JOURNAL OF VIROLOGY, June 1992, p. 3740–3748
0022-538X/92/063740-09$02.00/0
Copyright © 1992, American Society for Microbiology
Vol. 66, No. 6

Two AP1 Sites Binding JunB Are Essential for Human Papillomavirus Type 18 Transcription in Keratinocytes

FRANÇOISE THIERRY,[1]* GIANNIS SPYROU,[1] MOSHE YANIV,[1] AND PETER HOWLEY[2]

Unité des Virus Oncogènes, UA1149 CNRS, Institut Pasteur, 28 rue du Dr. Roux, 75724 Paris Cedex 15, France,[1] and Laboratory of Tumor Virus Biology, National Cancer Institute, Bethesda, Maryland 20892[2]

Received 27 December 1991/Accepted 12 March 1992

The activity and epithelial tropism of the human papillomavirus type 18 P_{105} early promoter, which directs the synthesis of the E6 and E7 transforming genes, are controlled by *cis* elements included in the viral long control region. To identify potential cellular regulators of this promoter, we mutagenized one or both of the 5′-TGACTAA-3′ *cis* elements capable of interacting with the AP1 transcription factor, which is composed either of homodimers or heterodimers of the Jun products or of heterodimers of Jun and Fos. Mutation of both elements completely abolished P_{105} promoter activity in human keratinocytes. We show that either AP1 site can interact efficiently in vitro with any of the three different Jun products as heterodimers with c-Fos. However, in nuclear extracts prepared from human keratinocytes, JunB was the predominant Jun component bound to the DNA probe containing this *cis* element. These results implicate JunB as an important factor in human papillomavirus type 18 transcription in keratinocytes and strongly suggest a potential role of this Jun gene product in the tissue-specific transcription of the genital papillomaviruses.

Several human papillomaviruses (HPVs), including types 16, 18, and 33, are associated with anogenital carcinomas. In many cases, the malignant cells express the viral transforming functions, which are encoded by the E6 and E7 open reading frames (42, 46, 48). These products are together necessary and sufficient for immortalization of human normal keratinocytes in culture, although in certain conditions, E6 alone or E7 alone can immortalize keratinocytes (2, 22, 32).

Transcription of the HPV-18 E6 and E7 RNAs initiates upstream of the E6 open reading frame at nucleotide (nt) 105, very close to the E6 initiation codon (42, 46, 50). The P_{105} promoter has been shown to exhibit stringent cell type specificity, since it can function only in certain human epithelial cells. These include human keratinocytes and cervical carcinoma cell lines, two cell types commonly used to study transcriptional regulation of genital papillomaviruses. A transcriptional enhancer, contained in a 200-bp subfragment of the upstream regulatory region, situated 230 bp upstream of the P_{105} initiation site and containing one AP1 binding site, also exhibits cell specificity, although not as stringently as does the whole promoter region (4, 17, 49, 52).

Binding sites for several nuclear proteins have been identified in the HPV-18 and HPV-16 regulatory regions by DNase I footprinting experiments (17, 21, 38, 47). Some of the protected sequences could be attributed to known factors either by sequence homology or by binding competitions. Among these known factors are the ubiquitous NF1/CTF, OCT1, AP1, and SP1 transcription factors. *cis* elements interacting with these factors are found in the regulatory regions of HPV-16, HPV-18, and HPV-11, although at different locations and in varying numbers. Functional analyses to investigate the role of these elements in the cell-type-specific transcription of this class of virus have indicated that these ubiquitous transcription factors may all be involved in a very complex interplay. Notably, an important role of AP1 in the enhancer activities of the HPV-16 (9, 12) and HPV-18 (34) regulatory regions has been suggested.

The HPV-18 regulatory region contains two putative AP1 binding sites, as shown by footprinting experiments using nuclear extracts from various cell types (17). The two binding sites, located at nt 7795 and 7613, at positions −171 and −349 with respect to the P_{105} start site, contain identical sequences 5′-TGACTAA-3′. Although in inverted orientations, these sequences are closely related to the AP1 binding consensus tetradecanoyl phorbol acetate (TPA)-responsive element (TRE) sequence. Binding to these sequences could be abolished by an excess of a DNA probe containing the consensus TRE sequence 5′-TGACTCA-3′ (17). Sequences similar to that of HPV-18 and binding AP1 (PEA1) were found in the polyomavirus or simian virus 40 (SV40) regulatory regions, where they play a crucial role in the control of viral transcription as well as replication (28, 31, 35). AP1, which mediates transcriptional stimulation by tumor promoters such as TPA, is a heterodimer of the products of two proto-oncogenes, *jun* and *fos*. Both *jun* and *fos* constitute multigene families; three and four different members, respectively, have been identified for each of them: c-*jun*, *junB*, and *junD*, and c-*fos*, *fosB*, *fra1*, and *fra2* (reviewed by Vogt and Bos [53]). While the different members of the *jun* and *fos* families seem to bind identical cognate sequences on the DNA with rather similar specificities, their regulation and tissue distribution are different.

In this study, we analyzed the role played by the AP1 factor binding to the *cis* elements contained in the regulatory region of HPV-18 in the transcriptional activity of the P_{105} promoter. Promoter regions mutagenized in their AP1 *cis* elements were assayed in human keratinocytes and cervical carcinoma cell lines for transient expression. These experiments revealed the crucial role of the AP1 sites for P_{105} transcription in these two cell types. The two *cis* elements of the HPV-18 regulatory region were shown to act as bona fide AP1 binding sequences in in vitro binding experiments as well as in transactivation assays. In human keratinocytes, the predominant Jun protein, determined by in vitro supershift experiments and Western immunoblot analysis, was

* Corresponding author.

JunB. Our results are thus consistent with a mechanism in which JunB is involved in the transcription of the HPV-18 transforming genes in keratinocytes. However, although essential for P_{105} transcription, this factor alone does not appear to be sufficient to confer the keratinocyte-restricted transcription of HPV-18.

MATERIALS AND METHODS

Cell culture, transfections, and CAT assays. C33, HaCaT (7), and HeLa cells were grown in Dulbecco's modified Eagle medium containing 7% fetal calf serum. Normal foreskin human keratinocytes were maintained as previously described (41). Cells were kept in KGM medium and passaged by trypsinization followed by centrifugation through medium containing serum for trypsin inactivation. C33, F9, and HaCaT cells were transfected by the standard calcium phosphate coprecipitation technique (55) in 6-cm dishes with 5 μg of chloramphenicol acetyltransferase (CAT) reporter plasmids. Human keratinocytes were transfected with Lipofectin (Bethesda Research Laboratories) as previously described (37). Cells were harvested 48 h after transfection for CAT and β-galactosidase analysis as described previously (50). Cotransfections were done with 4 μg of CAT reporter plasmids and 0.5 μg of each of the Jun and Fos expression plasmids.

