	1.7	
3-9	30.3	0.9	
23-0	23.8	1.0	
23-N(5')1	30.7	2.9	
23-N(5')5	106.9	45.5	
23-38(1)	21.8	1.1	
23-38(3)	24.9	12.7	
23-38(6)	40.1	13.3	
23-38M(3)	20.5	4.6	
23-GT4R	259.5	259.3	

FIG. 1. Relative CAT activity of deletion mutants of the HPV-11 URR E6 promoter linked to a promoterless CAT gene. Relative CAT activity was defined as the ratio of the percent acetylated chloramphenicol to that obtained with extracts made from cells cotransfected with pUR23-0 and pRSE2-11 under the same conditions. All numbers represent the average of two or more independent experiments, each with duplicate samples. The structure of each clone is diagrammed at the right. The nucleotide positions shown differ from those previously published (16) by two after position 7718 because an additional GC dinucleotide was found in the DNA (S. C. Dollard, T. R. Broker, and L. T. Chow, unpublished results).

RESULTS

Analysis of enhancer domains in conjunction with the E6 promoter in human cervical carcinoma cells. By transient expression of the CAT gene from the minimal SV40 early promoter in monkey CV-1 cells, we have previously identified two domains of the HPV-11 enhancer in the URR, one being E2 inducible and the other constitutive (28). We have also demonstrated that, in the absence of E2 proteins, the HPV-11 URR E6 promoter is relatively inactive in CV-1 cells but is highly active in human cervical carcinoma cell lines C-33A and HeLa (9). To identify the DNA sequences necessary for constitutive activation of the E6 promoter in these cell lines, we constructed a series of 5′ deletion mutants of the HPV-11 URR E6 promoter and assayed their ability to express the CAT reporter gene in C-33A cells (Fig. 1). When the sequences were deleted to nt 7700 (clone d7700), no effect was observed, but upon further deletion to and beyond nt 7730 (clone 14-0), a sharp drop in constitutive activity was observed. One copy of the most active constitutive domain identified in CV-1 cells (nt 7769 to 7831), which we now rename constitutive element I (CEI), was still present in deletion clone 14-0, demonstrating that one copy of CEI alone is not sufficient to confer full activity. These results suggested that a second constitutive domain existed in the region between nt 7700 and 7769. To test whether this segment of the genome could alone confer activity, we cloned a 71-bp sequence from nt 7677 to 7747 either as a monomer [clone 23-N(5′)1] or as a pentamer [clone 23-N(5′)5] into pUR23-0 (nt 7902 to 99), a minimal E6 promoter clone that does not contain CEI. Clone 23-N(5′)1 did not demonstrate enhanced expression. In contrast, clone 23-N(5′)5, containing five copies of this segment, led to a partial restoration of constitutive activity. Identical results were obtained in HeLa cells (data not shown). RNase protection analysis and primer extension demonstrated that this effect occurs at the level of transcription, with initiation at nt 99 (Fig. 2, lanes 1, 2, and 4; data not shown). Because there was no band of lower molecular weight that was unique in any of the lanes, we interpret the result to indicate the absence of a cryptic promoter in CEII in clone 23-N(5′)5. The message level for clone 14-0 was below the level of detection (lanes 2 and 3). These results indicate that the 71-bp genomic segment contains a transcriptional regulatory element, which we call constitutive element II or CEII. CEI, when placed as an inverted tetramer in the vector pUR23-0 (clone 23-GT4R), also functions as an enhancer element, in agreement with our previous result obtained in CV-1 cells (28) (Fig. 1). We conclude, therefore, that the HPV-11 enhancer consists of three distinct, independent functional domains, two being constitutive and the third E2 responsive.

