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Transcriptional Control of Human Papillomavirus (HPV) Oncogene Expression: Composition of the HPV Type 18 Upstream Regulatory Region

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The malignant transformation potential of high-risk human papillomaviruses (HPVs) is closely linked to the expression of the viral E6 and E7 genes. To elucidate the molecular mechanisms resulting in HPV oncogene expression, a systematic analysis of the *cis*-regulatory elements within the HPV type 18 (HPV18) upstream regulatory region (URR) which regulate the activity of the E6/E7 promoter was performed. As the functional behavior of a given *cis*-regulatory element can be strongly influenced by the overall composition of a transcriptional control region, individual elements were inactivated by site-directed mutagenesis in the physiological context of the complete HPV18 URR. Subsequently, the effects of these mutations on the activity of the E6/E7 promoter were assessed by transient transfection assays. We found that the transcriptional stimulation of the E6/E7 promoter largely depends on the integrity of *cis*-regulatory elements bound by AP1, Sp1, and in certain epithelial cells, KRF-1. In contrast to previous reports implying a key role for NF1 and Oct-1 recognition motifs in the stimulation of papillomavirus oncogene expression, the inactivation of these elements in the context of the HPV18 URR did not strongly affect the transcriptional activity of the E6/E7 promoter. Mutation of a promoter-proximal glucocorticoid response element completely abolished dexamethasone inducibility of the HPV18 E6/E7 promoter and resulted in an increase of its basal activity. Functional dissection of the HPV18 constitutive enhancer region indicates that its transcriptional activity is largely generated by functional synergism between a centrally located AP1 module and thus far undetected *cis*-active elements present in the 5′ flank of the enhancer. Furthermore, comparative analyses using homologous and heterologous promoters show that the transcriptional activity of HPV18 enhancer elements is influenced by the nature of the test promoter in a cell-type-specific manner.

Human papillomaviruses (HPVs) are causative agents of a variety of benign epithelial proliferative lesions in humans, such as skin warts and condyloma acuminata. In addition, specific HPV types have been closely associated with the development of human anogenital malignancies. The DNA of these so-called high-risk HPV types (in particular HPV type 16 [HPV16] and HPV18) is detectable in approximately 90% of cervical carcinoma biopsies, often integrated into the host chromosome (67). Although the viral genome can exhibit significant rearrangements and deletions, the E6 and E7 early genes are regularly preserved and transcribed in the tumorous tissue as well as in cell lines derived therefrom (51).

Both the E6 and the E7 genes of high-risk HPVs possess transforming potential in vitro (4, 23, 38, 52), and their expression has been shown to be required for the maintenance of the transformed phenotype of cervical cancer cells in vitro and in vivo (62, 63). Recent experiments indicate a potential molecular mechanism by which these viral functions might be involved in HPV-associated cell transformation, as both E6 and E7 proteins specifically complex with the products of cellular tumor suppressor genes. E6 binds to and induces the degradation of p53 protein in vitro by a ubiquitin-dependent proteolytic pathway (48, 64), while E7 interacts with the retinoblastoma gene product (17). It is possible that by complex formation, the HPV gene products interfere with the normal control functions of these cellular growth regulatory proteins in vivo and thus contribute to malignant transformation. Indeed, it has been shown that the E6 product of high-risk HPV16 E6, but not low-risk HPV6 or HPV11 E6, can efficiently repress p53-mediated transcriptional stimulation (26, 36), a biochemical property which has been proposed to be important for the antitumorigenic activity of wild-type p53 (61). Furthermore, E6 protein from high-risk HPVs has been shown to interfere with transcriptional repressor functions of p53 (32).

These results indicate that the expression of the E6 and E7 genes of high-risk HPV types is a prerequisite for the process of HPV-associated cell transformation. The loss of intracellular control functions regulating E6/E7 transcription and the concomitant increase of viral oncogene expression has been implicated as playing a key role in the development of cervical cancer (66, 67). It therefore is of particular interest to elucidate the molecular mechanisms participating in the activation of HPV transforming gene expression. Using the high-risk HPV18 as a model system, this study analyzes the cellular functions involved in the transcriptional control of the E6 and E7 genes.

HPV18 E6/E7 transcription is regulated by *cis*-active elements contained within the so-called upstream regulatory region (URR), which extends over 825 bp between the late and early open reading frames within the viral genome (Fig. 1). The HPV18 URR has been shown to be a major determinant of the transformation potential of the virus (45). A variety of cellular transcription factors, including NF1, AP1, KRF-1, Oct-1, Sp1, and the glucocorticoid receptor, have been proposed to bind to the HPV18 URR and to participate in the transcriptional regulation of the E6/E7 promoter located at the 3′ terminus of

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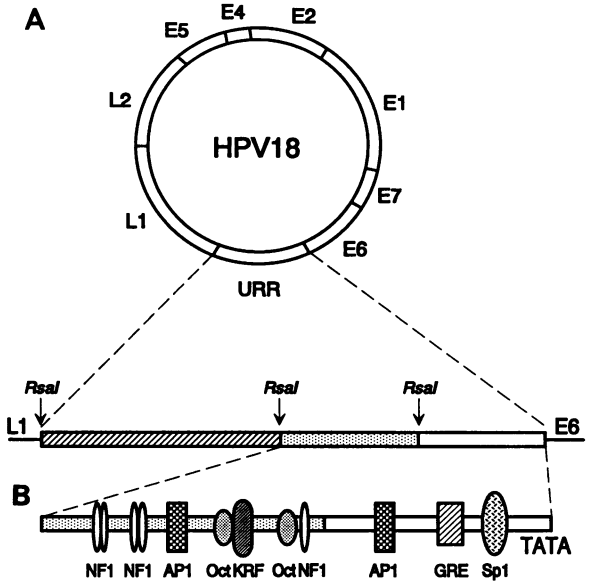

FIG. 1. (A) Schematic representation of HPV18 genome organization. The URR separates the late (L) from the early (E) genes and can be subdivided by *Rsa*I digestion (21) into (i) a 389-bp 5′-terminal region (dashed area), (ii) a centrally located 230-bp constitutive enhancer (stippled area), and (iii) a 206-bp promoter-proximal region (open area). (B) Symbolic representation of the factors binding to the HPV18 enhancer and promoter-proximal region (compiled from references 8, 20, 22, 24, and 33).