Recombinant DNAs. The proximal AP1 binding site to the P_{105} promoter was mutated by oligonucleotide reconstruction as already described (51). The distal AP1 binding site was mutated by the use of an oligonucleotide containing overlapping point mutations in the AP1 binding sequence as indicated. This oligonucleotide contains two point mutations in the AP1 DNA sequence and three additional point mutations creating a new endonuclease restriction site, *Apa*I (GGGCC/C); these oligonucleotides contain, respectively, 60 nucleotides (5′-TTGAACAATTG*GCGCGCGC*CTCTTTG GCGCATATA*GGGCCCC*ACCTGGTA*TTACTTA*TTTTCC -3′) and 23 nucleotides (5′-TCAAA*GCGCGC*CATAGTAT TGTG-3′) with unique *Bss*HII sites (G/CGCGC) used for cloning in the CAT expression plasmids. We designed an additional oligonucleotide which contains the mutations to create the new *Apa*I endonuclease restriction site but no mutations in the AP1 binding site. This oligonucleotide was used to construct the CAT plasmid p18APwt (5′-TTGAA CAATTGGCGCGCCTCTTTGGCGCATATA*GGGCCC*AC CTGGTA*TTAGTCA*TTTTCC-3′), used as the wild-type reference in the transfection experiments.

Reconstructed fragments have all been confirmed by sequence analysis.

Jun and Fos expression plasmids were previously described (24).

Nuclear extracts and gel shift assays. Nuclear extracts were prepared as previously described by Schreiber et al. (43). Briefly, confluent cells from a 10-cm petri dish were scraped in 1 ml of Tris-buffered saline, pelleted, and then resuspended in 400 μl of a buffer containing 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.9), 10 mM KCl, 0.1 mM EGTA, 1 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). Cells were gently resuspended on ice and allowed to swell on ice for 15 min, then 15 μl of a 10% solution of Nonidet NP-40 was added, and the tube was vigorously vortexed for 10 s. After centrifugation, the nuclear pellet was resuspended in 50 μl of ice-cold buffer containing 20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF, and the tube was vigorously rocked at 4°C for 15 min on a shaking platform. The nuclear extract was then centrifuged for 10 min at 4°C, and the supernatant was frozen in aliquots. Protein content was determined by Bradford assays and was usually around 5 μg of protein per μl of extract. Gel shift assays were performed according to the method of Fried and Crothers (15) with 1 or 2 μl of the nuclear extracts incubated with the A18 probe end labelled by filling with the Klenow fragment of *Escherichia coli* DNA polymerase I in the presence of [α-^{32}P]ATP. Extracts were preincubated in the presence of 3 μl of poly(dI-dC) and 1 μl of salmon sperm DNA for 30 min on ice before addition of the labelled probe for 5 min and loading of the gels. For supershift experiments in the presence of antisera, 1 or 2 μl of the antipeptide antiserum was added in the preincubated nuclear extracts and further incubated at room temperature for 15 min before addition of the labelled probe and loading of the gels. Induction was done by incubation of the cell culture in the presence of 1 ng of TPA per ml for 5 h.

The various Jun and Fos RNAs were synthesized by using T7 expression plasmids and T7 RNA polymerase, and the proteins were synthesized with a rabbit reticulocyte lysate system (Amersham N90) as described elsewhere (24). For the Jun/Fos heterodimers, both Jun and Fos RNAs were translated together in the same reaction mixture to ensure better formation of the heterodimers.

Antisera. Anti-Jun rabbit polyclonal antibodies were generated against the following peptides: c-Jun, SYGAAGLAF PSQPQQ; JunB, ISYLPHAPPFAGG; and JunD, GCQ LLPQHQVPAY. These peptides are 100% homologous in mouse and human Jun proteins for JunB and JunD. For c-Jun, only one amino acid differs in the 15-amino-acid-long peptide (S versus A at position 11). Prior to supershift experiments, the antisera were precipitated by ammonium sulfate.

Western blotting. Ten micrograms of nuclear extracts from HaCaT cells or primary human keratinocytes was fractionated on denaturing polyacrylamide gels and analyzed with specific antibodies after Western blot as previously described (13). Cell cultures were treated for 5 h with 1 ng of TPA per ml.

RESULTS

Mutations in the putative AP1 binding sites of the HPV-18 regulatory region. Mutations were introduced into the 230-bp proximal fragment of the P_{105} promoter in the CAT expression plasmid p18/42, which contains the entire long control region (LCR) of HPV-18 cloned in front of the bacterial CAT gene (50). Mutations of the wild-type 5′-TGACTAA-3′ sequence to 5′-TAAGTAA-3′ were introduced in the proximal AP1 binding site, 171 nt upstream of the P_{105} initiation site, by reconstructions with combinations of oligonucleotides as previously described (37, 51). Identical mutations were made in the distal AP1 binding site 5′-TTAGTCA-3′, which is the same sequence as the proximal 5′-TGACTAA-3′ on the opposite DNA strand, 349 nt upstream of the P_{105} initiation site, by polymerase chain reaction amplification using a 60-nt-long primer containing mutations in the overlapping AP1 binding sequence as shown in Fig. 1. The mutated distal AP1 binding site was introduced, either in the background of the wild-type P_{105} promoter sequences or in the promoter containing the mutated proximal AP1 binding site. These various combinations generated the four different CAT expression plasmids depicted in Fig. 1.

Basal transcriptional activities of the mutated P_{105} promoters in the human keratinocytes and cervical carcinoma cell

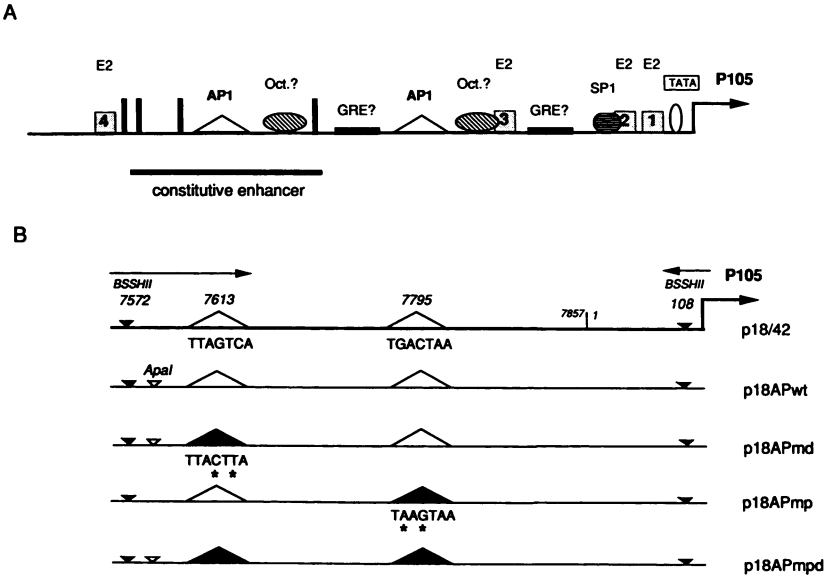

FIG. 1. (A) Schematic representation of the HPV-18 LCR. The sites of interaction with cellular and viral transcriptional factors are indicated. The four E2 binding sites are represented by gray squares, and NF1/CTF interactions are represented by vertical bars; SP1 (20) and the TATA box are indicated. The two horizontal black bars indicate putative glucocorticoid-responsive elements (GRE?) (10), and the gray ovals represent putative OCT1-binding elements (16, 38). The two putative AP1 binding sites (17) are represented by triangles. The 230-bp fragment (nt 5709 to 7739) that has been shown to contain an intrinsic enhancer activity restricted to human epithelial cells (49) is underlined. (B) Site-directed mutagenesis of the putative AP1 binding sites. The locations of the two oligonucleotides used for the polymerase chain reaction amplification are represented by arrows at the top. Triangles represent the two AP1 binding sites; the white triangles represent wild-type sequences, and the black triangles represent sequences mutated as indicated. The proximal AP1 binding site was mutated by oligonucleotide reconstruction (51). Sequences of the putative AP1 sites are given 5′ to 3′ on the upper DNA strand and are therefore identical in opposite orientations.

