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Identification and Characterization of Novel Promoters in the Genome of Human Papillomavirus Type 18

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Most studies on the regulation of gene expression in human papillomaviruses (HPV) have focused on the promoter for the early genes E6 and E7. This promoter is located at the junction between the long control region and the E6 open reading frame. RNA mapping studies have suggested that additional promoters may exist in other parts of the genome. In this study, we used a combination of transcription in vitro and an analysis of RNA produced in vivo in transfected cells to identify three novel promoters in the genome of human papillomavirus type 18. These promoters are located in front of the E2 gene (P_{2598}), within the E2 coding sequences (P_{3036}), and at the end of the L2 open reading frame (P_{5600}). They were active in HeLa cells, as shown by a chloramphenicol acetyltransferase assay. The activity of the P_{3036} promoter was stimulated by the bovine papillomavirus type 1 E2 protein.

Human papillomavirus type 18 (HPV-18) is one of the papillomaviruses associated with cervical carcinomas (13, 74). In such tumors and derived cell lines, the HPV-18 DNA is usually found integrated into the host cell genome (56, 57) and only the E6 and E7 early genes are expressed (4, 54, 58). The E6 and E7 gene products have oncogenic potential. Together, they are sufficient for immortalization of primary human epithelial cells (27, 48). They also interfere with the normal functions of the products of the cellular tumor suppressor genes p53 and retinoblastoma protein. E6 can bind p53 (39, 75) and promote its degradation (35, 55), whereas E7 forms a complex with the retinoblastoma protein (20, 28).

Transcripts coding for E6 and E7 are initiated in the long control region (LCR) of the viral genome, just upstream of the E6 open reading frame (ORF). The major mRNA start site is located at nucleotide (nt) 105 (69). Expression from the P_{105} promoter is controlled by cellular and viral proteins which interact with *cis*-acting responsive elements in the LCR (21, 23). A major viral regulatory protein is the E2 gene product (8, 62, 72). This protein is well conserved among papillomaviruses (8). E2 binds as a dimer (17, 22, 45) to the palindromic sequence $ACCN_6GGT$ (17), which is present four times within the regulatory region of HPV-18. Two of these sites are located 3 nt upstream of the TATA element of the P_{105} promoter. Binding of the E2 protein to these motifs results in repression of promoter activity by preventing formation of the initiation complex (16, 70). In contrast, when the E2-binding sites are placed farther upstream, they can function as an enhancer; in this case E2 acts as a transcriptional activator (8, 72). Several cellular factors interact with the HPV-18 regulatory region (21). At least two such factors (AP1 and KRF-1) are implicated in the cell-type specific activity of the P_{105} promoter (43, 50, 71). In contrast, transcription from P_{105} is repressed by the transcription factor Oct-1 (34) and by the transcriptional repressor YY1 (5).

A promoter in front of E6 is a common feature of all papillomaviruses studied so far. Additional promoters, or putative promoters, have been identified in the LCR or within the early regions of certain papillomaviruses. The 5′ end of an RNA has been mapped in front of E7 on the genomes of HPV-6 and HPV-11 (60). A spliced RNA encoding an E1/E4 fusion protein has its 5′ end within the E7 ORFs of HPV-6 (11), HPV-11 (49), HPV-16 (32), and HPV-31b (36). Promoters controlling the expression of the activator (62) and repressor (38) forms of the E2 protein as well as the E5 gene product have been found in bovine papillomavirus type 1 (BPV-1) (3, 53, 63, 73, 77). Finally, a late promoter located close to the junction of L1 and the LCR has been identified in the genomes of HPV-1 (51), HPV-8 (65), BPV-1 (3), and cottontail rabbit papillomavirus (76).

