Promoter activity of sequences located upstream of the human papillomavirus types 16 and 18 late regions

Caroline Geisen and Tomas Kahn

Deutsches Krebsforschungszentrum, Angewandte Tumorvirologie (ATV 0640), Im Neuenheimer Feld 242, 69120 Heidelberg, Germany

The regulation of human papillomavirus (HPV) late gene expression is difficult to analyse because the late proteins L1 and L2 are only produced in the upper layers of terminally differentiated keratinocytes. However, for the minor capsid protein L2 of HPV types 1, 6, 11 and 16, rare mRNAs or cDNAs starting 3' of the E5 open reading frame (ORF) were previously described. In order to analyse whether the DNA region preceding the late ORFs (late upstream region, LUR) of HPV-16 and HPV-18 has promoter activity, transient transfection assays employing luciferase reporter constructs were performed. The results show that the LUR of HPV-16 and HPV-18 exhibits an orientation-dependent promoter activity in different cells. By analysing 3'-deletion mutants of the HPV-16 LUR, we identified 78 bp within the sequence between the E5 and L2 ORFs to be critical for the promoter activity. Furthermore, the analysis of a 5'-deletion mutant revealed a negative cis-regulatory element located within the E2 ORF. The HPV-16 early poly(A) signal is located downstream of the critical promoter region. Inactivation of this element by site-directed mutagenesis strongly enhanced luciferase activity. However, mutation of two potential TATA-binding protein (TBP) sites located within the E2 ORF revealed a negative cis-regulatory element. The HPV-16 early poly(A) signal is located downstream of the critical promoter region. Together with the early poly(A) signal, this potential promoter might be involved in the differentiation-dependent regulation of late gene expression.

Introduction

Human papillomaviruses (HPV) are a group of small epitheliotropic DNA viruses containing more than 70 known types (de Villiers, 1994). They cause a variety of benign proliferative lesions in humans, including skin warts and anogenital condylomata (Shah & Howley, 1990). In addition, specific HPV types (in particular HPV-16 and HPV-18) are strongly associated with the development of human anogenital cancers as numerous epidemiological and molecular biology studies have revealed (Bosch et al., 1995).

During natural HPV infection, the basal cells of the epithelium appear to be the primary target cells for the virus (Shah & Howley, 1990). Its transcriptional activity within the epithelium is tightly linked to the differentiation state of the keratinocyte. Early gene transcription is already observed in basal cells and suprabasal cell layers. Transcription of the late genes with synthesis of the capsid proteins is restricted to the terminally differentiated keratinocytes close to the epithelial surface (Dürst et al., 1992; Stoler et al., 1989, 1992). However, late transcripts are also detectable in layers lower than this, but they remain in the nucleus due to a block in RNA processing (Beyer-Finkler et al., 1990; Stoler et al., 1992). The analysis of transcriptional regulation of the late genes is hampered due to the lack of an easily accessible experimental system. However, transcript maps of late mRNAs are available for HPV types 1, 6, 11, 16 and 31b (Chow et al., 1987a, b; Sherman et al., 1992; Hummel et al., 1995), and late gene promoters have been characterized for HPV-1 and HPV-8 (Palermo-Dilts et al., 1990; Stubenrauch et al., 1992) as well as for the animal papillomaviruses bovine papillomavirus type 1 (BPV-1) and cottontail rabbit papillomavirus (CRPV) (Baker & Howley, 1987; Wettstein et al., 1987). These studies indicated that most L1 transcripts are initiated at a promoter located within the E7 ORF or the upstream regulatory region (URR). For the minor capsid protein L2, the transcription start site is less well defined. In mRNAs recovered from HPV-1-infected plantar warts and subsequently amplified using PCR, Palermo-Dilts et al. (1990) found one L2-encoding RNA species that originated from the late promoter in the URR. By analysing raft cultures of the established cell line CIN612 (Bedell et al., 1991), which