lines. The activities of the four CAT plasmids described above were studied in the C33 and HeLa cervical carcinoma-derived cell lines, in the HaCaT keratinocyte cell line, and in normal human keratinocyte cultures. The activity of the reconstructed wild-type HPV-18 LCR plasmid was similar to the activity of the original wild-type expression plasmid p18/42 (51), which represents about 10% of the transcriptional activity of the SV40 early promoter-CAT plasmid. As shown in Fig. 2, mutations in the putative AP1 binding sites dramatically affected the basal level of transcription of the P_{105} promoter in each of the cervical carcinoma cell lines, in human foreskin keratinocytes, and in the HaCaT keratinocyte cell line. In the cervical carcinoma cell lines, plasmid p18APmpd, containing mutations in both AP1 binding sites, expressed about 10% of the wild-type transcriptional activity, while in keratinocytes, no residual activity above background could be detected. In agreement with these results, the plasmids containing single mutated sites, p18APmp and p18APmd, exhibited decreased transcriptional activities in each of the cell types, although the decrease was particularly marked in the HaCaT keratinocyte line and in normal foreskin keratinocytes (10 to 20% of the wild-type transcription level [Fig. 2]). Various keratinocyte cell lines which were either immortalized by HPV-16, HPV-18, or SV40 T antigen (2, 32, 41) were also assayed for their ability to support HPV-18 P_{105} expression. In each of these cell lines, the AP1 site mutations induced a drastic decrease of the basal activity of the P_{105} promoter comparable to the effects observed for the foreskin keratinocytes and the HaCaT cell line (data not shown).

The HPV18 5′-TGACTAA-3′ sequence is functionally equivalent to the consensus TRE. Both putative AP1-binding *cis* elements of the HPV-18 regulatory region contain the identical sequence (5′-TGACTAA-3′) referred to in this study as A18, which is close to the consensus TRE sequence (5′-TGACTCA-3′). This sequence has previously been shown to interact as efficiently as the consensus TRE with Jun/Fos complexes (36) or with a partially decreased affinity (23). Such differences are probably due to the effect of sequences flanking the heptamer. We compared binding of the three different Jun products, synthesized in vitro as heterodimers with c-Fos, to the two types of sequences and found that all three types of Jun/Fos heterodimer bound efficiently the A18 sequence, although three- to fourfold less efficiently than they bound the TRE consensus sequence in in vitro gel shift assays (Fig. 3A and B). When cloned in a reporter plasmid upstream of the thymidine kinase (TK) promoter, the A18 sequences increased the activity of the TK promoter in a variety of cell lines, including the cervical carcinoma cell lines and keratinocytes examined in this study (results not shown).

Cotransfections of the mouse F9 cell line, which expresses very low levels of endogenous Jun, with these plasmids and the three Jun expression plasmids together with a c-Fos expression plasmid resulted in a 30-fold activation of transcription of the TK promoter in the presence of five copies of the A18 binding sequences (Fig. 4). It is interesting to note that in the same cell line under similar experimental conditions, the HPV-18 P_{105} promoter was completely inactive and could not be activated by the various combinations of the Jun and Fos products. The differences could not be attributed to the number of copies of the A18 AP1 *cis* elements in the two promoters (two versus five), since TK chimeric plasmids, with one or two AP1 binding sites, were

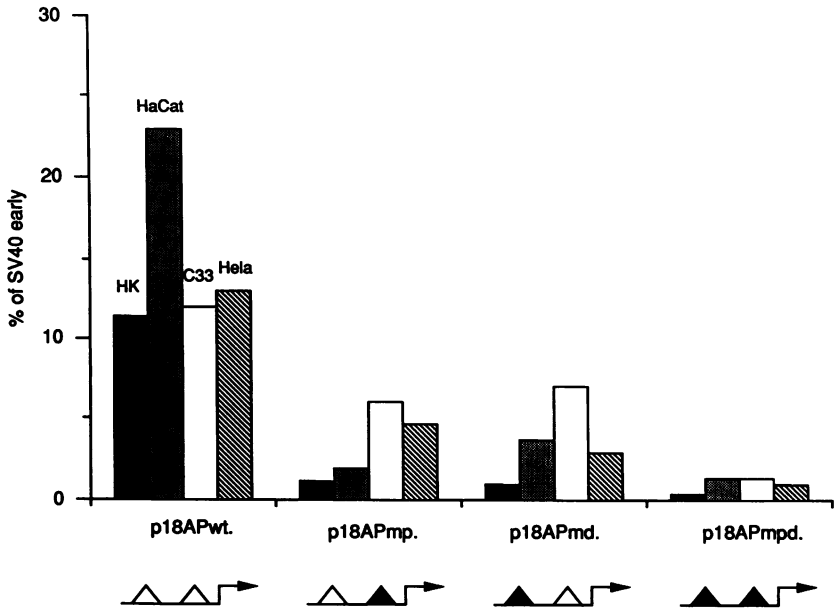

FIG. 2. Basal activities in various cell lines of the P_{105} promoters containing mutations in the AP1 binding sites. Five micrograms of each of the CAT expression plasmids was transfected in either HaCaT, HeLa, or C33 cells by the calcium phosphate coprecipitation technique or by lipofection in foreskin keratinocytes (HK). Transfection efficiencies were normalized by cotransfection with a Rous sarcoma virus–β-galactosidase plasmid, and the corrected CAT and β-galactosidase activities are given as percentages of the activity of SV40 early promoter for each cell type. The results are means of at least three independent transfection experiments. Schematic representations of the mutated promoters are indicated; the black triangles represent mutated AP1 binding sequences.

also efficiently activated (10- to 20-fold) in F9 cells (18). Promoters of genes such as the collagenase gene (24) containing a single AP1 binding site can also be efficiently activated by Jun/Fos in cotransfected F9 cells. The inability of the HPV-18 promoter to be activated in Jun/Fos-transfected F9 cells could reflect the location of the AP1 sites in the LCR, the effect of the other *cis* elements in the LCR which negatively regulate transcription in F9 cells, or the absence of other positive factors essential for P_{105} expression in this cell line. These results, however, are consistent with previous studies indicating a stringent cell specificity for HPV-18 P_{105} transcription.