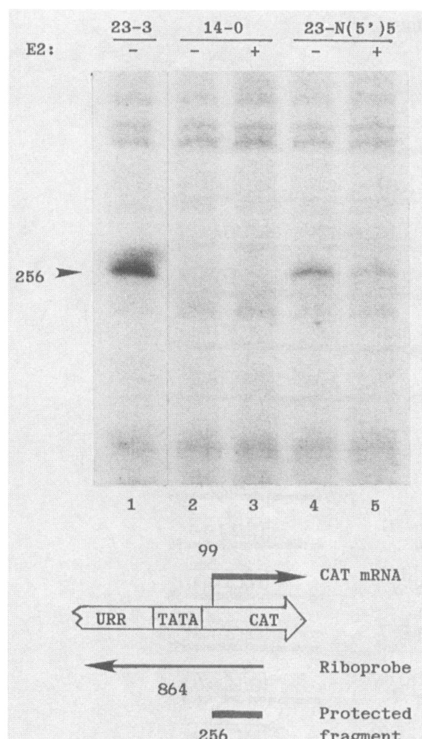


FIG. 2. RNase protection. An 864-nt ^{32}P-labeled riboprobe was synthesized as described in Materials and Methods. RNase protection assays were carried out with total RNA recovered from cells transfected with the indicated clones. The structures of the clones are shown in Fig. 1, and the mRNA, the riboprobe, and the protected fragment are diagrammed below. The arrowhead points to the 256-base protected band. The presence (+) or absence (−) of E2 protein is indicated.

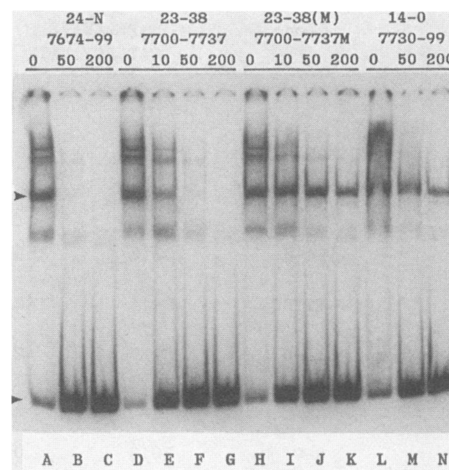


FIG. 3. Gel mobility shift DNA-binding assay. The gel shows protein-DNA complexes formed with a labeled 103-bp fragment containing the 71-bp CEII element (nt 7677 to 7747 plus flanking polylinker sequences) in the presence of unlabeled 24-N (lanes A to C), pUR23-38(1) (lanes D to G), 14-0 (lanes L to N), or pUR23-38M (lanes H to K) with the single-base-pair deletion mutation shown in Fig. 5. The fold molar excess of competitor DNA is indicated above each lane. Arrowheads point to the complex (top) and free DNA (bottom).

The effect of HPV-11 E2 protein. To investigate possible interactions among the full-length HPV-11 E2 protein and cellular factors that recognize CEI, CEII, and the E6 TATA motif, the same series of CAT expression clones containing 5′ deletion mutations of the HPV-11 URR E6 promoter as described above were cotransfected into C-33A cells with the HPV-11 E2 expression clone, pRSE2-11 (27) (Fig. 1). As was shown previously, HPV-11 E2 protein had little effect on pUR23-3 containing the full-length URR E6 promoter segment because of the high constitutive enhancer activity and low *trans*-activational activity of HPV-11 E2 protein (9, 27). The E2 protein had little effect on transcriptional activity of clones containing 5′ deletions up to nt 7700. Unexpectedly, when the deletions extended to and beyond nt 7730, the remaining basal activity was practically abolished by the presence of the HPV-11 E2 protein (Fig. 1). To the best of our knowledge, this is the first reported example of a full-length E2 protein suppressing its homologous E6 promoter. The basal activity of clone 23-N(5′)1, containing one copy of the 71-bp segment, was also suppressed by the HPV-11 E2 protein, whereas that of clone 23-N(5′)5, containing five copies of the 71-bp segment, was only partially suppressed. Clone 23-GT4R, containing four copies of CEI, was neither suppressed nor stimulated. Identical results were obtained in HeLa cells (data not shown). The repression by cotransfection with the E2-C protein expression vector was similar but quantitatively stronger in that the full-length enhancer (clone 23-3) was also repressed, as reported earlier (9; data not shown). RNase protection experiments demonstrated that the repression of clone 23-N(5′)5 by the HPV-11 E2 protein was at the level of transcription from the same cap site at nt 99 (Fig. 2, lanes 4 and 5). These results suggest complex interactions among the E2 proteins and cellular factors in the formation or destabilization of active transcription complexes.