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18NF1(7512)   5'-GTACGCTGGCACTATTGCAAAATTTAATC-3'
18NF1M(7512)  5'-GTACGCaGaCACTATTGtAtAATTTAATC-3'

18NF1(7568)   5'-CCATTGGCGCGCCTCTTTGGCGCATAC-3'
18NF1M(7568)  5'-CCATaGaCGCGCCTCTTaGaCGCATAC-3'

18AP1E        5'-TGGCGATACAAGGCGCACCTGGTATTAGTCATTTTCCTGTCC-3'
18AP1EM       5'-TGGCGATACAAGGCGCACCTGGTActagacTTTCCTGTCC3-3'

18KRF         5'-TGCTTGCATAACTATATCCACTCCCTATGT-3'
18KRFM        5'-TGCTtaacgAACTATATCCACTaaaTATGT-3'

18OCT/NFI     5'-TTAAGCTAATTGCATACTTGGCTTGTACAACTACTTTCATGTCCAACATTCTGT-3'
18OCTM        5'-TTAAGCTAAgTaaATACTTGGCTTGTACAACTACTTTCATGTCCAACATTCTGT-3'
18NF1M(7730)  5'-TTAAGCTAATTGCATACTTGGtaTGTACAACTACTTTCATGTCCAACATTCTGT-3'

18AP1P        5'-GAACTATAATATGACTAAGCTGTGC-3'
18AP1PM       5'-GAACTATAATATaAgcttGCTGTGC-3'

18GRE         5'-AGGTTGGGCAGCACATACTATACTTTTC-3'
18GREM        5'-AGGTTGGGCAGCggATACTATACTTTTC-3'

18SP1         5'-GTAGTATATAAAAAAGGGAGTGACCGA-3'
18SP1M        5'-GTAGTATATAAAAAAactAGTGACCGA-3'
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FIG. 2. DNA sequences of oligonucleotides derived from the HPV18 URR, which were used for binding analyses and in vitro mutagenesis. For ease of presentation, only the sense strand of each oligonucleotide is shown. Mutant oligonucleotides are indicated by the letter "M." Mutated sequences are indicated by lowercase letters. Binding motifs are highlighted by boxes. Oligonucleotides 18NF1(7512), 18NF1(7568), and 18OCT/NF1 contain the NF1 binding motifs (22) at HPV18 nucleotides 7512, 7568, and 7730, respectively (numbering according to reference 12) within the constitutive enhancer (18OCT/NF1 contains an additional octamer motif, AATTG CAT). 18AP1E and 18AP1P encompass the AP1 recognition sites (20) within the enhancer and the promoter-proximal region, respectively. 18KRF contains the binding motif for KRF-1 (33), while 18GRE and 18Sp1 carry the GRE (8) and the Sp1 recognition site (24), respectively, within the promoter-proximal region.

the URR (8, 20, 22, 24, 25, 33, 41, 60). With the exception of the HPV18 AP1 (60) and Sp1 (24) elements, most approaches to investigate the functional significance of cellular factors for the transcriptional regulation of HPV18 or HPV16 have been performed by analyzing the *cis* activity of isolated DNA-binding elements which have been removed from the natural context of the viral URR and cloned upstream of a heterologous promoter (5, 8, 11, 21, 22, 29, 33, 37). However, it is known that the activity of a given regulatory element can strongly depend on the overall composition of a transcriptional control region, i.e., on the nature of potentially cooperating *cis* motifs. Furthermore, the proper spacing between different *cis*-regulatory elements can be highly important for their functional cooperation (19, 42). Finally, the activity of *cis* elements can also be promoter dependent (39, 49). In view of these findings, we assessed the functional significance of individual *cis*-regulatory elements for HPV18 E6/E7 transcription by performing a site-specific mutational analysis of the transcriptional control elements within the physiological context of the HPV18 URR. The effects of single and combined mutations of specific *cis* elements on the transcriptional activity of the HPV18 URR were analyzed by transient transfection of luciferase reporter constructs into HeLa cervical carcinoma cells and into the spontaneously immortalized human keratinocyte cell line HaCaT (6).

Previous analyses of the HPV18 URR defined the constitutive enhancer region as being indispensable for efficient E6/E7 promoter stimulation (21, 28, 56, 58). To contribute to the understanding of the functional composition of the HPV18 enhancer and its cooperative interplay with promoter elements, a mutational analysis of the enhancer, either linked to the homologous HPV18 promoter region or upstream of the truncated, heterologous herpes simplex virus (HSV) thymidine kinase (TK) promoter, was also performed.

The results obtained in this study define the relative contribution of individual *cis*-regulatory elements to the transcriptional activity of the HPV18 URR, identify host cell factors involved in the control of HPV18 E6/E7 oncogene expression, and indicate potential regulatory circuits involved in the tissue specificity of the HPV18 URR. Compared with previous analyses investigating isolated subregions of the HPV16 or HPV18 URR upstream of heterologous promoters, several *cis*-regulatory elements exhibited a different functional behavior in the context of the complete HPV18 URR. The dissection of the HPV18 constitutive enhancer shows that its activity is dependent on a functional synergism between a centrally located AP1 element and heterologous enhancer elements contained in its flanking sequences. In addition, our results reveal that the nature of the test promoter influences the activity of the HPV18 enhancer in a cell-type-specific manner, underlining the need to examine the functional significance of *cis*-active elements in conjunction with the homologous viral promoter region.

MATERIALS AND METHODS

Plasmids and oligonucleotides. Oligonucleotides (nucleotide sequences are shown in Fig. 2) representing wild-type or mutated sequences of the HPV18 URR were synthesized with a Gene Assembler Plus (Pharmacia) and purified by polyacrylamide gel electrophoresis. Basic vector pBL and reporter plasmid p18URRL, which contains the HPV18 URR linked to the firefly *Photinus pyralis* luciferase gene, have been described in detail before (28). Transcription of the luciferase reporter gene in p18URRL is initiated from the HPV18 E6/E7 promoter (28). Deletion constructs of p18URRL were created by

restriction enzyme digestion and conventional cloning techniques (34).

Site-specific mutagenesis of p18URRL was performed by a polymerase chain reaction (PCR)-based protocol (24). For an individual mutation, four primers were used: two complementary internal primers carrying mutated HPV18 sequences (Fig. 2) and two outer primers carrying wild-type HPV sequences. Initially, two separate PCRs were performed, each using one internal and one external primer, resulting in a DNA product containing the desired mutation in either the 3′ or the 5′ end. After purification by agarose gel electrophoresis and staining with ethidium bromide, the amplification products were directly picked in the agar with a Pasteur pipet and combined into a third PCR using the two outer primers. The resulting amplification product, carrying the desired mutation, was subcloned into p18URRL. DNA sequences were verified by the dideoxynucleotide termination reaction (47).

Reporter plasmid ptk*L contains the HSV TK promoter (nucleotides −79 to 0 [35]) and has been described previously (25).

Cells. HeLa and C33A cervical carcinoma cells, HaCaT keratinocytes (kind gift of N. Fusenig), and primary human fibroblasts derived from oral mucosa (kind gift of E. M. de Villiers) were maintained in Dulbecco's minimal essential medium (pH 7.2) supplemented with 10% fetal calf serum. Primary human foreskin keratinocytes (kind gift of L. Shahabeddin) were grown in adenine-enriched F12 medium (15). For dexamethasone titration experiments, HeLa cells were grown after transfection for 30 h in Dulbecco's minimal essential medium containing 10% charcoal-stripped fetal calf serum, supplemented with various concentrations (see Fig. 7) of dexamethasone (Sigma).