Author for correspondence: Tomas Kahn.
Fax +49 6221 424852. e-mail t.kahn@dkfz-heidelberg.de
maintains episomal copies of HPV-31b, Hummel et al. (1995) detected a polycistronic L2-containing transcript initiating from a differentiation-dependent promoter (P742) located in the middle of the E7 ORF. However, Chow et al. (1987a, b) using the electron microscopic R-loop technique, demonstrated that upstream region (LUR), has a function for the differentiation-dependent regulation of late gene expression. This possibility was supported by sequence analyses demonstrating that transcribed HPV ES-like sequences from mice and humans (Wagner et al., 1991) show similarities not only to E5, but also to upstream regulatory regions of genes known to be expressed in a tissue- and differentiation-specific manner (Kahn et al., 1992; Geisen et al., 1995) show similarities to sequences at the 3' end of the E50RF preceding the late ORFs, designated in the following as late plasmid pBL. For sense orientation (s) they were ligated by T4 DNA ligase into BamHI/Smal-digested, calf intestinal alkaline phosphatase (CIP)-treated pBL DNA. For antisense orientation (as) the fragments were cloned into the BamHI and blunt-ended NotI sites of CIP-treated pBL vector. The name of the latter plasmids tested in this study are composed of the HPV type (16 or 18) from which the LUR insert was derived, the orientation of the insert, the orientation of the insert, the orientation of the insert, and in case of site-directed mutagenesis the corresponding mutation (M). Plasmid p16LUR665/5' delsL contains a 5' end deletion of the HPV-16 LUR (nucleotide positions 3535–3763). It was generated by digestion of pl16LUR665sL with BamHI and Ndel and religation of the remaining plasmid DNA, which had been blunt-ended by T4 DNA polymerase in advance.

In the present study transient transfection experiments with luciferase reporter constructs were performed in order to investigate whether the DNA region preceding the late ORFs of HPV-16 and HPV-18 has promoter activity. The results revealed that an orientation-dependent stimulation of luciferase activity is conferred by both LURs in different cells, thus providing evidence for a promoter located just upstream of the L2 ORF of HPV types 16 and 18. To further define important elements for the HPV-16 LUR promoter activity, mutational analyses were carried out. By analysing the 5' - and 3' -deletion mutants of the LUR, the critical promoter region was identified, and a negative cis-regulatory element could be located within the E2 ORF. The early HPV-16 poly(A) signal and two potential TATA-binding protein (TBP) sites located in the critical promoter region were mutated in the context of the complete HPV-16 LUR. Subsequently, the effect of these mutations on the promoter activity was assessed by transfection assays.

Methods

■ Generation of exonuclease III deletion mutants. An 801 bp DdeI fragment (nucleotide positions 3535–4336) of HPV-16 (which contains part of the E2 ORF, the complete E4 and E5 ORFs, the sequences located between the 3' end of E5 and the start codon from L2 and the first 99 bp from the L2 ORF) was cloned into the Smal site of pBluescript KS. The 3' end of this fragment was digested with exonuclease III (Pharmacia) according to the manufacturer's instructions, to create deletion mutants of the HPV-16 LUR. Suitable deletion mutants were selected by size and sequence analysis. The different clones were digested with KpnI, blunt-ended by treatment with T4 DNA polymerase and then digested with BamHI. The HPV-16 LUR inserts were separated from pBluescript DNA by agarose gel electrophoresis, isolated and finally cloned into the reporter plasmid pBL (see below).