Identification of a nuclear factor that binds specifically to CEII. To identify cellular factors that interact with CEII, we initially chose to prepare nuclear extracts from C-33A cells instead of HeLa cells, because C-33A cells have no apparent endogenous HPV genomes (40, 62). In contrast, HeLa cells contain multiple copies of HPV-18 DNA integrated into the chromosome, from which the E6, E6*, and E7 mRNAs or proteins are expressed (48, 49, 51); thus, the interpretation of the results obtained with HeLa cell extracts would be complicated. By using an end-labeled restriction fragment containing the 71-bp CEII element (nt 7677 to 7747), a CEII-specific complex was detected in gel shift assays. The complex was eliminated by competition with a 50-fold excess of unlabeled plasmid 24-N (nt 7674 to 99; Fig. 3, lanes A, B, and C). This clone exhibited high endogenous enhancer-promoter activity not repressed by the HPV-11 E2 protein, properties by which CEII was defined (Fig. 1). The unlabeled clone 14-0 (nt 7730 to 99) failed to compete (Fig. 3, lanes L, M, and N). Since this clone lacks the CEII element and has greatly reduced constitutive activity which is repressed by HPV-11 E2 protein, we concluded that the factor detected by gel shift analysis binds specifically to CEII. To define further the binding site, additional clones were used as competitors. The 5′ deletion clone 10-N (nt 7713 to 7906) did not compete for complex formation, nor did 3′ deletion clone 23-N(5′)U (nt 7677 to 7726 and 7902 to 99) (Fig. 4). On the basis of these results, 38-bp complementary oligonucleotides (5′ TGCCAACAACACACCTGGCGCCAGGGCGCGGTA TTGCA 3′) spanning nt 7700 to 7737 were synthesized and cloned upstream of the HPV-11 minimal E6 promoter (nt 7902 to 99) in CAT plasmid 23-0. The resulting clone (23-38) was tested in gel shift assays. As judged from the position of

Clone	Competition	Structure
23-3	yes	7072--------//----------------------7933/1-----99
24-N	yes	7674--------------------------------------99
d7700	yes	7700----------------------------------99
10-N	no	7713------------------7906
14-0	no	7730----------------------99
23-N(5')1	yes	7677------------7747··········7902---------99
23-38(1)	yes	7700--------7737··········7902---------99
23-38M	no	7700------*-7737··········7902---------99
23-N(5')U	no	7677-----------7726···········7902---------99
23-0	no	7902---------99
SV40	no	273----------1/5243----5172
HPV-1a	no	6578---------------//---------------------------7815/1-3
HPV-6b	weak	7674-------------------7842
HPV-7	no	6900----------//----------------------------------48
HPV-16	no	6359------------------//--------------------7905/1------280
HPV-18	no	6809-------------//--------------------7857/1------119
BPV-1	no	6959---------//---------------------------7945/1-3
DEII	no	TCGACTGCCAAGC
CK octamer	no	TCGACGAAGCCAAAGC

FIG. 4. Effect of unlabeled clones on specific protein binding to CEII. The relevant structures of various CAT expression clones and their ability to compete for specific binding to the CEII element at 200-fold molar excess is indicated. The deletion of an adenine residue at position 7732 (*) is shown. DEII and CK octamer were synthetic oligonucleotides cloned in one and two copies each in the pCAT-A vector. The relevant sequences are underlined.