Characterization of the specificity of the Jun antipeptide antisera. To examine the identity of the Jun product(s) present in nuclear extracts from keratinocytes and cervical carcinoma cell lines, we used antipeptide antisera. Rabbit polyclonal antibodies were raised against peptides unique to each of the three different mouse Jun proteins, c-Jun, JunB, and JunD. The amino acid sequences used are identical in the homologous human Jun proteins. The supershift experiments shown in Fig. 3C demonstrate the specificity of each antipeptide antiserum to its corresponding in vitro-translated Jun protein, since no cross-reactions could be detected. Efficiencies of the supershifts, however, were variable. While the interaction of the shifted complex containing JunD with its specific antiserum led to its complete disappearance, with part of the complex being supershifted high in the gel, the two other antisera were less efficient, and increased volumes of antiserum were necessary to fully shift the DNA/protein complexes. In both cases, however, a clear decrease in the intensity of the shifted complexes, after incubation with the specific antisera, was detected along with a partial supershift. These quantitative differences in the supershifts of in vitro DNA/protein-bound complexes could be due to a number of factors, including the affinity

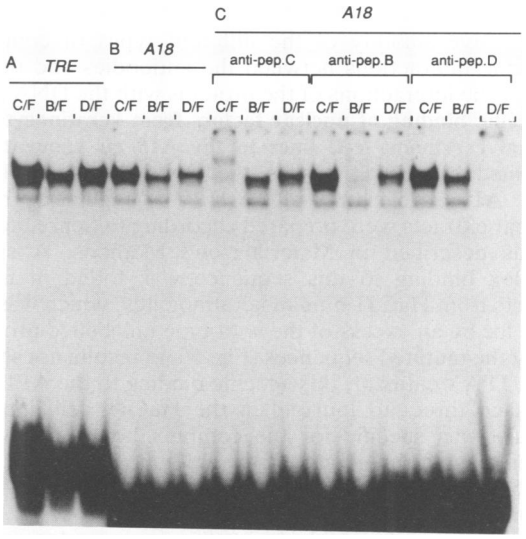

FIG. 3. (A and B) Comparative binding of the in vitro Jun/Fos heterodimers to the TRE and the HPV-18 AP1 binding sequences. Oligonucleotides containing the consensus TRE sequence (5′-TGACTCA-3′) and the putative HPV-18 AP1 binding sequence contained in a 17-mer oligonucleotide, AP18 (5′-CTAGAATAT GACTAAGCT-3′), were used in gel shift assays with in vitro-cotranslated Jun and Fos proteins (1 μl of the cotranslated Jun/Fos mixtures). Comparison of the intensities of the binding of the Jun/Fos complexes to the TRE probe (A) and the HPV-18 probe (B) indicates a three- to fourfold decrease in efficiency for the latter probe. (C) Analysis of the Jun antipeptide antibodies. Supershift experiments were done in the presence of specific Jun antipeptide antibodies added to incubation mixtures containing in vitro Jun and Fos proteins and the labelled HPV-18 AP1 probe. C, c-Jun; F, c-Fos; B, JunB; D, JunD.

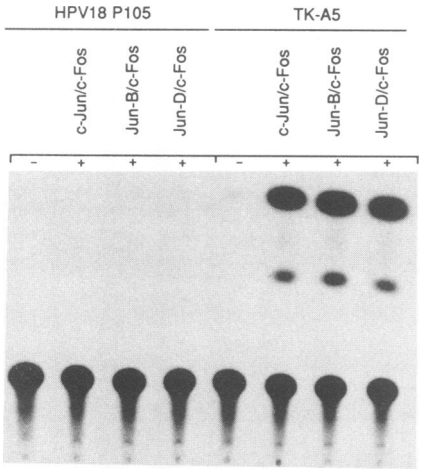

FIG. 4. Lack of Jun/Fos activation of transcription of the P_{105} promoter in mouse embryonic F9 cells. Cotransfection experiments in F9 cells were done by the calcium phosphate coprecipitation technique with 5 μg of the CAT expression plasmids and 0.5 μg of each of the Jun and Fos expression plasmids. HPV-18 P_{105} is the wild-type promoter linked to the CAT gene, and TK-A5 contains five copies of the HPV-18 AP1 binding sequence (5'-TGACTAA-3') cloned upstream of the TK promoter. Activations were 30-fold for either of the cotransfected TK-A5 plasmids with the c-Jun/c-Fos, JunB/c-Fos, or JunD/c-Fos combinations. The CAT assays shown are representative of several independent transfection experiments leading to comparable high activation of the TK plasmid by the three Jun/Fos combinations and no detectable effect on P_{105} promoter activity.

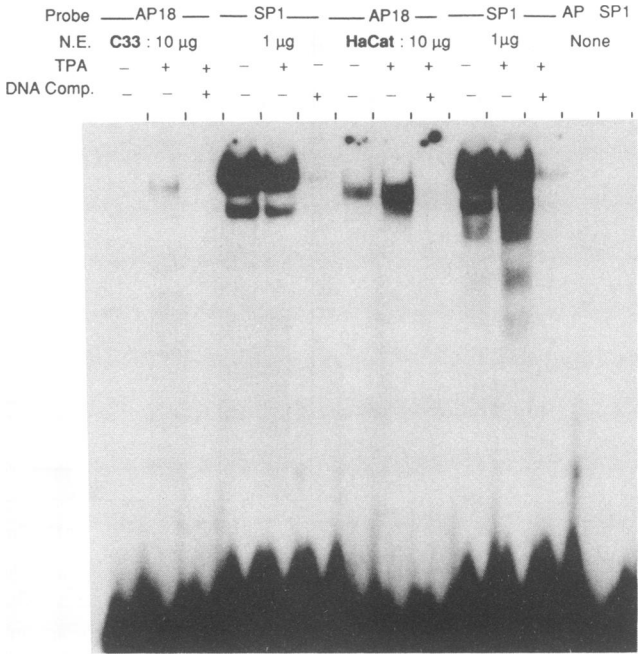

FIG. 5. Gel shift assays with HaCaT and C33 nuclear extracts. Two DNA probes were used. AP18 contains the HPV-18 putative AP1 binding sequence in a 17-mer oligonucleotide (5'-CTAGAA TATGACTAAGCT-3'), and SP1 contains the TK distal SP1 binding site in a 20-mer oligonucleotide (5'-GATCTAAACCCCGCCCA GCG-3'). Nuclear extracts (N.E.) were studied at similar protein concentrations (indicated above the lanes), whether or not the cells were treated with TPA. A 10-fold-higher concentration of proteins was needed for AP1 binding than for SP1 binding, as indicated. Specific binding competitions were done with a 20-fold excess of the corresponding unlabelled oligonucleotides.

and relative stability of the different types of complexes involving interactions between the antibodies and the proteins or the interactions of the proteins with the DNA probe.