In HPV-18, only the E6 promoter has been characterized. The identification of additional promoters has so far been difficult for two reasons. First, in cervical cancers and derived cell lines, integration of viral DNA is characterized by the loss of part of the viral genome, and thus only the early transcripts initiated at the P_{105} promoter are detected. Second, a productive papillomavirus infection occurs only in squamous epithelial cells undergoing terminal differentiation, suggesting that certain promoters controlling viral gene expression are activated during keratinocyte differentiation (52). The lack of a productive system for HPV-18 has not permitted the identification of such promoters. The aim of the work reported here was to screen for potential promoters outside the LCR. DNA fragments spanning the coding sequences of the HPV-18 genome were isolated and used as templates for in vitro runoff transcription experiments. We found promoter activities in the E2 region and in the middle of the late region. Primer extension and RNase protection analysis allowed us to identify three start sites, one in front of E2 (P_{2598}), one within the E2 coding sequences (P_{3036}), and one at the end of the L2 ORF (P_{5600}). These initiation sites corresponded to promoters which were all active in HeLa cells as shown by chloramphenicol acetyltransferase (CAT) assays. We also investigated the importance of a putative Sp1-binding site as well as the effect of E2 on P_{3036} activity.

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MATERIALS AND METHODS

Cell extract. HeLa whole-cell extracts were prepared as described previously by Manley et al. (44). HeLa cells (strain S3) were grown in suspension culture in Dulbecco's minimal essential medium with 5% fetal bovine serum.

Transcription in vitro. Transcription reactions with HeLa whole-cell extracts (about 150 μg of protein in a reaction volume of 50 μl) were carried out as described previously (6). ^{32}P-labeled RNA products were denatured with glyoxal and were analyzed by agarose gel electrophoresis (3% NuSieve agarose; FMC BioProducts). The DNA templates (0.5 μg per reaction) are described in the legends to Fig. 1 and 3. For primer extension analysis, transcription reactions were carried out with nonlabeled nucleoside triphosphates. For competition studies, the in vitro transcription mix was incubated on ice without DNA template for 5 min with a 20-fold molar excess of competing oligonucleotide. The in vitro transcription was started by addition of the DNA template and transfer to 30°C.

Plasmids. To generate the pST1CAT construct, the *Eco*RI (nt 2440)-*Alu*I (nt 3042) fragment was excised from the HPV-18 genome (HPV-18 DNA was a gift from Harald zur Hausen) and inserted via intermediate cloning into the unique *Sal*I site of the CAT vector pBLCAT3 (42). The plasmid pST2CAT was made by inserting the *Hin*dIII (site in the polylinker)-*Nsi*I (nt 2707) fragment of pST1CAT between the *Hin*dIII and *Pst*I sites of pBLCAT3. The plasmid pST3CAT was obtained by cutting pST2CAT at the *Eco*72I site (nt 2514) and *Xho*I (site in the polylinker), filling the ends with the T4 DNA polymerase, and religating. The pST1CAT construct was digested with *Hin*dIII and *Nsi*I (nt 2707). The ends were made blunt and were religated to generate the pST4CAT plasmid. The *Dra*III (nt 2999)-*Xho*I (site in the polylinker) fragment was deleted in this latter construct to give pST5CAT. To prepare pST4CAT-DraIII, the pST4CAT plasmid was digested with *Dra*III which cuts at position 2999. The ends were made blunt with T4 DNA polymerase and were religated. A new *Dra*III site was formed, and by a second cycle of digestion, filling, and ligation, a deletion of 6 nt from nt 2997 to 3002 was created.

The *Nsi*I-*Xba*I fragment (nt 4031 to 5730) was isolated from HPV-18 and was cloned into pBLCAT3 between the *Pst*I and *Xba*I restriction sites to generate pST6CAT-5730. pST6CAT-5250 and pST7CAT-5730 were obtained by removing from pST6CAT-5730 the *Pst*I-*Xba*I (nt 5250 to 5730) and *Hin*dIII (site in the polylinker)-*Eco*RV (nt 4669) fragments, respectively. The deletion of the *Pst*I-*Xba*I fragment (nt 5250 to 5730) of pST7CAT-5730 results in pST7CAT-5250. The plasmid pST2βG was obtained by inserting the *Hin*dIII (site in polylinker)-*Nsi*I (nt 2707) fragment from pST1CAT between the *Hin*dIII and *Pst*I sites of pGβG(−). The plasmid pGβG(−) (a gift from Markus Nabholz) contains the 1,664-bp-long DNA fragment encoding the entire β-globin gene cloned into the *Hin*dII site of pGEM3zf(+) (Promega), with the 3′ end of the gene inserted proximal to the T7 promoter.