■ Reporter plasmids and oligonucleotides used for in vitro mutagenesis. All reporter plasmids are based on basic vector pBL, which was kindly provided by Andrea Klotzbücher and Martin Rentrop (DKFZ, Heidelberg). As described in Hoppe-Seyler et al. (1991), pBL contains the firefly (Phoitus pyralis) luciferase gene (DeWet et al., 1987) fused to the SV40 poly(A) signal derived from pBLCAT2 (Lucow & Schütz, 1987), and cloned into the polylinker of pBluescript KSM13 + (Stratagene). The HPV-16 LUR deletion mutants were cloned in both orientations into the multiple cloning site of the enhancer- and promoter-less plasmid pBL. For sense orientation (s) they were ligated by T4 DNA ligase into BamHI/Smal-digested, calf intestinal alkaline phosphatase (CIP)-treated pBL DNA. For antisense orientation (as) the fragments were cloned into the BamHI and blunt-ended NotI sites of CIP-treated pBL vector. The names of all the LUR constructs tested in this study are composed of the HPV type (16 or 18) from which the LUR insert was derived, the orientation of the insert, the orientation of the insert, and in case of site-directed mutagenesis the corresponding mutation (M).

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To create the reporter plasmids p18LUR258sL and p18LUR258asL, a 258 bp SacI fragment of HPV-18 (nucleotide positions 3930–4188) was cloned in sense and antisense orientation into the Smal-digested, CIP-treated pBL vector.

Plasmid p16URRL was constructed by cloning the HPV-16 URR (nucleotide positions 7007–102) into the pBl and BamHI sites of pBL. Plasmid p18URRL, kindly provided by Karin Butz and Felix Hoppe-Seyler (DKFZ, Heidelberg), has been described in detail previously (Hoppe-Seyler et al., 1991).

Plasmids p16LUR697(A)MsL, p16LUR665TATA1MsL, p16LUR665TATA2MsL and p16LUR665TATA1/2MsL, containing the mutated poly(A) signal and the mutated potential TBP sites of the HPV-16 LUR respectively, were generated by in vitro mutagenesis. Site-specific mutagenesis of p16LUR697MsL, p16LUR665MsL and p16LUR665TATA2MsL was performed by a PCR-based protocol (Hoppe-Seyler & Butz, 1992). For each individual mutation, four primers were used: two complementary internal primers containing mutated HPV-16 sequences, and two outer non-mutated vector-specific primers. 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 from the agar with a Pasteur pipette and combined into a third PCR using the two outer primers. The amplification product, carrying the desired mutation, was subcloned into p16LUR697sL, p16LUR665sL or p16LUR665TATA2sL. The DNAs of all mutants were fully sequenced. For the in vitro mutagenesis the following primers were used: M13 primer 5' GTAAAACGACGGCAGT 3' and 5' CCGTCGACGTAATTCTTAG 3'.
the pBL primer 3' AGCTCCACTTGAGTGCATG 5' as vector-specific outer primers. As internal primers to create the mutation of the poly(A) sequence at nucleotide positions 4215–4220, the sense primer 5' GTTTGTTTTTTAATACGCTTTATTgg 3' and its complementary equivalent were used. Besides HPV-16-specific and mutated sequences, both oligonucleotides contain four vector-specific nucleotides, indicated in lower case. The primers 5' GTAATTTACAGCTTTTGTTG-3' and 5' CTTTTTTATTTTCAGCTGTAATTTTTTTTTTG 3' and their complementary equivalents were used as internal primers to generate the mutations of the putative TFII sites at nucleotide positions 4130–4135 and 4180–4185, respectively. All mutated positions are indicated in bold.

### Transient transfection and luciferase reporter analysis

Transfection of cells was performed by calcium phosphate precipitation according to Chen & Okayama (1987). Briefly, 3 μg of the respective promoter–luciferase reporter plasmid, and 1 μg pAc-Gal plasmid for internal standardization, were mixed and adjusted to 6.5 μg by addition of PBBlueScript DNA (pAc-Gal contains the 4.3 kb EcoRI–AflI fragment of the human β-actin promoter linked to the Escherichia coli β-galactosidase gene). 2 x 10⁵ cells in 6 cm Petri dishes were incubated with the calcium phosphate/DNA mixture for 16–18 h at 35°C under 3% CO₂ and then washed twice with medium. After 24 h at 37°C and 5% CO₂, cells were harvested and luciferase activity was measured according to Brasier et al. (1989). β-Galactosidase activity of cotransfected pAc-Gal was measured as described (Maniatis et al., 1989) to account for variation in transfection efficiency. Relative luciferase activity (RLA) was calculated as the ratio of standardized luciferase activity of reporter plasmid to standardized luciferase activity of promoterless pBL plasmid. 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. Standard deviations for every experiment were determined and generally did not exceed 15%.