the complex in the gel, the same specific complex formed with CEII using the labeled 71-bp fragment was eliminated by competition with a 50-fold molar excess of this unlabeled 38-bp synthetic oligonucleotide (Fig. 3, lanes D through G). pUR23-38M, which contained a deletion of a single adenine residue within the TATTGC sequence at the 3′ end of the synthetic oligonucleotide, did not compete, even at 200-fold molar excess (Fig. 3, lanes H through K). Gel shift experiments using labeled DNA containing only the 38-bp segment gave identical results when specific [23-3 and 23-38(1)] or nonspecific (14-0 and 23-38M) competitor DNAs were added. These data suggest that the fragment spanning nucleotides 7677 to 7747 which exhibits enhancer activity when multimerized contains a transcription factor-binding site located within the region defined by the synthetic oligonucleotide (between nt 7700 and 7737). Clones containing the 38-bp synthetic oligonucleotide were also tested in CAT assays. The oligonucleotide did not stimulate CAT activity when present as a monomer [clone 23-38(1)] in the forward orientation. Although six copies in the inverted orientation [clone 23-38(6)] stimulated E2-independent activity only slightly, the transcriptional repression by E2 was partially blocked (Fig. 1), as was seen with the clone containing five copies of the 71-bp fragment [clone 23-N(5′)5]. At present, we are not certain as to why the enhancer activity of the 38-bp fragment in the absence of the E2 protein is lower than that of the 71-bp element (discussed further below). Three copies of the 38M mutant in the forward orientation did not stimulate E2-independent activity, and its activity was repressed to a greater extent (two- to threefold) than by either three or six copies of the 38-bp wild-type sequence (Fig. 1). The residual activity is probably due to the sequence GTTT GCAT, created by the adenine deletion that matches in seven of eight positions the SV40 octamer sequence CTTTG CAT. A factor that binds this sequence is known to be present in HeLa cells (60).

To determine whether the cellular protein specific for CEII is important for other viral systems, we performed gel mobility shift competition experiments, using the regulatory regions of the viruses shown in Fig. 4. The SV40 enhancer-early promoter and the URRs of BPV-1, HPV-1, and HPV-7 and of the genital papillomaviruses HPV-16 or HPV-18 did not compete for binding of this CEII-specific factor (Fig. 4), indicating that it probably does not play a direct role in their regulation. HPV-6b competed for binding with an approximately 10-fold lower affinity (Fig. 4; data not shown). In HPV-6b, the corresponding region has both divergent nucleotides and a 19-bp insertion (Fig. 5). These findings indicate that the regulation of the genital HPVs by cellular factors may be less similar than expected. In addition, a clone containing the synthetic oligonucleotide 5′ TCGACTGC CAAGC 3′ (nt 7700 to 7705), a half site for CTF/NF-1, did not compete, either. These results indicate that this CEII factor is distinct from those that bind to the broad-host-range SV40 virus, such as Sp1, AP-1, AP-2, AP-3, AP-4, and C/EBP, and that it is distinct from CTF/NF-1 (summarized in reference 60), lending credence to our hypothesis that this factor is different from any that has previously been described. Since some regulatory proteins such as C/EBP have been shown to bind to two unrelated sequences (34), we addressed the possibility that this factor may be related to one that might recognize a consensus motif, 5′ AANCCAAA 3′, found upstream of several cytokeratin genes (5). We performed competition experiments, using clones containing one or two copies of the synthetic oligonucleotide 5′ TCGA CGAAGCCAAAGC 3′. No competition was detected at 200- to 400-fold molar excess (Fig. 4).

Estimation of the CEII factor molecular weight by UV cross-linking. To determine the molecular weight of this CEII-specific factor, the 71-bp CEII DNA was labeled with [^{32}P]dCTP in the presence of 5-bromodeoxyuridine, as described in Materials and Methods. The CEII factor was found to bind to 5-bromodeoxyuridine-substituted DNA with no apparent loss of affinity or specificity, as determined by gel shift assays (data not shown). After the crude nuclear lysates were incubated with the labeled DNA, cross-linked complexes were generated by UV irradiation. The complexes were then treated with DNase I and run on sodium dodecyl sulfate-polyacrylamide gels. A major broad band of approximately 44 kilodaltons (kDa) and a minor band of 34