C59 is a plasmid which expresses the full-length E2 protein from BPV-1 (a gift from Peter Howley). C59KpnTTL (kindly provided by Moshe Yaniv) is a construct in which a translation termination linker has been inserted into the *Kpn*I site of C59 and which expresses only the N-terminal domain of E2 (N-E2).

Cell transfection and CAT assays. HeLa cells were grown in Dulbecco's minimal essential medium containing 5% fetal bovine serum. They were transfected in 100-mm culture dishes at 50 to 60% confluency by standard calcium phosphate techniques (2). For transfection, 20 μg of CAT reporter plasmid (5 μg of pSV2CAT) was used per plate. For E2 *trans*-activation studies, 5 μg of C59, C59KpnTTL, or sonicated salmon sperm DNA was cotransfected with the CAT reporter plasmid. The cells were incubated for 45 min at 37°C, with the transfection solution added directly onto the cells. Fresh medium was added to the transfection solution, and the cells were further incubated overnight at 37°C. The transfected cells were washed with a phosphate-buffered saline solution and were refed with fresh medium. Cell extracts were prepared 40 h after transfection, and CAT activity was analyzed as described previously by Doerig et al. (14).

RNA analysis. Total cytoplasmic RNA was extracted from transfected cells according to the Nonidet P-40 lysis method described by Ausubel et al. (2). For primer extension analysis, in vitro RNA transcripts or total cytoplasmic RNAs (30 μg) were hybridized to 20 fmol of oligonucleotide that had been labeled at the 5′ end with [γ-^{32}P]ATP and polynucleotide kinase. The hybridization reaction (20 μl) was carried out in 250 mM KCl–10 mM Tris-HCl (pH 7.5)–1 mM EDTA, incubated for 60 min at 65°C, and then cooled slowly to 42°C. Extension was initiated by adding 6 μl of 10× reverse transcriptase buffer (100 mM $MgCl_2$, 200 mM Tris-HCl [pH 8.3], 100 mM dithiothreitol, 5 mM dATP, 5 mM dCTP, 5 mM dGTP, 5 mM dTTP), 32 μl of H_2O, and 20 U of avian myeloblastosis virus reverse transcriptase (Promega). Reactions were incubated for 90 min at 42°C and were stopped by extraction with phenol-chloroform.

The probe used for RNase protection analysis was prepared as follows. The *Acc*I-*Sma*I fragment, which contains the 3′ end of the β-globin coding sequence, was deleted in pST2βG. This deletion places the T7 promoter of pGEM3zf (+) adjacent to the *Acc*I site. A T7 probe was synthesized by transcription of this template linearized at the *Hin*dIII site (see Fig. 8). T7 in vitro transcription was carried out as described by the supplier (Promega). Hybridization with 5×10^5 cpm of T7 probe and 30 μg of total cytoplasmic RNA was carried out at 48°C overnight (46). RNase digestion was performed as described by Thierry et al. (69).

Dideoxy sequencing ladders were generated by using plasmid DNA, and the same oligonucleotide used for the primer extension reactions. Sequencing of double-stranded DNA was carried out according to the protocol described in the Sequenase version 2.0 DNA sequencing kit (United States Biochemical Corporation).

Primer extension, RNase protection, and sequencing reaction products were analyzed on 6% polyacrylamide–7 M urea sequencing gels.

DNA mobility shift experiments. Standard binding reaction mixes (20 μl) contained 30 μg of whole-cell protein (44) or 7.5 ng of Sp1 protein (>90% pure; Promega), 50 mM KCl, 8 mM $MgCl_2$, 11 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.9] 11% glycerol, 0.1 mM EDTA, 1 mM dithiothreitol, 1 μg of single-stranded *Escherichia coli* DNA, and 20 fmol of end-labeled oligonucleotide probe. Probe preparation, protein-DNA binding reactions, competitive binding studies, and analysis by polyacrylamide gel electrophoresis were performed as described by Offord and Beard (50).