Transfections and transient expression assays. Approximately 5×10^5 cells were transfected by calcium phosphate coprecipitation (9). Transfection mixtures usually contained 3 μg of the HPV18-luciferase reporter plasmid and 0.5 μg of the internal standard pAc-Gal (28) to account for variations in transfection efficiency and were adjusted to a total of 6.5 μg by the addition of Bluescript DNA. For the determination of luciferase and β-galactosidase activities, cells were directly lysed on the plate and processed as described previously (27, 34). Luciferase activities were quantitated by using a Lumat luminometer (Berthold). Values represent the means of at least four independent transfections performed in triplicate, using at least two different plasmid preparations, each purified by two subsequent CsCl gradient ultracentrifugations. Results from individual transfections varied by less than 20%.

Nuclear extracts and gel retardation assays. Nuclear extracts were prepared by the method of Dignam et al. (14) and analyzed in gel shift assays essentially as described before (24). Polyclonal anti-Oct-1 antiserum (44) was kindly provided by I. W. Mattaj (European Molecular Biology Laboratory, Heidelberg, Germany) and included in the binding reaction mixture before addition of the radiolabeled probe. Purified NF1 protein from rat liver was kindly provided by J. Schmitt and H. Stunnenberg (European Molecular Biology Laboratory). For NF1 gel shifts, purified NF1 protein was mixed with 1 μg of poly(dI-dC) in a 25-μl reaction volume containing 10% glycerol, 50 mM NaCl, 1.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 1% Triton X-100. Following a 5-min incubation on ice, 12,000 cpm of [γ-^{32}P]ATP-5′-end-labeled double-stranded oligonucleotide probe (10 to 20 fmol) was added, and incubation was continued on ice for 15 min. After separation of DNA-protein complexes from unbound probe on a 4% (29:1 cross-linking ratio) nondenaturing polyacrylamide gel containing 0.1% Triton X-100, dried gels were exposed overnight to Kodak X-Omat film.

RESULTS

Functional significance of individual *cis*-regulatory elements for E6/E7 promoter activation in the context of the complete HPV18 URR. A number of cellular transcription factors have been implicated as binding to potential *cis*-regulatory elements within the HPV18 URR (Fig. 1). Their individual recognition motifs were specifically mutated in the natural context of the complete HPV18 URR (Fig. 2 shows wild-type and mutated sequences) by using a PCR-based protocol for site-directed mutagenesis (24). By comparison with the transcriptional activity of the wild-type HPV18 URR, the effects of these mutations were monitored by transient transfection assays in HaCaT and HeLa cells. In addition, gel retardation assays were performed to characterize the factors binding to the various subregions of the HPV18 URR and to verify that the introduced mutations interfered with the binding of the respective regulatory factors to their recognition sites.

(i) The NF1 elements within the constitutive enhancer region only marginally contribute to E6/E7 promoter activity. By analyzing isolated subfragments of the closely related HPV16 URR linked to a heterologous promoter, NF1 elements have been implicated as playing a key role in the transcriptional stimulation of the E6/E7 promoter of oncogenic HPV types (10, 11). On the basis of footprinting experiments with purified NF1 protein, it has been proposed that the HPV18 URR contains three binding sites for the cellular transcription factor NF1 (22), all located within its constitutive enhancer portion (Fig. 1). Compared with the NF1 consensus motif TTGGCT(N)$_3$AGCCAA (31), the two upstream elements [18NF1(7512) and 18NF1(7568)] contain sequence-aberrant recognition sites (Fig. 2), while the downstream element [18NF1(7730)] contains only one half-site recognition motif (TTGGCT). As shown in Fig. 3A, gel shift analyses indicate that all three putative HPV18 NF1 elements are bound by purified NF1 protein in vitro, while the mutation of each NF1 recognition motif abolished complex formation.

To assess the contribution of each of the NF1 elements to the transcriptional activity of the HPV18 E6/E7 promoter, these mutations were individually introduced into the complete context of the HPV18 URR. However, neither one of the NF1 binding site mutations, although abolishing NF1 binding, strongly reduced the activity of the HPV18 URR in HeLa or HaCaT cells (Fig. 4), indicating that these elements by themselves are dispensable for efficient E6/E7 promoter stimulation.

As it was possible that the inactivation of a single NF1 module is functionally compensated for by the remaining two intact NF1 elements within the constitutive enhancer, the effect of a triple mutation which simultaneously inactivated the three NF1 elements was examined. However, as observed for the single mutations, the combined inactivation of all three NF1 recognition elements did not lead to a strong reduction in the transcriptional activity of the HPV18 URR (Fig. 3B). Taken together, these results indicate that the NF1 elements within the HPV18 URR do not play a crucial role in activation of the E6/E7 promoter.

(ii) The integrity of the AP1 elements is indispensable for efficient HPV18 E6/E7 promoter activity. The HPV18 URR contains two AP1 elements, one located within the constitutive enhancer region and one contained within the promoter-proximal region (Fig. 1). Each AP1 element was mutated in the

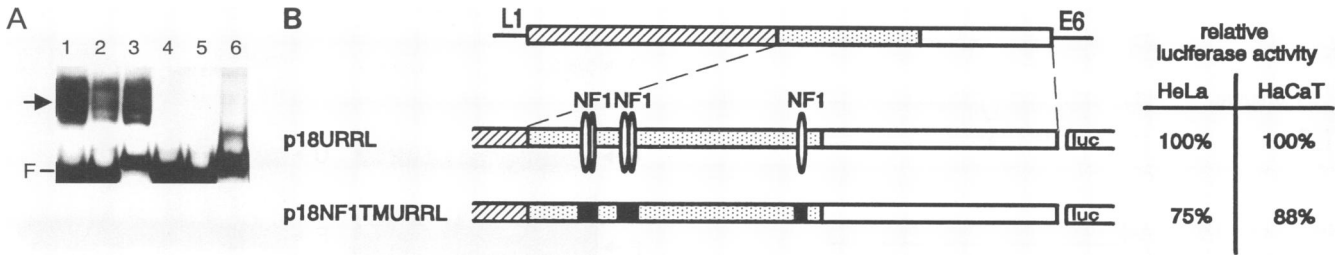

FIG. 3. Binding of NF1 to the HPV18 enhancer. (A) Purified NF1 protein (arrow) binds to three oligonucleotides derived from HPV18 constitutive enhancer in gel retardation analysis. Lanes: 1 to 3, interaction of NF1 with wild-type oligonucleotides 18NF1(7512), 18NF1(7568), and 18OCT/NF1, respectively; 4 to 6, loss of NF1 binding after mutation of the NF1 recognition sequences in 18NF1M(7512), 18NF1M(7568), and 18NF1M(7730), respectively. F, free probe. (B) Transcriptional activity of the HPV18 URR after simultaneous mutation (represented by a solid bar) of all three NF1 elements (p18NF1TMURRL) in percent relative to the activity of the wild-type URR (p18URRL) after transfection into HeLa and HaCaT cells.

context of the complete URR, resulting in the loss of AP1 binding in vitro as assessed by gel retardation assays (not shown). In contrast to the minor effects of the inactivation of the NF1 elements, the specific mutation of either one of the AP1 elements within the HPV18 URR led to a strong reduction of E6/E7 promoter activity in HeLa cells and an even stronger reduction in HaCaT cells (Fig. 4). These results indicate that AP1 factors play a key role in activation of the HPV18 E6/E7 promoter. Furthermore, they show that the inactivation of one of the two AP1 elements within the HPV18 URR cannot be functionally compensated for by the remaining wild-type element, indicating that the integrity of both AP1 elements is required for efficient activation of the E6/E7 promoter.