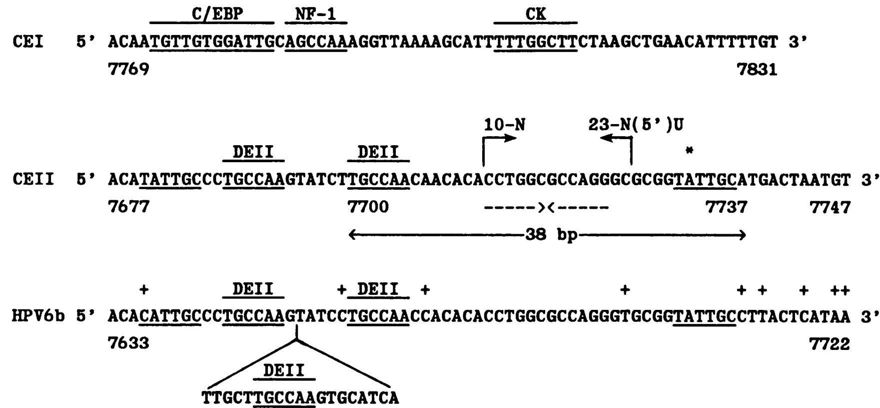


FIG. 5. Sequences and notable features of CEI and CEII. CEI was defined by Hirochika et al. (28). CEII is defined by the experiments described in Fig. 1, 2, and 3. The first and last positions of both segments in the HPV-11 DNA are indicated. The direction of deletion and the ends of deletion mutations in CEII that did not compete for factor binding in gel shift assays (↱) are indicated. The nucleotide deleted in clone 23-38M (*) which did not compete for factor binding (Fig. 3 and 4) is shown. Sequences with a twofold symmetry (– – →) and the 38-bp segment of CEII that binds a major nuclear protein of 44 kDa and a minor nuclear protein of 34 kDa (→) are shown. The homologous region of HPV-6b (nt 7633 to 7722), with divergent nucleotides (+) and the site of a 19-bp insertion, is shown. The divergent nucleotides were confirmed by DNA sequence determination in the particular clone used for competition. Homology to recognition sequences of known transcription factors is over- and underlined. CK, Consensus (in the inverted orientation) to an octamer found in the upstream region of some cytokeratin genes; DEII, a consensus element found upstream of the rat albumin gene.

kDa were visualized (Fig. 6). The proteins represented by both bands were reduced at equal rates when unlabeled competitor DNA containing one copy of the 38-bp sequence was added to the incubation mixture prior to UV irradiation. Neither was affected when the competitor DNA used was clone 38M, which also failed to compete in the gel shift assay (Fig. 3). Proteins of identical size and binding characteristics were detected in HeLa extracts (data not shown). The 34-kDa band could arise from proteolytic cleavage of the 44-kDa protein during the isolation of the cross-linked complex, because only one specific DNA-protein complex was detected in gel shift assays when competitor DNAs spanning different regions of the 71-bp fragment were used. Alternatively, they may be two distinct proteins that form a heterodimer or are highly cooperative in their binding to their target sequences.

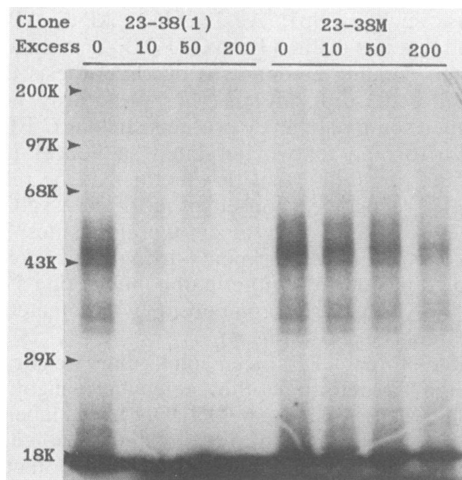


FIG. 6. UV cross-linking of CEII factor to its cognate sequence. The CEII factor was bound to a [^{32}P]dCTP-labeled 103-bp fragment containing CEII in the presence of unlabeled pUR23-38(1) or pUR23-38M. After UV cross-linking and DNase I treatment, the protein was run on a 10% sodium dodecyl sulfate-polyacrylamide gel, dried, and autoradiographed. The fold molar excess of competitor DNA is indicated above each lane. Molecular weights (in thousands) are given on the left.