RESULTS

The only promoter identified so far in the genome of HPV-18 is P_{105}. This promoter functions both in vivo (69)

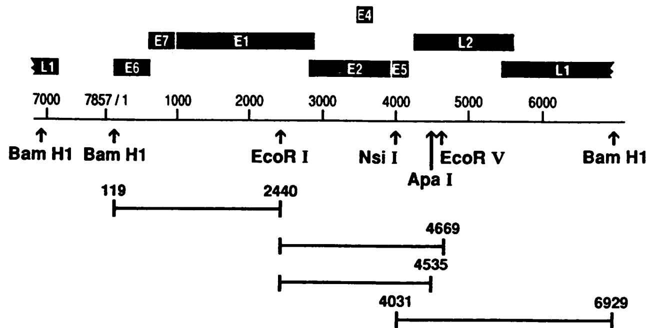

FIG. 1. HPV-18 genome organization and templates used for in vitro runoff transcription experiments. The HPV-18 genome open at the *Bam*HI site 6929 is shown with early (E) and late (L) ORFs (12). The fragments *Bam*HI-*Eco*RI (nt 119 to 2440), *Eco*RI-*Eco*RV (RI-RV, nt 2440 to 4669), *Eco*RI-*Apa*I (RI-ApaI, nt 2440 to 4535) and *Nsi*I-*Bam*HI (nt 4031 to 6929) were isolated and used as templates for in vitro runoff transcription assays.

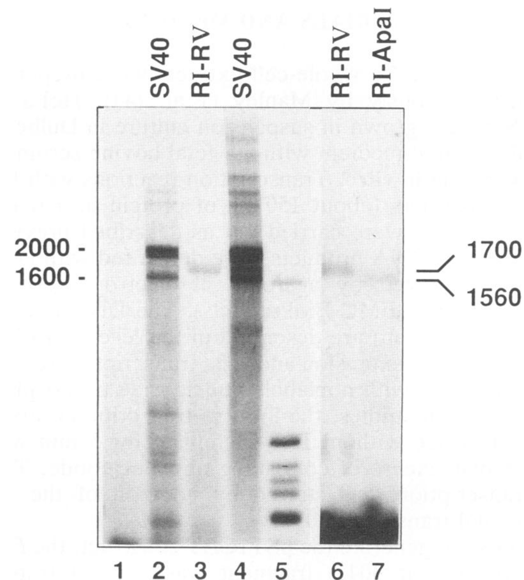

FIG. 2. Analysis of the HPV-18 E2 region by transcription in vitro. HPV-18 and SV40 DNA were transcribed by using a whole-cell extract from HeLa cells. DNA templates (0.5 μg) were the HPV-18 fragments RI-RV (lanes 3 and 6), RI-ApaI (lane 7), and SV40 cut with *Pst*I (lanes 2 and 4). Lane 1 is a reaction which contains no DNA template. The runoff RNA transcripts were glyoxylated and analyzed by agarose (3% NuSieve) gel electrophoresis. The size markers (lane 5) were radiolabeled pBR322 *Hin*fI restriction fragments (1631, 516/506, 396, 344, 298, 221/220, 154, and 75 bp). The sizes (nucleotides) of the SV40 early and late transcripts are indicated on the left, and the sizes of the HPV-18 transcripts are indicated on the right.

and in vitro (50). Our first approach to screen for promoters outside the LCR was to isolate a series of DNA fragments covering the HPV-18 coding region (Fig. 1) and to test their activities as transcriptional templates.

Detection of promoter activity in the E2 region of HPV-18 by transcription in vitro. The DNA fragment covering the HPV-18 genome from the *Eco*RI site at position 2440 to the *Eco*RV site at position 4669 was isolated (Fig. 1). This fragment (RI-RV), which includes the 3′ end of E1, the entire E2, E4, and E5 ORFs, and the 5′ end of L2, was used as template for in vitro runoff transcription experiments with a whole-cell extract (44) of HeLa cells. The resulting transcripts were compared with those from simian virus 40 (SV40) DNA cut with *Pst*I. The results are shown in Fig. 2. One transcript of about 1,700 nt was observed with the RI-RV template (Fig. 2, lanes 3 and 6). A reaction carried out without DNA showed that no endogenous transcript was produced from the extract (lane 1). Lanes 2 and 4 show the results of in vitro transcription of SV40 DNA cut with *Pst*I. The two 2-kb early and 1.6-kb late transcripts of SV40 (66) are indicated. The 1.7-kb transcript seen in lanes 3 and 6 was not observed when SV40 DNA was transcribed or when a reaction without DNA was carried out. Thus, the HeLa extract transcribed specifically the HPV-18 DNA template.