### Cell lines and cell culture conditions

The human cervical carcinoma cells HeLa, SiHa and C33 as well as HepG2 and Cos-7 cells were grown in tissue culture in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), glutamine, 100 U penicillin and 1000 U streptomycin. HaCaT keratinocytes (kind gift of N. Fusenig) were maintained in 4 x MEM also supplemented with 10% FCS, glutamine, 100 U penicillin and 1000 U streptomycin.

### DNA sequencing

All subcloned HPV fragments were controlled for orientation by sequencing, using vector-based or internal oligonucleotide primers. All primers were synthesized at the DKFZ (by H. Delius) and purified by polyacrylamide gel electrophoresis. The double-stranded DNA was sequenced by the dideoxynucleotide chain-termination method using a kit (Pharmacia).

All nucleotide positions mentioned in the text were calculated according to the published HPV-18 (Cole & Danos, 1987) and HPV-16 sequences (Seedorf et al., 1985; Halbert & Galloway, 1988).

### Computer analysis of DNA sequences

Sequence data were analysed using the Heidelberg Unix Sequence Analysis Resources (HUSAR) program library, a software package developed in part at the German Cancer Research Center, with contributions from the University of Wisconsin Genetics Computer Group, the University of California at San Diego (Doolittle) and EMBL (Vingrom, Zehetner). EMBL release 45.0 and Transfac (transcription factor sites, GBF Braunschweig, 10/95) were scrutinized for nucleotide or transcription factor similarities, respectively.

### Results

#### Promoter activity within the LUR

In order to test the genomic region immediately upstream of the late genes of HPV-16 and HPV-18 for a promoter activity, transient transfection luciferase assays were performed in the HPV-18-positive human cervical carcinoma cell line HeLa, using reporter constructs containing LUR fragments of these HPV types. As controls, the transcriptional activities of the early promoter/enhancer of HPV-16 and HPV-18 (URR) were determined in parallel. The LUR of HPV-16 was cloned in sense and antisense orientation directly upstream of the luciferase gene of the enhancer- and promoter-less plasmid pBL. To define elements critical for HPV-16 LUR promoter activity, several 3' end exonuclease III deletion mutants of the LUR were generated and cloned in both orientations into the same reporter plasmid.

As shown in Fig. 1, the HPV-16 LUR (positions 3535–4200) as well as the HPV-18 LUR (positions 3930–4188) induced luciferase expression when tested in sense orientation. However, in antisense orientation both fragments were inactive. These results demonstrate that the LURs of both HPV types exhibit a promoter activity. The strong activity of the HPV-16 LUR fragment is even higher than the activity of the HPV-16 URR, and is comparable to the activity of the HPV-18 URR. On the other hand, the stimulation of transcription conferred by the HPV-18 LUR construct tested is much lower compared with that of the HPV-16 LUR (see Discussion). These results could be confirmed with a slightly shorter HPV-16 LUR fragment, which ends at position 4196 (not shown in Fig. 1). When sequences at the 3' end of the HPV-16 LUR were deleted further (to nucleotide positions 4118, 4014 and 3985, respectively), the luciferase activity was almost completely abolished (Fig. 1). Thus, the 78 bp located between nucleotide positions 4118–4196 contain elements critical for the HPV-16 LUR promoter activity (see below). In addition, deletion of sequences at the 5' end of the HPV-16 LUR (from nucleotide positions 3535–3763), further stimulated luciferase expression (Fig. 1). This effect suggests the existence of a negative cis-regulatory element in this portion of the HPV-16 LUR fragment.