(iii) Cell-type-specific activity of the KRF-1 motif. The HPV18 constitutive enhancer region has been shown to interact with an epithelial factor designated KRF-1, which binds to a recognition motif overlapping a low-affinity Oct-1 binding site. In reporter assays analyzing subregions of the HPV18 URR upstream of a heterologous promoter, this complex regulatory element has been shown to contribute strongly to the activity of the HPV18 constitutive enhancer in epithelial cells through the binding of KRF-1 (33).

As indicated in Fig. 4, a mutational inactivation of the KRF-1 binding motif (33) in the physiological context of the complete HPV18 URR had a strong effect on E6/E7 promoter activity when tested after transfection into HaCaT keratinocytes. In contrast, the same mutation led to a much weaker

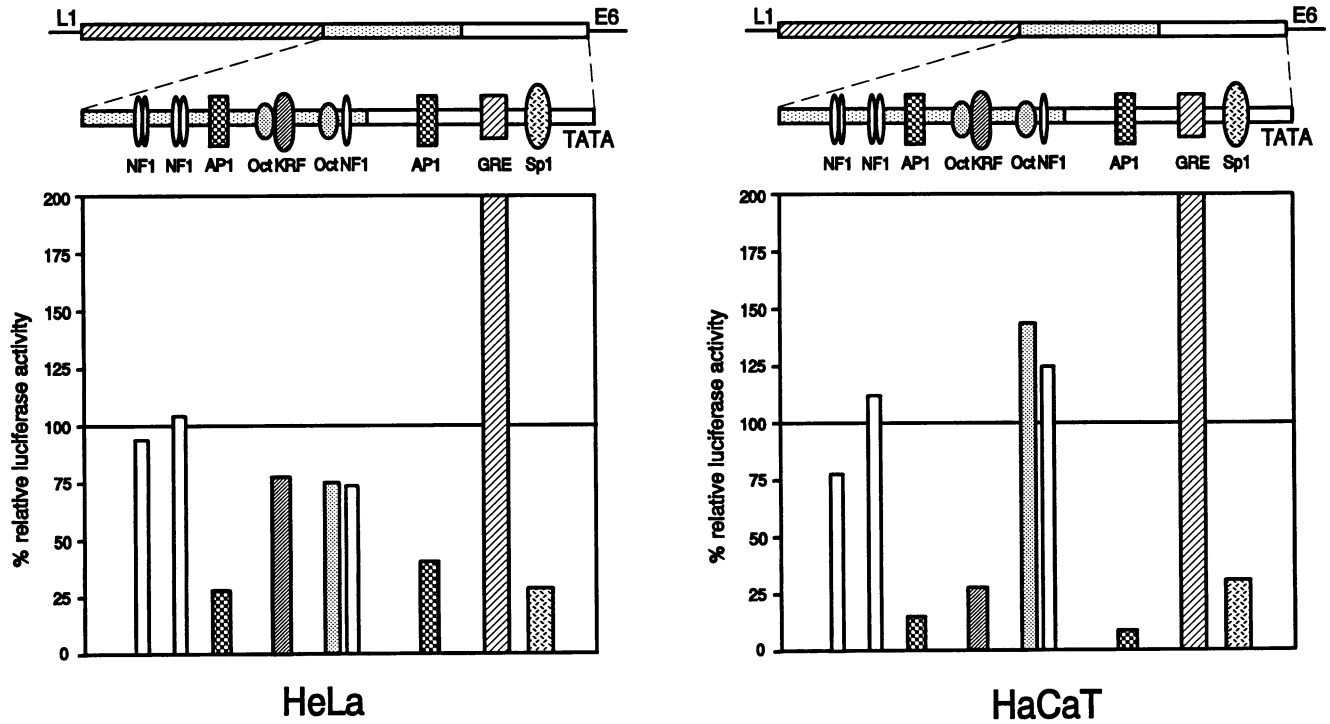

FIG. 4. Site-directed mutational analysis of the HPV18 URR. The HPV18 URR and the factors binding to the constitutive enhancer (stippled area) or the promoter-proximal region (open area) are indicated schematically. Columns below each transcription factor represent the relative activity of the E6/E7 promoter in HeLa or HaCaT cells after mutation of the respective binding site in the context of the URR. Activities are shown in percent relative to the activity of the wild-type URR, which was set 100% (represented by the horizontal line).

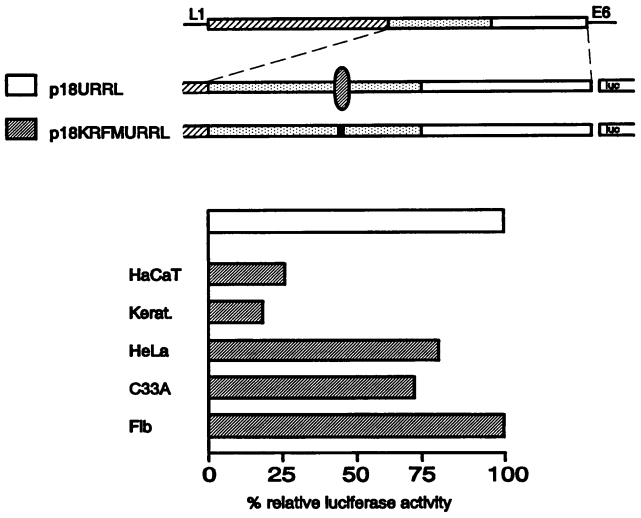

FIG. 5. Cell-type-dependent activity of the KRF-1 element. Transcriptional activity of the HPV18 URR after mutation of the KRF-1 binding motif, represented by a solid bar in plasmid p18KRFMURRL, is shown in percent (striped columns) relative to the activity of the wild-type URR in p18URRL (open column) after transfection into HaCaT keratinocytes, primary human foreskin keratinocytes (Kerat.), HeLa and C33A cervical carcinoma cells, and primary human dermal fibroblasts (Fib).