Specific binding of nuclear factors from keratinocytes and cervical carcinoma cell lines to the A18 *cis* elements. We examined, by gel shift assays, the binding of nuclear factors to the A18 *cis* elements of the HPV-18 regulatory region. Nuclear extracts were prepared according to Schreiber et al. (43) as described in Materials and Methods. A specific complex binding to this sequence was found in nuclear extracts from HaCaT human keratinocytes, which was competed for by an excess of the wild-type unlabelled probe but not by the mutated sequences (Fig. 5 and results not shown). Upon TPA treatment, this specific binding to the AP1 probe increased three- to fourfold in the HaCaT cell line. This increase was specific for the complex bound to the A18 sequence, since no change in the amount of complex bound to an SP1 probe could be detected upon identical experimental conditions (Fig. 5).

The specific antipeptide antibodies were used in gel shift experiments to determine the type of Jun present in the AP1 complexes binding A18. With the human HaCaT keratinocytes, the JunB antipeptide antiserum significantly displaced part of the complex (Fig. 6B). A clear decrease in the intensity of the retarded band was reproducibly observed, which corresponded to more than half of the total shifted complex, regardless of whether the cells were TPA treated (Fig. 6B and results not shown). Although very faint, the supershifted band was often visible (arrow in Fig. 6A). In contrast, with the c-Jun antipeptide antibody, no decrease in the intensity of the bound complex and no appearance of a supershifted complex could be detected. A slight but reproducible decrease was observed with the anti JunD antipeptide antibody. When both anti-JunB and anti-JunD antibodies were used together, the retarded complex was almost completely displaced (Fig. 6). We conclude from these experiments that JunB is the predominant Jun component associated with Fos in human keratinocytes. Only a small portion of the bound complex could be attributed to JunD, and little or no c-Jun could be detected.

In the HPV-negative cervical carcinoma cell line C33, the AP1 complex bound to A18 sequence was very faint, about 20 times less abundant than in keratinocytes when compared with the binding of an SP1 probe (Fig. 5). TPA treatment induced a severalfold increase in DNA binding specifically to the A18 probe compared with the SP1 probe (Fig. 5). Studies using the antipeptide antibodies (not shown) indicated that the retarded complex was more clearly displaced by both antipeptide C and antipeptide D and less by antipeptide B. However, it was very difficult to define a precise pattern of Jun expression in this cell line, and we tentatively propose that the three Jun factors are expressed at low levels in this cell line. In contrast, we found that keratinocyte cell lines transformed by HPV-18 or by the SV40 T antigen, as well as the cervical carcinoma cell line HeLa, which contains HPV-18 sequences, exhibited high levels of JunB, although c-Jun and JunD were also present (data not shown).

To confirm the predominance of JunB in keratinocytes, we examined the Jun proteins present in nuclear extracts prepared from HaCaT cells or from normal foreskin human keratinocytes by Western blot analysis. The JunB antipep-

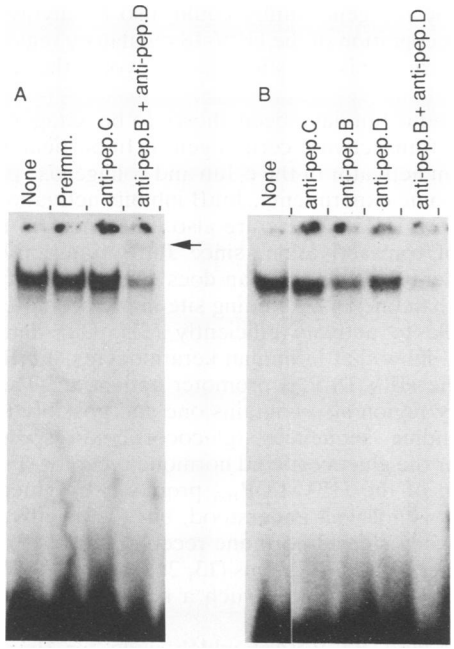

FIG. 6. Gel shift assays with HaCaT nuclear extracts in the presence of specific Jun antipeptide antibodies. Two different experiments are presented. The probe used is the HPV-18 AP1 sequence (A18), incubated with 5 μg of nuclear proteins from HaCaT cells. Cells were either not treated (A) or pretreated with TPA (1 ng/ml) for 5 h (B). Incubations were done in the presence of the indicated Jun antipeptide antibodies. The two gels are from two independent experiments, and the times of exposure of the autoradiograms are different. The arrow indicates the new supershifted band corresponding to the triple antibody/AP1/DNA complex.

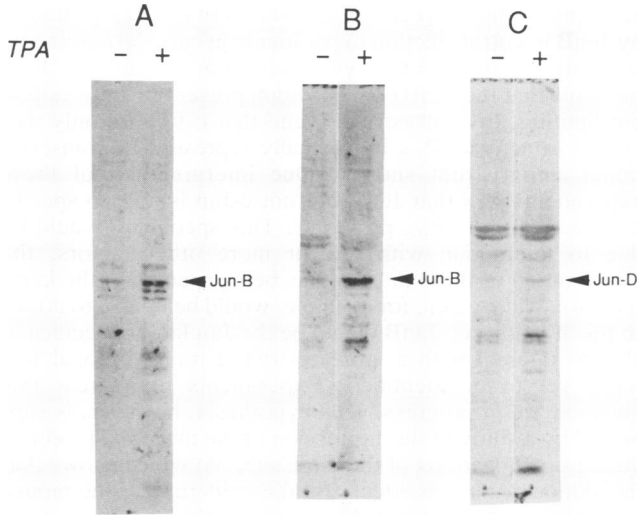

FIG. 7. Western blot analysis of Jun in keratinocyte nuclear extracts with the specific antipeptide antibodies. (A) Western blot with 10 μg of HaCaT nuclear extract in the absence (−) or presence (+) of TPA treatment, revealed with the anti-JunB antibody. (B) Western blot with 10 μg of total cellular extract from primary foreskin keratinocytes in the absence (−) or presence (+) of TPA treatment. The antibody used to reveal the blot is the specific JunB antipeptide antiserum. (C) Same as panel B, but the antibody used is the JunD antipeptide antiserum. Arrowheads indicate the specific Jun bands.

tide antiserum revealed a detectable band in these cells which was further increased by TPA treatment (Fig. 7). A faint band could also be seen with JunD antipeptide antibody, with a weaker activation by TPA. c-Jun could not be detected in these extracts (results not shown). Western blots using nuclear extracts from NIH 3T3 cells have indicated that the three Jun antipeptide antibodies were able to detect their corresponding Jun product with similar efficiencies (results not shown). These results are consistent with those of the antibody supershift experiments shown in Fig. 6 and implicate JunB in the regulation of HPV-18 P_{105} promoter transcription in human keratinocytes, since it is the Jun family member present at a high level in these cells.