#### Effect of the early poly(A) signal on the promoter activity

Immediately upstream of their late genes, papillomaviruses have a poly(A) signal (AATAAA) responsible for the polyadenylation of the early genes (nucleotide positions 4215–4220 in case of HPV-16). As shown in Fig. 1, HPV-16 LUR fragments that contain the early poly(A) signal (reporter constructs p16LUR697sL and p16LUR717sL, respectively) had a markedly reduced activity compared to the LUR fragment without the poly(A) site (reporter construct p16LUR665sL). In order to investigate whether the presence

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Fig. 1. Activity of different HPV-16 and HPV-18 LUR reporter constructs in HeLa cells. Luciferase assays were performed after transient transfection of HeLa cells. Each transfection contained 3 μg of the construct to be analysed (schematically presented on the left side), 1 μg of pAc-Gal as an internal control, and 2.5 μg of pBluescript DNA. Promoter activities were determined by calculating the luciferase/β-galactosidase ratios thereby accounting for variation in transfection efficiency (see Methods). The average relative luciferase activity (RLA) of the different constructs (fold activation above basal pBL activity) are given by the horizontal bars on the right-hand side. Standard deviations are indicated. Luciferase activities for each reporter plasmid were determined in at least four independent experiments. As control the transcriptional activity of the URR of HPV-16 and HPV-18 was determined in parallel.

Fig. 2. Influence of the early poly(A) signal on the promoter activity of the HPV-16 LUR in HeLa cells. Schematic representation of the HPV-16 reporter construct used for the mutational analysis of the early HPV-16 poly(A) signal on the left-hand side. Transient transfection assays were performed in HeLa cells. The activities of the constructs relative to that of basic vector pBL are given by the horizontal bars on the right side. Standard deviations are indicated. For calculation see legend to Fig. 1.

of the early poly(A) site is responsible for the reduced luciferase activity, the poly(A) sequence AATAAA was changed into AATCAG by PCR-mediated site-directed in vitro mutagenesis, thus creating plasmid p16LUR697(A)MsL. The luciferase activity of the wild-type and the mutant clone were determined after transient transfection of HeLa cells. The mutation resulted
in a strong increase of the luciferase activity to a value comparable to the activity of the LUR construct p16LUR665sL (Fig. 2). This result suggests that the poly(A) signal itself is involved in the observed reduction of luciferase activity.

Analysis of the promoter activity of the HPV-16 and HPV-18 LURs in SiHa, C33A, HaCaT, Cos-7 and HepG2 cells

To investigate whether the activities of the HPV-16 and HPV-18 LURs observed in HeLa cells can also be detected in other cell types, transfection experiments were performed in cells of the spontaneously immortalized human keratinocyte cell line HaCaT, the cervical carcinoma cell lines SiHa (HPV-16-positive) and C33A (HPV-negative), the hepatoma-derived cell line HepG2, as well as in the SV40-transformed epithelial renal monkey cell line Cos-7. In all these cells, an orientation-dependent stimulation of luciferase activity was measured for both the HPV-16 and the HPV-18 LUR when cloned in promoter configuration (Fig. 3). Moreover, the repressive effect of the HPV-16 LUR poly(A) signal was seen in all cell types. Interestingly, both LURs exhibited a promoter activity in HepG2 cells, in which the HPV-18 URR is almost transcriptionally silent (Hoppe-Seyler & Butz, 1992) (Fig. 3).