In order to determine the polarity of the transcript generated by the RI-RV template, a second fragment was isolated by cutting the HPV-18 DNA at *Eco*RI (nt 2440) and *Apa*I (nt 4635). This second template (RI-ApaI) has a common 5′ end with the RI-RV fragment but is 134 bp shorter (Fig. 1). If transcription proceeds from left to right on the map shown in Fig. 1, one would expect that the RNA transcript produced by the RI-ApaI fragment would be approximately 130 nt shorter than the transcript observed with the RI-RV template. These two fragments were analyzed by in vitro transcription (Fig. 2, lanes 6 and 7). As predicted for rightward transcription, a slightly shorter transcript was observed with the RI-ApaI. These results allowed us to map the in vitro start site around position 2960 in the E2 ORF of the HPV-18 genome.

Promoter activities in vivo in the E2 region. To test the activity in vivo of the promoter detected in the E2 region, we prepared a series of plasmids by inserting DNA fragments spanning the HPV-18 genome from the *Eco*RI site (nt 2440) to the *Alu*I site (nt 3042) into the polylinker of pBLCAT3 (42), upstream of the *cat* reporter gene (Fig. 3A). These constructs were transfected into HeLa cells, and their promoter activities were tested by CAT assays. The results are summarized in Fig. 3B. Transfections with two plasmids (pST1CAT, lane 2, and pST4CAT, lane 5), both containing the E2 region where the in vitro promoter activity was mapped, led to the expression of the *cat* gene. These results confirmed the presence of an RNA start site within or immediately upstream of the E2 ORF. The removal of a short sequence (45 bp) at the downstream end of the HPV-18 sequence in pST4CAT completely abolished this activity (pST5CAT, lane 6), suggesting that the RNA initiation site is located in this small fragment, close to the internal initiation codon (ATG 3057) of the E2 ORF.

After deletion of a fragment which contains essentially the E2 coding sequences (construct pST2CAT), we still observed a strong CAT activity (about 20% of the activity of pSV2CAT in lane 3, Fig. 3B), suggesting the presence of a second promoter which was not detected by in vitro transcription. The activity of the pST2CAT construct was reduced by a factor of 50 when the sequences between the *Eco* 72I and *Nsi*I sites (nt 2514 to 2707) were removed (plasmid pST3CAT, lane 4, Fig. 3B). This result confirmed the presence of an additional start site upstream of the E2 ORF in the HPV-18 genome.

Analysis of the transcripts initiating within the E2 ORF: mapping of the P_{3036} promoter. The promoter activity detected within the E2 ORF of HPV-18 was mapped close to the ATG at 3057 (by in vitro transcription and CAT assay). The 5′ termini of the RNAs initiating at this promoter were