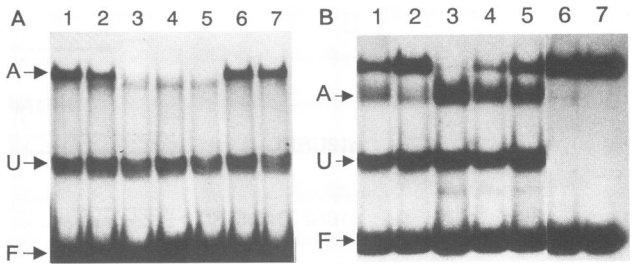

FIG. 6. Sequence-specific binding of Oct-1 to the 3′ portion of the HPV18 enhancer. (A) Gel retardation assay of p18OCT/NF1 with HeLa crude cell extract. Lanes: 1, Oct-1 binding to p18OCT/NF1 (complex A); 2 and 3, competition with 10- and 100-fold molar excesses of unlabeled self-oligonucleotide, respectively; 4 and 5, competition with a 100-fold molar excess of an oligonucleotide containing the Oct-1 consensus sequence derived from the human histone H2B promoter (18) and by an oligonucleotide containing four copies of only the octamer motif within p18OCT1/NF1, respectively; 6 and 7, competition with 10- and 100-fold molar excesses of a heterologous oligonucleotide containing the NF1 binding sequence of the H-*ras* promoter (31), respectively. F, free probe. Complex U represents a nonspecific DNA-binding activity in HeLa cell nuclear extract. (B) Immunological identification of Oct-1. Complex A (lane 3) is caused by Oct-1, as complex formation is inhibited by inclusion of 1 and 2 μl of anti-Oct-1 immune serum (lanes 1 and 2, respectively) but not by the same volumes of preimmune serum (lanes 4 and 5, respectively). Both the preimmune and immune sera contain a nonspecific DNA-binding activity resulting in a slowly migrating complex (lanes 6 and 7, respectively). F, free probe. Note that under the conditions used to detect Oct-1 binding to the octamer motif, the interaction of NF1 with the adjacent NF1 half-site in 18OCT/NF1 is not detectable in HeLa cell crude nuclear extract.

reduction of activity in HeLa cells (Fig. 4). To investigate whether this differential functional behavior is reflected by differences in the pattern of proteins binding to the wild-type and mutated KRF-1 recognition motifs, gel retardation assays using HeLa and HaCaT nuclear extracts were performed. However, no significant differences in binding to the KRF-1 motif could be detected between these cell types (not shown).

The much stronger reduction in E6/E7 promoter activity after mutational inactivation of the KRF-1 binding motif in HaCaT cells than in HeLa cells indicates that the activity of the KRF-1 element can vary significantly between different epithelial cell types. In agreement with this hypothesis, mutation of the KRF-1 recognition motif within the complete HPV18 URR led to a much stronger reduction in E6/E7 promoter activity in primary human keratinocytes than in C33A cervical carcinoma cells (Fig. 5). Consistent with the postulated epithelial cell specificity of KRF-1 (33), the mutation of its recognition site did not result in any measurable effect on the transcriptional activity of the HPV18 URR in primary dermal fibroblasts (Fig. 5).

(iv) The octamer motif within the 3′ terminus of the constitutive enhancer is not required for efficient stimulation of the E6/E7 promoter. It has been demonstrated that the HPV16 enhancer contains a sequence-aberrant Oct-1 binding site located close to a half-palindromic NF1 recognition element (10). These HPV16 sequences have been reported to confer transcriptional stimulation on a heterologous promoter when tested as isolated fragments in transient reporter assays in HeLa cells (37). A corresponding sequence (AATTGCAT) is located adjacent to the half-palindromic NF1 recognition motif at the 3′ terminus of the HPV18 constitutive enhancer region (Fig. 1 and 2). Gel retardation assays indicate that Oct-1 protein contained in HeLa cell crude nuclear extract binds to the HPV18 motif in a sequence-specific manner (Fig. 6A). Furthermore, complex formation is abolished by the inclusion in the binding reaction mixture of polyclonal Oct-1 antiserum but not preimmune serum (Fig. 6B). These data demonstrate that the 3′ portion of the HPV18 constitutive enhancer contains a sequence-aberrant Oct-1 recognition element.

Functional analysis of this octamer element within the HPV18 URR reveals that the introduction of a specific mutation abolishing Oct-1 binding (not shown) did not lead to a strong modulation of E6/E7 promoter activity (Fig. 4). These findings indicate that within its physiological context, the integrity of this Oct-1 element is not crucial for the activation of the E6/E7 promoter in either HeLa or HaCaT cells.

(v) Mutation of the GRE leads to an upregulation of basal HPV18 E6/E7 promoter activity. The HPV18 URR carries a sequence, AGCACATACTATACT, in its promoter-proximal region which has been shown to confer dexamethasone and progesterone inducibility when tested as an isolated oligonucleotide upstream of the heterologous HSV TK promoter (8). To assess the functional significance of this potential glucocorticoid response element (GRE) for regulation of the homologous HPV18 E6/E7 promoter activity, the element was specifically mutated in the context of the complete HPV18 URR. As indicated in Fig. 4, mutation of the GRE motif led to a consistent upregulation of the basal activity of the HPV18 URR by a factor 2 to 3.

Titration experiments were performed to analyze the role of the GRE in conferring the response of the HPV18 E6/E7 promoter to varied levels of glucocorticoid hormones. While the wild-type HPV18 URR exhibited a dose-dependent, dexamethasone-induced increase in transcriptional activity, mutation of the GRE completely abolished hormone-dependent stimulation of the E6/E7 promoter (Fig. 7). These results show that the GRE within the promoter-proximal portion mediates the response of the E6/E7 promoter to glucocorticoids. Furthermore, this activity cannot be functionally compensated for

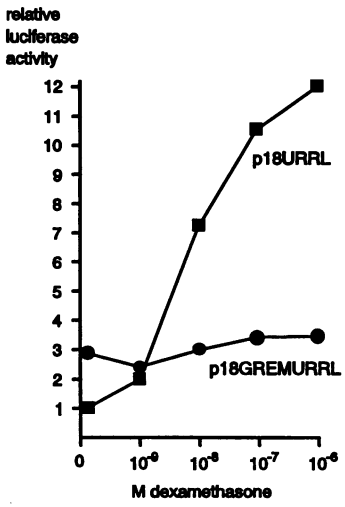

FIG. 7. Hormone induction of the HPV18 E6/E7 promoter. Squares, transcriptional activity of the wild-type HPV18 URR in p18URRL under the influence of increasing amounts of dexamethasone; circles, activity of the E6/E7 promoter after mutation of the GRE (p18GREMURRL) within the context of the complete HPV18 URR.