DISCUSSION

Transcription of the HPV-18 P_{105} promoter, which controls expression of the E6 and E7 transforming genes, is regulated by a combination of viral and cellular factors. This transcription depends upon keratinocyte differentiation in a way that is not yet understood. This very tight relationship between viral gene expression and cell differentiation is reflected by the cell specificity of the P_{105} promoter, which is active only in certain human epithelial cells. We show here that the ubiquitous transcription factor AP1 is essential for the activity of this promoter. Mutations which abolish AP1 binding drastically reduce transcription from this promoter in keratinocytes. Results of these experiments indicate that both of the AP1 binding sites of the HPV-18 regulatory region contribute equally to the final level of transcription despite their different locations within the control region, one site being located in the cell-type-specific enhancer (Fig. 1). Multiple molecular species constitute the AP1 factor, which has been implicated in the control of fibroblast proliferation. The fact that different combinations of Jun homodimers or of Jun/Fos heterodimers can be formed in the cell and bind to the same cognate sequence creates an important diversity. The three members of the Jun family, c-Jun, JunB, and JunD, were described in, and isolated from, mouse cells (25, 29, 39, 40) and human cells (1, 3, 6, 45). The different members of the Jun family are coexpressed in many tissues, although at different levels. The three Jun proteins, c-Jun, JunB, and JunD, have about 50% overall homology, and their N-terminal regions contain the transcriptional activation domains (24). As heterodimers with Fos, these three Jun products appear to be comparably efficient transcriptional transactivators. The finding that the *jun* genes differ in their sites of expression in adult mice (23, 39) raises the possibility that their products contribute to tissue-specific expression of target genes regulated by AP1.

Here we have shown that although AP1 is necessary for the basal level of expression of the HPV-18 P_{105} promoter, it is not sufficient since no activity could be detected in Jun/Fos-cotransfected mouse embryonic F9 cells. An additional cell-type-specific factor or factors that remain to be identified must be involved. The effect of mutations of the AP1 sites on P_{105} transcriptional activity was particularly drastic in keratinocytes, raising the question as to which Jun product(s) constituted the AP1 complex in keratinocytes. In the spontaneously immortalized human keratinocyte cell line HaCaT, the result was quite clear, since the major Jun expressed is JunB, with very little JunD and no detectable c-Jun. Furthermore, JunB was the major Jun product activated by TPA, as shown by retardation assays as well as by Western blot analysis of the nuclear extracts. However, our

attempts to show direct transactivation of the P_{105} promoter by JunB in cotransfection experiments in cervical carcinoma cell lines or in keratinocytes were not successful. It is probable that the JunB concentration present in these cells is not limiting. In contrast, we found that c-Jun not only was unable to activate P_{105} but actually repressed its transcriptional activity (not shown). One interpretation of these experiments was that JunB but not c-Jun is able to specifically activate the P_{105} promoter. This specificity would be due to interaction with one or more other factors, the combination of these interactions being specific to the keratinocyte, while c-Jun, for example, would be unable to do so. In this hypothesis, JunB would be the Jun factor specifically able to interact with a more restricted transcriptional factor(s) yet to be identified. Experiments to address this question are in progress. This hypothesis, however, is supported by a study of the expression of the three Jun products during organogenesis of the mouse (5, 56) which shows that the skin and, most particularly, the epidermis of the mouse specifically express JunB. Furthermore, this specific expression is linked to terminal differentiation of the epidermis, since JunB appears more abundant in the upper layer, which consists of terminally differentiated keratinocytes. This finding would be in accordance with a critical role of JunB in the tissue-specific expression of the genital papillomaviruses, since their expression appears to be dependent on terminal differentiation of the keratinocyte.

An interesting situation, however, stems from the study of the transcription of this promoter in the C33 cervical carcinoma cell line. We found that only a very low amount of AP1 was capable of binding to the A18 DNA sequences in this cell line and yet, despite this low expression of AP1, and especially of JunB, the P_{105} promoter was active in C33 cells. Mutations in one or the other of the *cis* elements in the upstream regulatory region resulted in only a 50% loss of transcriptional activity in C33 cells, which is much less drastic than the effect observed in keratinocytes. Altogether, these results indicate that efficient transcriptional activity of P_{105} could be achieved with low amounts of the AP1 complex and that other factors can substitute for AP1 in this environment.

The importance of AP1 in the keratinocyte-specific transcriptional activity of P_{105} was suggested by results of an in vitro transcription study (34). The authors concluded from their experiments that AP1 was more abundant in extracts from human epithelial cells than in extracts from fibroblasts in which the promoter is inactive and that these quantitative differences would explain the cell specificity. We show in this study that despite a low amount of AP1, the P_{105} promoter is active in the C33 cell line, while it is inactive in F9 cells expressing excess AP1, indicating that quantitative differences are not sufficient to explain the cell type specificity. More precisely, these results indicate that the cell specificity of the P_{105} promoter is a multifactor phenomenon. Factors interacting with the AP1 binding sites are essential for the transcriptional activity of the promoter in keratinocytes, but some other factor(s) is also necessary. One interpretation of the differences between keratinocytes and the C33 carcinoma cell line would be that the other factors involved in P_{105} transcription play a more predominant role in C33 cells than in keratinocytes. It is easy to interpret these results by the presence of different subsets of transcriptional factors in both cell types, P_{105} being activated by different combinations of factors in C33 cells or in keratinocytes. In conclusion, AP1 could be considered as necessary but not sufficient for specific activation of the P_{105} promoter. Members of the *fos* gene family could also be involved in this specific recognition of the HPV-18 regulatory region by AP1, but answering this question goes beyond the aim of this study.

JunB and c-Jun have been shown to be antagonists in the way they transactivate certain genes. In particular, c-Jun is an efficient activator of the c-Jun and collagenase promoters, while in some experiments, JunB inhibits activation of these promoters (11, 45). There are also differences in the mechanisms of transactivation, since JunB transactivates in a more synergistic manner than does c-Jun, requiring binding to more than one DNA binding site in the promoter in order to be able to activate efficiently (11). Our data, on the contrary, show that in human keratinocytes, JunB is essential for the HPV-18 P_{105} promoter activation. The HPV-18 regulatory region also contains one and possibly two of the DNA binding sequences (glucocorticoid-responsive elements) for the glucocorticoid hormone receptor (Fig. 1). The regulation of the HPV-18 P_{105} promoter by glucocorticoid hormones is not well understood, but the negative interference between steroid hormone receptors and AP1 has been documented in other systems (13, 26, 30, 44, 57). We cannot rule out the possibility of such a negative interference for P_{105} regulation.

The E6 and E7 RNAs, which code for the two viral oncoproteins, are transcribed from the HPV-18 P_{105} promoter. HPV infection induces cell proliferation which is probably due, at least in part, to specific interactions of the viral E6 and E7 products with the cellular p53 and Rb proteins, respectively (14, 32, 33, 54). On the other hand, cell proliferation is associated with elevated AP1 activity, which will activate promoters containing AP1-responsive elements (8, 27). It is therefore probable that cell proliferation, induced by E6/E7 gene expression will, in turn, activate AP1, which is a positive transactivator of E6/E7 P_{105} promoter transcription, thus creating a positive regulatory loop during the viral cycle.