DISCUSSION

We have shown that the HPV-11 enhancer-E6 promoter has a second constitutive domain localized to a 71-bp segment (nt 7677 to 7747), designated CEII, in addition to the constitutive domain (designated CEI) and the E2-inducible domain previously identified in monkey CV-1 cells when the minimal SV40 early promoter is employed (28). Both CEI and CEII are required for high E2-independent E6 promoter activity in cervical carcinoma cell lines C-33A and HeLa, but only CEI is also active in CV-1 cells (28; this study). Furthermore, we have shown, for the first time, that the HPV-11 E2 protein represses the homologous E6 promoter in the absence of one or both of the constitutive domains. Multiple copies of CEII not only restore partially the constitutive enhancer-E6 promoter activity, but also partially overcome the suppression of the E6 promoter by the HPV-11 E2 protein. RNase protection demonstrates that CEII in multiple copies increases transcription from the E6 cap site at nt 99, verifying that it is a genuine transcriptional regulatory element and does not contain a cryptic promoter. Furthermore, E2 repression resulted in the reduction or elimination of mRNA initiation from the cap site (Fig. 2). We have demonstrated that an approximately 44-kDa nuclear protein in C-33A cells binds specifically to CEII (Fig. 6). Since CEII contains little homology to any of the known transcriptional factor recognition sequences, we believe that the 44-kDa protein represents a novel transcriptional regulatory protein.

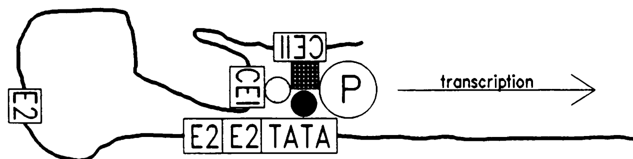
As shown in Fig. 5 and as described previously (28), CEI contains sequence motifs homologous to those that bind C/EBP (34) and CTF/NF-I (29). In addition, we note that it contains the motif 5′ TTTGGCTT 3′, found in the keratinocyte-dependent enhancer of HPV-16 (15) and in the URRs of all the other genital HPVs that have been sequenced (13,

14, 49). This motif is effectively identical to the consensus sequence 5′ AANCCAAA 3′ located upstream of a number of human and bovine cytokeratin genes (5), although there is no evidence that it alone functions as an enhancer in these genes, nor did it stimulate CAT activity in C-33A when present in multiple copies (data not shown). It is possible that the presence of several different enhancer motifs within this CEI domain contributed to its activity in cells as diverse as CV-1 and C-33A.