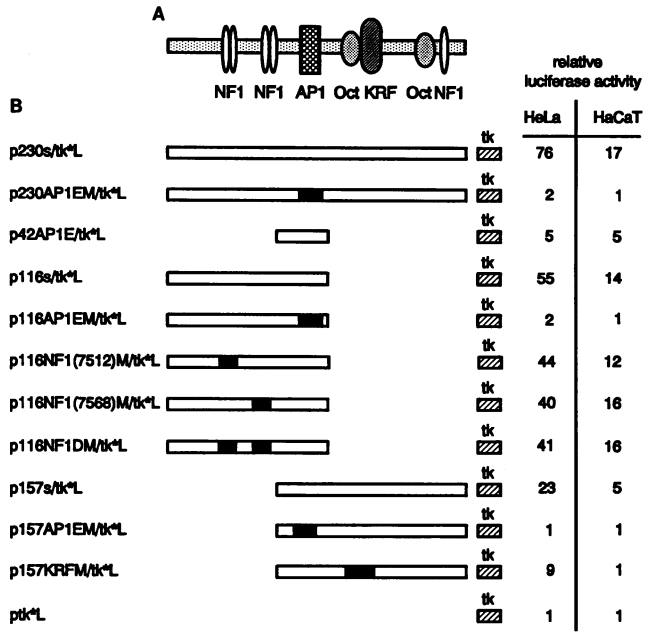

FIG. 8. Functional composition of the HPV18 enhancer. (A) Schematic representation of cellular transcription factors binding to the 230-bp constitutive enhancer region. (B) Mutations of factor binding sites are indicated by solid bars below the respective symbols. HPV18 regulatory sequences were cloned upstream the truncated (nucleotides −79 to 0 [35]) HSV TK promoter, designated tk* (25). The following HPV nucleotides (numbering according to reference 12) were contained in the reporter constructs: p230s/tk*L, 7510 to 7739; p116s/tk*L, 7510 to 7625; p157s/tk*L, 7583 to 7739; and p42AP1E/tk*L, 7583 to 7624. Columns at the right show activities of the constructs relative to that of basic vector ptk*L after transfection into HeLa cells and HaCaT keratinocytes.

by other regulatory sequences within the HPV18 URR, arguing against the presence of additional GREs within the HPV18 URR which contribute to the hormone response of the E6/E7 promoter.

(vi) The promoter-proximal Sp1-binding element is a major determinant of the transcriptional activity of the HPV18 E6/E7 promoter. Downstream of the GRE, the HPV18 URR contains a sequence-aberrant Sp1 recognition element, GG GAGT, located approximately 40 bp upstream of the TATA box of the E6/E7 promoter (24). As shown in Fig. 4, mutation of this element led to a strong reduction of the transcriptional activity of the HPV18 URR in both HaCaT keratinocytes and HeLa cells, strongly suggesting that it is important for the transcriptional activation of E6/E7 oncogene expression. Since after removal of the constitutive enhancer the HPV18 promoter-proximal region by itself does not exhibit transcription-activating potential (28, 58), the promoter-proximal Sp1 element generates its transcription-stimulating activity by functionally cooperating with *cis* elements contained within the viral enhancer.

Interplay between the constitutive enhancer region and promoter elements within the HPV18 URR. The HPV18 URR (Fig. 1) has been subdivided by *Rsa*I digestion into three functional units (21): (i) a 5′-terminal 389-bp region of unknown function, encompassing almost half of the URR but contributing only marginally to the transcriptional stimulation of the E6/E7 promoter (25, 58); (ii) a 230-bp constitutive enhancer region that plays a key role in the efficient activation of HPV18 E6/E7 transcription (21, 28, 56, 58); and (iii) a 206-bp promoter-proximal region containing the E6/E7 promoter at its 3′ terminus (59). To analyze the molecular mechanisms resulting in the generation of enhancer activity, a mutational analysis of *cis* elements within the context of the 230-bp constitutive enhancer region was performed. The functional consequences of these mutations on HPV18 enhancer activity were first analyzed upstream of a well-defined minimal HSV TK promoter (35) and then compared with the activity of the enhancer when linked to its homologous promoter-proximal region.

(i) Composition of the HPV18 constitutive enhancer. As shown in Fig. 8, the 230-bp enhancer significantly stimulated the HSV TK promoter in both HeLa and HaCaT cells. Enhancer activity was abolished in both cell types by a mutation of the centrally located AP1 recognition motif (p230AP1EM/tk*L), indicating a crucial role for this motif for the generation of enhancer activity. In contrast, an oligonucleotide containing the isolated AP1 element (p42AP1E/tk*L) only weakly stimulated the TK promoter in HeLa and HaCaT cells. The almost complete loss of enhancer function after mutation of the AP1 element within the 230-bp enhancer and the observation that the AP1 element by itself only weakly activates transcription indicate that the HPV18 constitutive enhancer generates its transcription-stimulating activity by a functional cooperation between the AP1 module and additional *cis* elements present in this region.

To map these elements, we created sets of reporter constructs (Fig. 8) which contain the AP1 recognition site linked to either its 5′ (p116s/tk*L) or its 3′ (p157s/tk*L) flank, conserving the spacing between the *cis* elements as present in the natural context of the 230-bp enhancer. Functional analysis of these constructs revealed that the AP1 element can synergistically interact with elements present in both portions of the enhancer, consistently showing a stronger activation when linked to its 5′ flank than when in conjunction with its 3′ flank. Mutation of the AP1 element resulted in loss of enhancer activity in both types of constructs (p116AP1EM/tk*L) and p157AP1EM/tk*L). A strong drop in enhancer activity of the

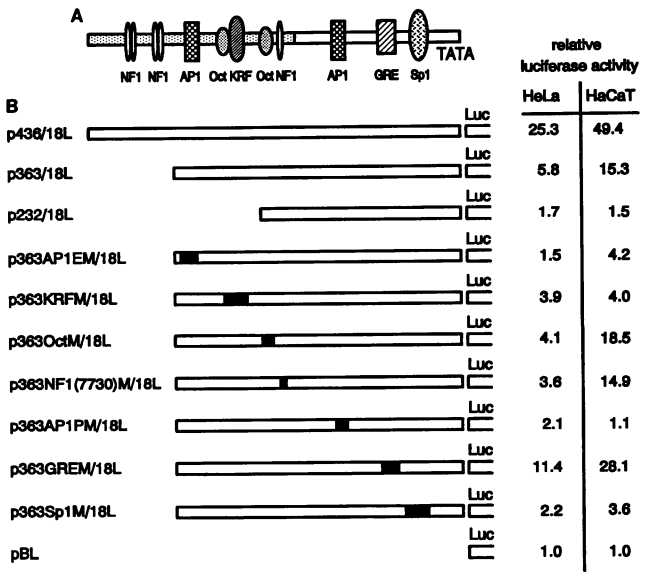

FIG. 9. Interaction between the HPV18 enhancer and its homologous promoter-proximal region. (A) Schematic representation of the cellular transcription factors binding to the HPV18 enhancer (stippled area) and the promoter-proximal region (open area). (B) Mutations of factor binding sites are indicated by solid bars below the respective symbols. Transcription of the constructs is under control of the homologous HPV18 E6/E7 promoter. Columns at the right show activities of the constructs relative to that of basic vector pBL after transfection in HeLa cells and HaCaT keratinocytes.

construct bearing the AP1 module linked to the 3′ flank of the enhancer was also observed after mutation of the KRF-1 binding site (compare p157s/tk*L with p157KRFM/tk*L), indicating that at least part of this activity stems from the functional interplay between AP1 and KRF-1. On the other hand, neither a single nor the combined inactivation of the two NF1 binding sites present in the 5′ flank of the AP1 element resulted in a significant loss of enhancer activity exerted by the 5′ portion of the enhancer [compare p116NF1(7512)M/tk*L, p116NF1(7568)M/tk*L, and p116NF1DM/tk*L with p116s/tk*L]. These data indicate that the synergistic generation of enhancer activity between the AP1 module and *cis* elements within the 5′ flank of the constitutive enhancer does not stem from an interplay between AP1 and NF1 but rather indicates the existence of a yet uncharacterized *cis*-regulatory element in this portion of the enhancer which functionally cooperates with AP1.