ACKNOWLEDGMENTS

We thank N. Fusenig for the HaCaT cell line, Y. Barrandon for foreskin keratinocytes, Janet Byrne for oligonucleotide synthesis, and Françoise Arnos for technical assistance. We thank John Ham for critical reading of the manuscript.

F.T. was supported in part by an E. Roosevelt fellowship from the International Union against Cancer and by an NCI-EORTC fellowship. This work was supported by grants from INSERM, ARC, LNFCC, and FRM and by a NATO grant for International Collaboration in Research to P.H. and M.Y.

ADDENDUM IN PROOF

After the completion of this work, Mack and Laimins (D. H. Mack and L. A. Laimins, Proc. Natl. Acad. Sci. USA **88:**9102–9106, 1991) described a keratinocyte-specific factor interacting with AP1 to activate HPV-18 transcription in keratinocytes.

REFERENCES

1. **Angel, P., E. A. Allegretto, S. T. Okino, K. Hattori, W. J. Boyle, T. Hunter, and M. Karin.** 1988. Oncogene Jun encodes a sequence-specific transactivator similar to AP-1. Nature (London) **332:**166–171.
2. **Barbosa, M. S., and R. Schlegel.** 1989. The E6 and E7 genes of HPV18 are sufficient for inducing two-stage in vitro transformation of human keratinocytes. Oncogene **4:**1529–1532.
3. **Berger, I., and Y. Shaul.** 1991. Structure and function of human jun-D. Oncogene **6:**561–566.

4. **Bernard, B. A., C. Bailly, M. C. M. Lenoir, M. Darmon, F. Thierry, and M. Yaniv.** 1989. The HPV18 E2 gene product is a repressor of the HPV18 regulatory region in human keratinocytes. J. Virol. **63**:4317–4324.
5. **Besserant, J. L.** Personal communication.
6. **Bohmann, D., T. J. Bos, A. Admon, T. Nishimura, and P. K. Vogt.** 1987. Human proto-oncogene c-Jun encodes a DNA binding protein with structural and functional properties of transcription factor. Science **238**:1386–1392.
7. **Boukamp, P., R. T. Petrussevska, D. Breitkreutz, J. Hornung, A. Markham, and N. E. Fusenig.** 1988. Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. J. Cell Biol. **106**:761–771.
8. **Carter, R., C. S. Cozenda, A. Pena, K. Lipson, A. Robert Soprano, and K. J. Soprano.** 1991. Potential role for c-Jun in cell cycle progression through late G1 and S. Oncogene **6**:229–235.
9. **Chan, W. K., T. Chong, H. U. Bernard, and G. Klock.** 1990. Transcription of the transforming genes of the oncogenic human papillomavirus-16 is stimulated by tumor promoters through AP1 binding sites. Nucleic Acids Res. **18**:763–769.
10. **Chan, W. K., G. Klock, and H. U. Bernard.** 1989. Progesterone and glucocorticoid response elements occur in the long control regions of several human papillomaviruses involved in anogenital neoplasia. J. Virol. **63**:3261–3269.
11. **Chiu, R., P. Angel, and M. Karin.** 1989. Jun-B differs in its biological properties from, and is a negative regulator of, c-Jun. Cell **59**:979–986.
12. **Cripe, T. P., A. Alderborn, R. D. Anderson, S. Pakkinen, T. Bergman, H. Haugen, V. Petterson, and L. P. Turek.** 1990. Transcriptional activation of the human papillomavirus-16 P97 promoter by an 88-nucleotide enhancer containing distinct cell-dependent and AP1-responsive modules. New Biol. **2**:450–463.
13. **Doucas, V., G. Spyrou, and M. Yaniv.** 1991. Unregulated expression of c-Jun or c-Fos proteins but not JunD inhibits oestrogen receptor activity in human breast cancer derived cells. EMBO J. **10**:2237–2245.
14. **Dyson, N., P. M. Howley, K. Münger, and E. Harlow.** 1989. The human papillomavirus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. Science **243**:934–937.
15. **Fried, M., and D. M. Crothers.** 1981. Equilibria and kinetics of lac repressor-operator interactions by polyacrylamide gel electrophoresis. Nucleic Acids Res. **9**:6505–6525.
16. **Garcia-Carranca, A.** Personal communication.
17. **Garcia-Carranca, A., F. Thierry, and M. Yaniv.** 1988. Interplay of viral and cellular proteins along the long control region of human papillomavirus 18. J. Virol. **63**:4321–4330.
18. **Gauthier, J. M., N. Dostatni, M. Lusky, and M. Yaniv.** 1990. Two DNA-bound E2 dimers are required for strong transcriptional activation and for cooperation with cellular factors in most cells. New Biol. **3**:498–509.
19. **Gius, D., S. Grossmann, M. A. Bedell, and L. A. Laimins.** 1988. Inducible and constitutive enhancer domains in the noncoding region of human papillomavirus type 18. J. Virol. **62**:665–672.
20. **Gloss, B., and H. U. Bernard.** 1990. The E6/E7 promoter of human papillomavirus type 16 is activated in the absence of E2 proteins by a sequence-aberrant Sp1 distal element. J. Virol. **64**:5577–5584.
21. **Gloss, B., T. Chong, and H. U. Bernard.** 1989. Numerous nuclear factors bind the long control region of human papillomavirus type 16: a subset of 6 out of 23 DNase I-protected segments coincides with the location of the cell-type-specific enhancer. J. Virol. **63**:1142–1152.
22. **Hawley-Nelson, P., K. H. Vousden, N. L. Hubbert, D. R. Lowy, and J. Schiller.** 1989. HPV16 E6 and E7 cooperate to immortalize human foreskin keratinocytes. EMBO J. **8**:3905–3910.
23. **Hirai, S., and M. Yaniv.** 1989. Jun DNA-binding is modulated by mutations between the leucines or direct interactions of Fos with the TGACTCA sequence. New Biol. **1**:181–191.
24. **Hirai, S. I., B. Bourachot, and M. Yaniv.** 1990. Both Jun and Fos contribute to transcription activation by the heterodimer. Oncogene **5**:39–46.
25. **Hirai, S. I., R. P. Ryseck, F. Mechta, R. Bravo, and M. Yaniv.** 1989. Characterization of Jun D: a new member of the *jun* proto-oncogene family. EMBO J. **8**:1433–1439.
26. **Jonat, C., H. J. Rahmsdorf, K. K. Park, A. C. B. Cato, S. Gebel, H. Ponta, and P. Herrlich.** 1990. Antitumor promotion and antiinflammation: down-modulation of AP1 (Fos/Jun) activity by glucocorticoid hormone. Cell **62**:1189–1204.
27. **Kovary, K., and R. Bravo.** 1991. Expression of different Jun and Fos proteins during the G_0-to-G_1 transition in mouse fibroblast: in vitro and in vivo associations. Mol. Cell. Biol. **11**:2451–2459.
28. **Kryszke, M. H., J. Piette, and M. Yaniv.** 1987. Induction of a factor that binds to the polyoma virus A enhancer on differentiation of embryonal carcinoma cells. Nature (London) **328**:254–256.
29. **Lamph, W. W., P. Wamsley, P. Sassone-Corsi, and I. Verma.** 1988. Induction of proto-oncogene Jun/AP1 by serum and TPA. Nature (London) **334**:629–631.
30. **Lucibello, F. C., E. P. Slater, K. U. Jooss, M. Beato, and R. Müller.** 1990. Mutual transrepression of Fos and the glucocorticoid receptor: involvement of a functional domain in Fos which is absent in FosB. EMBO J. **9**:2827–2834.
31. **Martin, M. E., J. Piette, M. Yaniv, and W. R. Folk.** 1988. Activation of the polyoma-virus enhancer by murine AP1 homolog and two contiguous proteins. Proc. Natl. Acad. Sci. USA **85**:5839–5843.
32. **Münger, K., W. C. Phelps, V. Bubb, P. M. Howley, and R. Schlegel.** 1989. The E6 and E7 genes of the human papillomavirus type 16 together are necessary and sufficient for transformation of primary human keratinocytes. J. Virol. **63**:4417–4421.
33. **Münger, K., B. A. Werness, N. Dyson, W. C. Phelps, E. Harlow, and P. M. Howley.** 1989. Complex formation of human papillomavirus E7 proteins with the retinoblastoma tumor suppressor gene product. EMBO J. **8**:4099–4105.
34. **Offord, E. A., and P. Beard.** 1990. A member of the activator protein 1 family found in keratinocytes but not in fibroblasts required for transcription from a human papillomavirus type 18 promoter. J. Virol. **64**:4792–4798.
35. **Piette, J., and M. Yaniv.** 1987. Two different factors bind to the β-domain of the polyomavirus enhancer, one of which also interacts with the SV40 and *c-Fos* enhancers. EMBO J. **6**:1331–1337.
36. **Risse, G., K. Joss, M. Neuberg, H.-J. Brüller, and R. Müller.** 1989. Asymmetrical recognition of the palindromic AP1 binding site (TRE) by Fos protein complexes. EMBO J. **8**:3825–3832.
37. **Romanczuk, H., F. Thierry, and P. M. Howley.** 1990. Mutational analysis of *cis* elements involved in E2 modulation of human papillomavirus type 16 P_{97} and type 18 P_{105} promoters. J. Virol. **64**:2849–2859.
38. **Royer, H. D., M. P. Freyaldenhoven, I. Napierski, D. D. Spitkovsky, T. Bauknecht, and N. Dathan.** 1991. Delineation of human papillomavirus type 18 enhancer binding proteins: the intracellular distribution of a novel octamer binding protein p92 is cell cycle regulated. Nucleic Acids Res. **19**:2363–2371.
39. **Ryder, K., A. Lanahan, E. Perez-Albuerne, and D. Nathans.** 1989. Jun D: a third member of the Jun gene family. Proc. Natl. Acad. Sci. USA **86**:1500–1503.
40. **Ryseck, R. P., S. Hirai, M. Yaniv, and R. Bravo.** 1988. Transcriptional activation of c-Jun during the G0/G1 transition in mouse fibroblasts. Nature (London) **334**:535–537.
41. **Schlegel, R., W. C. Phelps, Y.-L. Zhang, and M. Barbosa.** 1988. Quantitative keratinocyte assay detects two biological activities of human papillomavirus DNA and identifies viral types associated with cervical cancer. EMBO J. **7**:3181–3187.
42. **Schneider-Gädicke, A., and E. Schwarz.** 1986. Different human cervical carcinoma cell lines show similar transcription patterns of human papillomavirus type 18 early genes. EMBO J. **5**:2285–2292.
43. **Schreiber, E., P. Matthias, M. M. Müller, and W. Schaffner.** 1989. Rapid detection of octamer binding proteins with "mini-extracts" prepared from a small number of cells. Nucleic Acids Res. **15**:6419.
44. **Schüle, R., P. Rangarajan, S. Kliewer, L. J. Ransone, J. Bolado, N. Yang, I. M. Verma, and R. M. Evans.** 1990. Functional antagonism between oncoprotein c-Jun and the glucocorticoid receptor. Cell **62**:1217–1226.