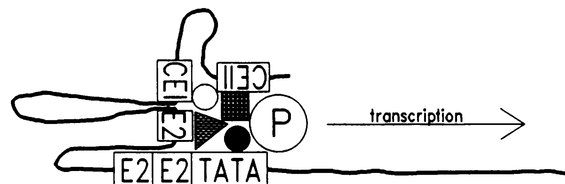
In contrast, CEII (Fig. 5) contains very little sequence homology to any of the known transcription factor-binding sites (summarized in reference 60). There are two copies of the sequence TGCCAA, which has been found in the reverse orientation in the distal element II (DEII) of the rat albumin promoter and was shown to bind an NF-1-like factor (8). Multiple copies of a consensus motif, YGCCAA, have been noted in the URRs of many HPVs, including HPV-11 (32). However, the cloned synthetic oligonucleotide 5′ TCGAC TGCCAAGC 3′ failed to compete in the gel shift assays (Fig. 4), nor did multiple copies stimulate CAT expression (data not shown). There are also two copies of the sequence TATTGC, which is part of a 19-bp insertion in the URR of HPV-6vc, relative to HPV-6b. This particular HPV-6 isolate is reported to have a more active enhancer than HPV-6b (44). The TATTGC sequence might be critical for CEII activity, because clone 38M, in which the A residue has been deleted from this sequence, can no longer compete for specific protein binding (Fig. 3, 4, and 6). The only other notable feature is a 12-bp palindrome (5′ CCTGGCGC CAGG 3′) in the center. None of these elements can, however, independently account for the binding activity of this segment, because the deletion mutation (clone 10-N), which affected neither the 12-bp palindrome nor the TAT TGC sequence, nonetheless abolished the ability of the clone to compete in the gel shift experiment. The deletion mutations 23-N(5′)U and 23-38M, in which the palindrome and the TGCCAA sequence were intact, did not compete, either (Fig. 4). Thus it appears that the recognition sequence for this factor constitutes the major portion of the 38-bp segment and is at least 25 to 30 bases in length. The reason that the 38-bp synthetic oligonucleotide multimer in the reverse orientation functions in overcoming E2 repression and in DNA-binding assays but only weakly in enhancer stimulation (Fig. 1; data not shown) may reflect the fact that the recognition sequence constitutes most of the oligonucleotide, and therefore the close positioning of tandem binding sites may prevent occupancy by multiple CEII proteins required for high activity. The CEII element may function better in the sense configuration and therefore may be a transcriptional regulatory element but not a classical enhancer element. Alternatively, factors not detected in our gel shift assay may bind outside the 38-bp sequence and augment the activity of the 44-kDa factor. Because CEII is active in C-33A (Fig. 1 and 2), HeLa (data not shown), and SCC-25, a cell line derived from a squamous cell carcinoma of the tongue (ATCC CRL1628), but not in CV-1 (28) or HepG2, a human hepatoblastoma cell line (ATCC HB8065) (S. C. Dollard, T. R. Broker, and L. T. Chow, unpublished results), we believe that this domain is one of the determinants of HPV-11 epithelial cell type specificity.

Our earlier studies have shown that the E2 proteins encoded by different papillomaviruses *trans* activate the URR-E6 promoter or the URR SV40 early promoter by binding to the E2-RS in the HPV URR (9, 27, 28). In contrast, the protein derived from the carboxyl-terminal half of the HPV-11 E2 ORF negatively regulates the E2-dependent activities from these promoters and the E2-independent activity of the URR-E6 promoter (9). We have postulated that the E2-C repression of the E2-independent activity is a result of steric interference with the association of a TATA motif-binding factor. This current study has demonstrated that the HPV-11 E2 protein can also function as a repressor when factors for CEI or CEII are absent. On the basis of these data, we propose a model for the transcriptional regulation of the E6 promoter in HPVs trophic for the genital and oral mucosa, all of which have similar proximity of the E2-RS elements to the E6 TATA motif (Fig. 7). We suggest that the constitutive activity of the HPV-11 enhancer in C-33A arises by a looping mechanism (reviewed in reference 43) and that CEI and CEII factors can facilitate the binding of the TATA factor, as has been proposed for adenovirus major late transcription factor and pseudorabies virus immediate-early protein (1, 45). The resulting complex would then recruit RNA polymerase II and lead to transcription. We propose that E2 activates transcription by binding to an E2-RS element and promoting the formation of a stable transcriptional complex, when the cellular factors are low in abundance. Of the four E2-RS elements in the HPV-11 URR, the second copy near nt 7900 alone is sufficient for E2 *trans*

A: HIGH CELLULAR FACTORS



B: LOW E2



C: HIGH E2 OR E2-C

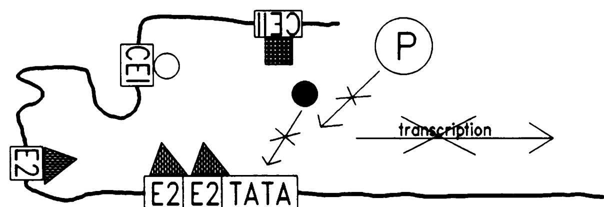


FIG. 7. A model for enhancer and E6 promoter regulation.