Mutational analysis of the two potential TBP sites in the HPV-16 LUR

By analysing the 3′ deletion mutants of the HPV-16 LUR we could localize the active promoter element to a 78 bp DNA fragment in the intervening sequence between the E5 and L2 ORFs. This region contains two potential binding sites for TBP: TATAAT at nucleotide positions 4130–4135 and TATATA at positions 4180–4185, respectively. Function of the latter site, which completely fits the consensus sequence TATAa/tA (Damell et al., 1990), would be in line with the results of Rohlf et al. (1991). Their analysis of cDNAs derived from the HPV-16-immortalized human keratinocyte cell line HPK suggested rare L2^L1 mRNAs starting immediately upstream of the L20RF at nucleotide position 4199. Mutations or deletions of functional TBP sites mostly result in a significant reduction of the transcriptional activity of the corresponding promoter (Maniatis et al., 1987; Guarente, 1987). Thus, mutants of the potential TBP sites within the HPV-16 LUR were created and tested for luciferase activity. The sequence TATAAT was changed into GCTGAT, and the sequence TATATA was changed into GCTGTA by PCR-mediated site-directed mutagenesis within the context of the reporter construct p16LUR665sL. The effects on luciferase activity of the individual mutations and that of the double mutation were then analysed by transient transfection assays in HeLa cells (Fig. 4). Compared with the activity of the wild-type construct, mutation of the potential TBP site at nucleotide positions 4130–4135 had no effect on luciferase activity. Surprisingly, mutation of the potential TBP site at nucleotide position 4180–4185, as well as double mutation of both sites, did not reduce but actually stimulated luciferase activity 2- to 3-fold compared to the wild-type construct. These results suggest that the potential TBP sites analysed are not essential for the promoter activity of the HPV-16 LUR.
Discussion

Previous observations based on transcription and sequence analyses had suggested a promoter element located immediately upstream of the L2 ORF of different human and animal papillomaviruses (Chow et al., 1987a,b; Rohlfis et al., 1991; Baker & Howley, 1987; Kahn et al., 1992; Geisen, 1993; Geisen et al., 1995; Karlen et al., 1996). In order to search for such a LUR promoter element, we performed transient transfection luciferase assays using reporter constructs containing the corresponding fragments of HPV types 16 and 18. The results revealed that both LURs exhibit promoter activity in all six cell lines tested. By using 3' and 5' deletion mutants, as well as point mutations in the natural context, we could define a fragment of 78 bp to be the critical HPV-16 LUR promoter element, and characterize the role of the early poly(A) signal and that of two putative TBP sites located within this element.

Transfection experiments in HeLa cells showed that the transcriptional activity of the HPV-16 LUR fragment is comparable to the activity of the well characterized HPV-18 URR. However, the activity of the HPV-18 LUR was much weaker not only in HeLa cells, but in all cell lines tested. This could be due to the fact that the HPV-18 LUR fragment tested is shorter at its 5' and 3' end than the HPV-16 LUR constructs with strong promoter activity (i.e. p16LUR665sL and p16LUR665/5'delsL). Recently, Maki et al. (1996) provided direct evidence for a promoter element located immediately upstream of the L2 ORF of HPV types 16, 18 and 33, using a CAT reporter system. They also observed a stronger activity of the HPV-16 promoter when compared to the corresponding HPV-18 region. It should be noted that the HPV-18 construct they used was 48 bp longer at its 3' end than p18LUR258sL. Thus it contains sequences up to the early poly(A) signal like the HPV-16 LUR in our analysis. The data presented here extend the findings by Maki et al. (1996) by several important additional aspects. The analysis of 3' exonuclease III deletion mutants of the HPV-16 LUR permitted the definition of the critical promoter element, 78 bp in length (nucleotide positions 4118–4196). This promoter element is part of the region analysed by Maki et al. (1996) (nucleotide positions 3982–4211). The result is in line with the transcription initiation site around nucleotide position 4199 suggested by Rohlfis et al. (1991). Truncation of the 5' end of the HPV-16 URR further enhanced luciferase activity to levels higher than those observed for the HPV-18 URR (Fig. 1), thus providing strong evidence for a negative regulatory element located between nucleotide positions 3535 and 3763, at the 3' end of the E2 ORF. This observation demonstrates that sequences located far upstream of the defined critical promoter element are important for its activity, confirming the sequence analysis predictions.