45. **Schütte, J., J. Viallet, M. Nau, S. Segal, J. Fedorko, and J. Minna.** 1989. Jun-B inhibits and c-Fos stimulates the transforming and trans-activating activities of c-Jun. Cell **59:**987–997.
46. **Schwarz, E., U. K. Freese, L. Gissmann, W. Mayer, B. Roggenbuck, A. Stremlau, and H. zur Hausen.** 1985. Structure and transcription of human papillomavirus sequences in cervical carcinoma cells. Nature (London) **314:**111–114.
47. **Sibbet, G. J., and S. Campo.** 1990. Multiple interactions between cellular factors and the non-coding region of human papillomavirus type 16. J. Gen. Virol. **71:**2699–2707.
48. **Smotkin, D., and F. O. Wettstein.** 1986. Transcription of human papillomavirus type 16 early genes in a cervical cancer and a cancer derived cell line and identification of the E7 protein. Proc. Natl. Acad. Sci. USA **83:**4680–4684.
49. **Thierry, F., A. Garcia-Carranca, and M. Yaniv.** 1987. Elements that control the transcription of genital papillomavirus type 18. Cancer Cells **5:**23–32.
50. **Thierry, F., J. M. Heard, K. Dartmann, and M. Yaniv.** 1987. Characterization of a transcriptional promoter of human papillomavirus 18 and modulation of its expression by simian virus 40 and adenovirus early antigens. J. Virol. **61:**134–142.
51. **Thierry, F., and P. M. Howley.** 1991. Functional analysis of E2-mediated repression of the HPV18 P_{105} promoter. New Biol. **3:**90–100.
52. **Thierry, F., and M. Yaniv.** 1987. The BPV1 E2 trans-acting protein can be either an activator or a repressor of the HPV18 regulatory region EMBO J. **6:**3391–3397.
53. **Vogt, P. K. V., and T. J. Bos.** 1990. Jun oncogene and transcription factor. Adv. Cancer Res. **55:**1–35.
54. **Werness, B. A., A. J. Levine, and P. M. Howley.** 1990. Association of human papillomavirus types 16 and 18 E6 proteins with p53. Science **248:**76–79.
55. **Wigler, M. S., S. Silverstein, L. S. Lee, A. Pellicer, Y. Cheng, and R. Axel.** 1977. Transfer of purified herpes simplex thymidine kinase genes to cultured mouse cells. Cell **11:**223–232.
56. **Wilkinson, D. G., S. Bhatt, R. P. Ryseck, and R. Bravo.** 1989. Tissue specific expression of c-Jun and JunB during organogenesis in the mouse. Development **106:**465–471.
57. **Yang-Yen, H. F., J. C. Chambard, Y. L. Sun, T. Smeal, T. J. Schmidt, J. Drouin, and M. Karin.** 1990. Transcriptional interference between c-jun and the glucocorticoid receptor: mutual inhibition of DNA binding due to direct protein-protein interaction. Cell **62:**1205–1215.