(ii) Influence of HPV18 promoter-proximal elements on the activity of the constitutive enhancer. To investigate the function of the HPV18 constitutive enhancer region in conjunction with its homologous promoter-proximal region, the 5′-terminal 389-bp portion of the URR was deleted. The resulting construct, p436/18L (Fig. 9), thus contains the 230-bp constitutive enhancer linked to the promoter-proximal fragment of the URR, without changes in the natural spacing between these elements. Importantly, and opposite the activity of the 230-bp enhancer upstream the heterologous TK promoter (p230s/tk*L; Fig. 8), p436/18L exhibited a stronger activity in HaCaT than in HeLa cells (Fig. 9), demonstrating that the interaction of the HPV18 constitutive enhancer with its homologous promoter-proximal region results in a relative increase in enhancer activity in HaCaT keratinocytes compared with HeLa cells.

A strong drop in E6/E7 promoter activity was observed after deletion of the 5′-terminal 73 bp of the constitutive enhancer region (p363/18L), removing the sequences 5′ to the AP1 binding site located in the center of the 230-bp constitutive enhancer region. In agreement with the results obtained from analysis of subfragments of the HPV18 enhancer (Fig. 8), these findings again indicate the presence of important *cis*-activating elements within the 5′ portion of the constitutive enhancer, which contribute to the transcriptional stimulation of the E6/E7 promoter. The activity of the E6/E7 promoter was almost completely abolished after further deletion of 141 bp of the enhancer which contain the AP1 and the KRF-1/Oct-1 modules, indicating that the promoter-proximal region (p232/18L) of the HPV18 URR does not possess *cis*-stimulatory activity by itself, despite the presence of binding sites for transcriptional activators such as AP1, the glucocorticoid receptor, or Sp1.

The smallest HPV18 URR deletion construct exhibiting significant stimulation of the E6/E7 promoter was p363/18L, containing the 157-bp 3′-terminal portion of the enhancer linked to the promoter-proximal region. As observed for the functional composition of the complete HPV18 URR, mutation of either one of the AP1 recognition sites in this minimal transcriptional regulatory unit led to a strong loss of E6/E7 promoter activity (p363AP1EM/18L and p363AP1PM/18L), while mutation of the KRF element (p363KRFM/18L) resulted in a much stronger reduction of activity in HaCaT keratinocytes than in HeLa cells. The activity of the E6/E7 promoter was only marginally altered by mutation of either the octamer binding motif or the NF1 half-site located at the 3′ terminus of the constitutive enhancer [p363OctM/18L and p363NF1(7730)M/18L] but strongly reduced after inactivation of the Sp1 element within the promoter-proximal portion of the URR (p363Sp1M/18L). Finally, mutation of the GRE resulted in an increase in E6/E7 promoter activity (p363GREM/18L).

DISCUSSION

The transcriptional activity of the HPV18 E6/E7 promoter is modulated by *cis* elements contained within the viral URR. However, many questions regarding the cellular control mechanisms involved in this regulation are still open. Which are the key factors for the efficient stimulation of E6/E7 transcription? Which factors determine the epithelial cell-specific activity of the HPV18 URR? Which elements mediate the hormone responsiveness of the E6/E7 promoter? Which role is played by individual *cis*-regulatory elements during the cooperative generation of the transcriptional stimulatory activity exerted by the viral URR? To answer these questions, a mutational analysis of the HPV18 URR was performed. Care was taken to investigate the biological significance of individual *cis*-regulatory elements within the physiological context of the complete HPV18 URR.

Transient transfection experiments comparing the transcriptional activity of the wild-type HPV18 URR with activities of mutated templates indicate that efficient transcriptional stimulation of the HPV18 E6/E7 promoter is largely dependent on the integrity of binding sites for AP1, Sp1, and in certain epithelial cells, KRF-1. While Sp1 is considered a rather ubiquitous factor playing an important role in the transcriptional activation of a wide array of cellular and viral promoters, the composition of the heterogeneous family of AP1 proteins has been shown to be cell type dependent (1). AP1 factors are dimeric protein complexes formed by members of the *jun* and *fos* multigene families and belong to the group of bZip proteins

characterized by a basic DNA binding domain and an adjacent leucine zipper domain required for protein dimerization. The transcriptional activity of AP1 proteins can depend both on their individual structural composition and on the particular context of a transcriptional control region on which they are acting. For example, subtle sequence variations from the AP1 consensus binding motif are preferentially recognized by specific subsets of AP1 proteins or other bZip factors, such as ATF or CREB (54). Furthermore, it has been shown that c-*jun*-containing AP1 complexes can activate control regions containing single AP1-binding elements, while *junB* has been reported to require multiple recognition elements for transcriptional activation (1). Interestingly, many HPVs, including HPV16 and HPV18, possess more than one potential AP1 recognition motif within their respective URRs (11, 13, 41, 60). These structural features raise the possibility that *junB* is important for stimulation of the E6/E7 promoter.

As high amounts of *junB* have been found in the upper, differentiated cells of the epidermis (65), which correspond to the layers of the epithelium where efficient viral E6/E7 transcription can be detected by in situ hybridization (16, 55), it is tempting to speculate that *junB* is involved in the tissue specificity of HPV transcription. AP1 factors have been proposed before to contribute to the epithelial cell-specific activity of the HPV18 URR (41). Recently, it was shown that *junB* represents the major *jun* component binding to the HPV18 AP1 motifs in HaCaT and HeLa cells and that AP1 elements, in agreement with the present study, are important for the activation of the HPV18 E6/E7 promoter in the context of the complete URR (60). If the HPV18 URR were predominantly activated by *junB* and if efficient transactivation by *junB* required more than one AP1 response element, the strong loss in transcriptional activity after mutation of either one of the two AP1 motifs in the context of the complete URR could be explained.

Surprisingly, we did not obtain any experimental evidence that NF1 elements play a major role in stimulation of the HPV18 E6/E7 promoter in the natural context of the HPV18 URR, nor did we observe a strong reduction in the transcriptional activity of HPV18 enhancer fragments upstream of a heterologous promoter after mutational inactivation of the NF1 recognition motifs. These results are in contrast to reports investigating the role of NF1 elements in subfragments of the HPV16 enhancer upstream of a heterologous promoter, in which NF1 elements had been implicated, by functionally cooperating with AP1 elements, to represent major determinants for the stimulation of E6/E7 transcription (10, 11). Although we cannot exclude the possibility that the NF1 motifs within the HPV16 URR and HPV18 URR are of different functional significance, we feel that given the similar compositions of these two regulatory regions, this possibility is rather unlikely. Thus, at this point we cannot provide an explanation for these conflicting results. However, it seems possible that the NF1 elements contribute to the regulation of other, yet unidentified promoters within the HPV18 URR or are involved in viral replication, as shown for adenoviruses (46). It is noteworthy that in gel retardation assays, the affinity of NF1 for the sequence-aberrant HPV18 recognition motifs is much lower than its affinity for an NF1 consensus oligonucleotide (7). This observation might explain why we did not detect NF1 binding to oligonucleotide 18OCT/NF1 in crude nuclear extract (Fig. 6B).