activation when the constitutive domain is present (28). As has been observed for the bacteriophage 434 repressor (31) and for the E2 protein of BPV-1 (39), we surmise that variations in the central 4 nt of the different E2-RS elements confer differential affinity for the HPV-11 E2 protein. When the E2 protein is synthesized in small amounts, our model predicts that it would bind to the site near nt 7900 (ACCG GTTTCGGT), resulting in transcription stimulation from the E6 promoter to give rise to, among others, the polycistronic message containing E6, E7, E2, and E5 ORFs, from which functional E2 protein is made (12; Rotenberg et al., submitted). As the concentration of E2 increases, it would also bind to the sites adjacent to the E6 promoter (ACCGAAAAGGT, or ACCGTTTTCGGT in the reverse orientation), preventing the putative TATA factor from binding, as has been proposed for E2-C on the basis of the observation that the DNase I footprints of E2 and E2-C overlap the E6 TATA motif (9, 28). Thus E2 would suppress the E6 promoter and, in doing so, would repress its own synthesis, as well as that of the transformation proteins. Our data are consistent with this hypothesis, and further experimentation in the area of site-directed mutagenesis and in vitro transcription using purified factors is needed to test this method.

Although our data do not reveal whether E2 proteins merely recruit cellular factors or are themselves part of the complex, the following arguments favor the latter viewpoint, as depicted in Fig. 7. The amphipathic helix of the GAL4 protein has been demonstrated to be important for the activation of the *GAL1* gene in *Saccharomyces cerevisiae* cells (20) and is thought to interact directly with RNA polymerase (summarized in reference 52). We have examined the sequences of the E2 proteins of all the papillomaviruses and found that each contains an amphipathic alpha helix with an acidic face at the amino terminus. Because the amino-terminal domain is required for *trans* activation but not repression (9, 15, 27, 33), it is probable that the E2 proteins are part of the transcription complex.

The biological significance of these various levels of HPV-11 enhancer regulation is best understood if one considers that all viruses are molecular parasites. Activation by cellular factors in the appropriate tissue facilitates the initial as well as subsequent viral gene expression. Onset of E2 synthesis would then increase transcription of viral genes to allow the establishment of infection. Further increases in the levels of E2 or E2-C would then result in attenuation of viral gene expression, preventing premature killing of the host cells before viral reproduction is accomplished. This autoregulation could, however, be overcome when the host factors for CEI and CEII or other *cis* elements yet to be identified are abundant, for instance, when the cells differentiate while migrating upward in the epithelium. The autoregulation of E2 expression would also explain the observation that in cervical carcinoma cell lines and in a few carcinomas examined, the integrated HPV DNAs are always interrupted near the junction of the E1 and E2 ORFs (10, 35, 50), dissociating both E2- and E2-C-coding regions from their respective promoters (11, 53). The loss of both repressors would then allow constitutive overexpression of the E6 and E7 transformation genes from the E6 promoter (3, 48, 56), eventually leading to cellular transformation. This hypothesis has previously been proposed on the basis of the observation that the BPV-1 E2 protein can suppress the E6 promoter of the genital HPVs (9, 59). We have now shown that HPV-11 E2 protein can indeed function as a repressor of its homologous E6 promoter, thus substantiating the hypothesis. In conclusion, we have demonstrated that the transcriptional activity from the HPV enhancer-E6 promoter is dependent on the delicate balance among positive and negative regulatory influences exerted by the host and the virus itself.

ACKNOWLEDGMENTS

This research was supported by Public Health Service grant CA36200 from the National Institutes of Health. M.T.C. is the recipient of Public Health Service Medical Scientist Training Program grant GM 07356 from the National Institutes of Health and a March of Dimes Predoctoral Fellowship.

We thank Hirohiko Hirochika for constructing three of the URR deletion clones used in this study and Melissa Colbert and Meirav Chovav for useful discussions on RNase protection and UV cross-linking, respectively.

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