Fig. 4. Functional analysis of the two potential TBP sites located in the critical HPV-16 LUR promoter region. Schematic representation of the HPV-16 reporter constructs used for the mutational analysis of the potential TBP sites on the left side. Sequences of the wild-type, as well as both single mutations, and the double mutation are indicated in the boxes. The relative activities of the constructs after transfection in HeLa cells are given by the horizontal bars on the right side. Standard deviations are indicated. For calculation see legend to Fig. 1.
The activities of the HPV-16 and HPV-18 LURs were tested in different HPV-positive or HPV-negative, immortal or tumorigenic cells. In all cell types, the LUR elements were active in sense and almost inactive in antisense orientation, with a stronger activity of the HPV-16 LUR when the poly(A) signal was deleted. This demonstrates that the observed promoter activity is not a peculiarity of HeLa cells. Nevertheless, some quantitative differences were observed when the relative activities of the URRs were compared to those of the LURs in a given cell line (Fig. 3). Since HPV-negative cells like HaCaT, C33A, Cos-7 and HepG2 are capable of supporting strong LUR activities, no HPV proteins (at least not the E6 and E7 gene products) seem to be required for LUR activation. Moreover, HepG2 cells, in which the HPV-18 URR is almost inactive, show LUR activity. This raises the possibility that factors other than those essential for the early HPV-18 promoter are required for LUR-driven transcription.

Most eukaryotic promoter elements contain canonical TBP sites at a distance of about 20 nucleotides upstream of the transcription start site. In the case of the HPV-16 LUR, Maki et al. (1996) detected transcription initiation from a number of different sites. However, after co-transfection with a liver-activating protein [pCMV-LAP (Descombes et al., 1990)], they could enhance one of the potential TBP sites suggesting a transcription start site about 20 bp downstream of a potential TBP site, and about 40 bp upstream of the L2 AUG around nucleotide position 4199. This probable start site is in good agreement with the 5' end of an HPV-16 L2-encoding cDNA described by Rohlfs et al. (1991). In our own preliminary RNAse protection and primer extension experiments, multiple start sites, including those shown by Maki et al. (1996) could be detected (not shown). Since the critical HPV-16 LUR promoter element of 78 bp contains two potential TBP sites, mutational analysis of these sites was performed to provide direct evidence for their function. It was necessary to analyse both sites, since the inactivation of a major TBP site may induce the use of otherwise minor TBP sites, leading to confusing results. As expected, mutation of the more distal potential TBP site at position 4130 had no effect on the LUR activity (Fig. 4). Most surprisingly, mutation of the canonical TATATA sequence (position 4180), supposed to be the TBP site used by the LUR promoter element, did not reduce LUR activity. On the contrary, the activity increased by more than twofold compared with the activity of the wild-type construct, rendering the highest HPV-16 LUR activity ever measured. When both sites were mutated simultaneously, the same two- to threefold stimulation of luciferase activity was observed (Fig. 4). These results suggest that the LUR element may be a TATA-less promoter. Promoters without TBP sites have been described for many late viral genes, including those for the L1 gene of CRPV, BPV-1 and HPV-8 (Wettstein et al., 1987; Baker & Howley, 1987; Stubenrauch et al., 1992). However, all these promoters are located in the L1 proximal part of the URR, their messages include a short URR exon, and all drive L1 but not L2 expression. As an alternative interpretation it may be assumed that the putative TBP sites tested are inactive in cultured cells, but become active during keratinocyte differentiation by the expression of adequate regulatory factors. Papillomavirus late gene expression is extremely complex, as described in detail by Hummel et al. (1995), who analysed HPV-31b transcription in a raft culture system. The different pathways include not only differentiation-dependent induction, but also the use of alternative splice sites, post-transcriptional changes, and down-regulation of splicing factors influencing the use of the late polyadenylation signal.

From the data presented in this study, together with the results obtained by the different mRNA mapping experiments, cDNA analyses and the functional assays performed by Maki et al. (1996), we conclude that there is strong evidence for the existence of LUR promoter elements in different HPV genomes. Future experiments will be necessary to analyse the possible role of the LUR elements in transcription of the late genes and to identify the protein factors involved in the regulation of LUR promoter activity during keratinocyte differentiation.

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