Although the inactivation of the two NF1 motifs in the 5′ portion of the constitutive enhancer only marginally reduced its transcriptional activity, deletion of this fragment led to a strong loss of enhancer activity when the fragment was cloned upstream of either homologous and heterologous promoters. Furthermore, this portion of the enhancer can functionally cooperate with the AP1 element in the center of the enhancer, independently of the integrity of the NF1 motifs. These data suggest that the 5′ flank of the constitutive enhancer is a target for hitherto undetected *trans*-active factors which can synergistically interact with AP1 for the generation of enhancer activity. It should be noted that this part of the enhancer contains sequences related to the binding motif GCATACTT for the cellular transcription factor TEF-1 (42), which has recently been reported to bind to and activate the HPV16 enhancer (29). More work is required to identify the regulatory units contained in this portion of the HPV18 enhancer and to investigate the binding of TEF-1 to the HPV18 URR. Furthermore, it should be noted that the HPV16 enhancer contains a *cis*-active 63-bp enhancer core, which does not contain AP1 elements (13). In contrast, we did not obtain evidence for AP1-independent enhancer activity for HPV18, as both the complete 230-bp constitutive enhancer as well as subfragments derived therefrom strictly required intact AP1 elements for significant *cis*-stimulating activity.

Recent experiments ascribed an important *cis*-activating role to the octamer motif located at the 3′ end of the constitutive enhancer when tested upstream of the heterologous TK promoter in HeLa cells (37). However, although we found that this motif is bound by Oct-1 in a sequence-specific manner, we did not measure a strong alteration of E6/E7 promoter activity after mutation of this sequence in the physiological context of the HPV18 URR. Observations that the transcriptional activity of octamer elements can depend on the distance to and the context of a test promoter (43, 49, 57) could provide an explanation for the differing results obtained in analyzing the transcriptional activity of this element by these different approaches. We also cannot exclude the possibility that additional octamer elements within the HPV18 URR can functionally compensate for the mutation of an individual motif.

The inactivation of the GRE within the promoter-proximal portion of the URR abolished the dexamethasone response of the E6/E7 promoter, demonstrating that this element mediates the effects of glucocorticoid hormones on E6/E7 oncogene transcription. The finding that hormone responsiveness of the E6/E7 promoter is completely lost after mutation of this element provides strong evidence that other GREs potentially present in the HPV18 URR do not contribute considerably to the hormone induction of E6/E7 transcription.

Interestingly, mutation of the GRE also led to a consistent upregulation of the basal E6/E7 promoter activity. At present, we do not know the molecular basis for this mechanism. However, it is possible that the mutation resulted in the inactivation of a negative regulatory element within this portion of the URR. Whether this mechanism involves the glucocorticoid receptor itself or possibly a regulatory factor binding to an overlapping recognition motif remains to be clarified. The presence of negative regulatory elements in the promoter-proximal portion of the HPV18 URR is also suggested by the observation that this region by itself, despite containing potential *cis*-active elements such as AP1 or Sp1 recognition motifs, does not exhibit any intrinsic enhancer activity. Recently, the cellular transcription factor YY1 has been proposed to be involved in a negative control of E6/E7 promoter activity by binding to a recognition motif in this portion of the URR (3).

Analyzing the interplay between the HPV18 enhancer and promoter elements, we found that in HaCaT cells, the HPV18 constitutive enhancer region is more active when linked to its homologous promoter region than when in conjunction with

the heterologous TK promoter, while in HeLa cells, the enhancer shows the opposite behavior. This finding could be explained by the presence of transcriptional elements in the promoter-proximal portion of the URR, which more efficiently interact with enhancer elements in HaCaT than in HeLa cells. Alternatively, the promoter-proximal region might contain negative regulatory elements which are less active in HaCaT cells. In addition, it is possible that a positive factor which plays a role in activation of the enhancer linked to its homologous promoter region is limiting in HeLa cells. These findings show that the activity of HPV18 enhancer elements can be influenced by the nature of the promoter in a cell-type-dependent manner, which underlines the importance of analyzing HPV transcriptional control elements in conjunction with their homologous HPV promoter regions.

By cooperating with AP1, the cellular transcription factor KRF-1 has been shown to exhibit epithelial cell-specific transactivation activity when tested in HPV18 enhancer fragments upstream of the heterologous simian virus 40 promoter in SCC-13 keratinocytes or HeLa cells (33). In our experiments, we found that both in the natural context of the HPV18 URR as well as in constructs carrying the constitutive enhancer linked to a heterologous TK promoter or to its homologous promoter region, inactivation of the KRF-1 binding motif resulted in a much stronger reduction of E6/E7 promoter activity in HaCaT cells or primary human keratinocytes than in HeLa or C33A cells. It is possible that in the latter cell types, the loss of KRF-1 activity is functionally compensated for by another factor binding elsewhere in the URR. Taken together with the observed promoter-dependent differential regulation of the viral enhancer in epithelial cells, these results indicate that the regulatory mechanisms resulting in the transcriptional activation of the HPV E6 and E7 genes can significantly differ among epithelial cells. While a number of candidate proteins have been implicated as contributing to the tissue-specific activity of the HPV18 or HPV16 URR (5, 10, 24, 33, 40, 41, 53), it seems unlikely that the epithelial cell-specific activity of the HPV18 URR stems simply from a common, epithelial transactivating factor but rather may result from alternate regulatory pathways in different epithelial cells.

Transcription factors themselves are modified in their activities by distinct biochemical pathways. For example, activities of certain AP1 factors can be stimulated by phorbol esters, specific growth factors as epidermal growth factor or transforming growth factor beta, and the products of proto-oncogenes such as c-*mos*, c-*src*, or c-Ha-*ras* (1). On the other hand, the activity of AP1 elements can be inhibited by steroid hormone receptors (30, 50), and a recent report demonstrated repression of the HPV18 E6/E7 promoter by retinoic acid receptor beta (2). It is conceivable that the malfunction of biochemical pathways regulating the activity of crucial transcription factors can contribute to the deregulated stimulation of E6/E7 transcription during HPV-associated carcinogenesis (66, 67). The definition in this work of the biological significance of regulatory proteins for the transcriptional control of HPV18 E6/E7 oncogene expression thus provides an experimental basis for the investigation of specific cellular mechanisms resulting in the activation of HPV oncogene expression during HPV-associated carcinogenesis.

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