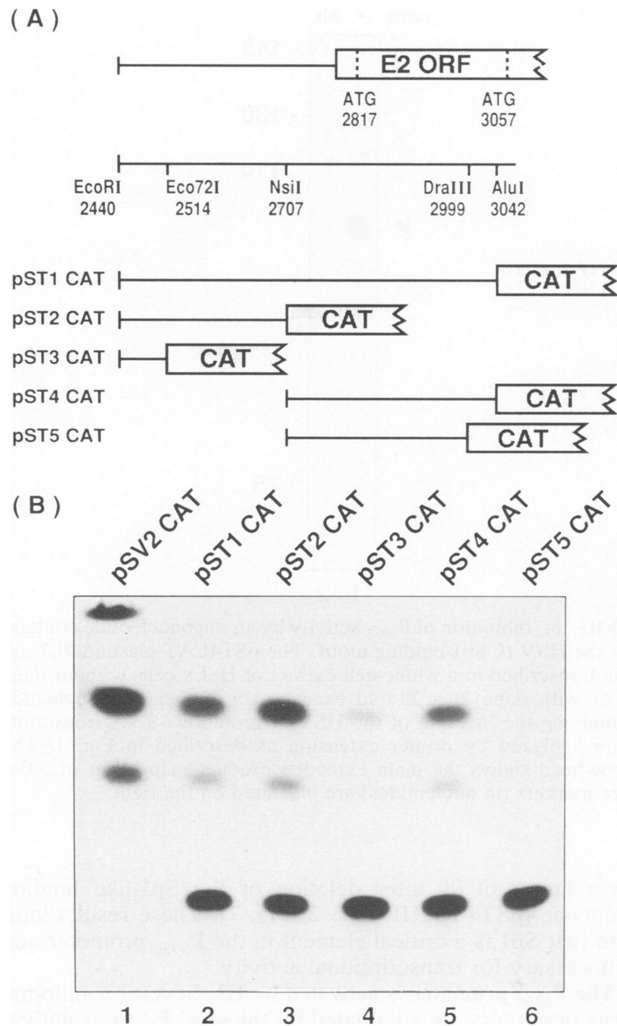

FIG. 3. CAT activities expressed from the E2 region. (A) DNA fragments spanning the HPV-18 genome from the *Eco*RI site at position 2440 to the *Alu*I site at nt 3042 were cloned into the polylinker of pBLCAT3 upstream of the *cat* reporter gene. (B) HeLa cells were transfected with 20 μg of the CAT construct, and the CAT assay was carried out 40 h later, as described in Materials and Methods, with a reaction time of 1 h. Lane 1 is a reaction with 5 μg of pSV2CAT DNA as a positive control.

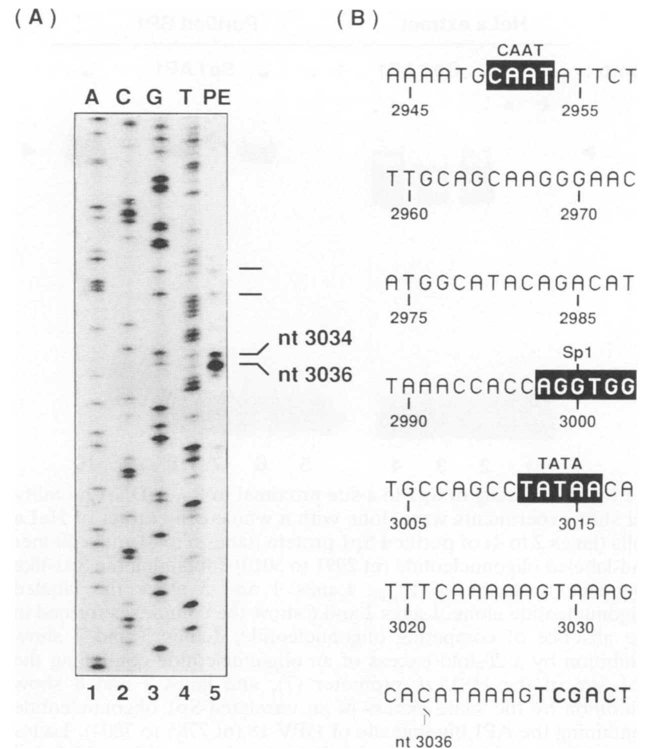

FIG. 4. Primer extension analysis of in vitro transcripts initiated in the E2 region. (A) The pST4CAT construct was linearized at the *Sma*I site and transcribed in vitro with unlabeled nucleoside triphosphates. Transcripts were hybridized to a 5′-^{32}P-radiolabeled oligonucleotide (nt 523 in the pBLCAT3 sequence). The extended products were analyzed on a sequencing gel (6% polyacrylamide–7 M urea). The marker is a sequencing reaction with the same oligonucleotide as a primer and pST4CAT DNA as a template. The positions and nucleotide numbers of the main extended products are indicated on the right. (B) Sequence analysis of the P_{3036} region. The sequences matching TATA, CAAT, and Spl motifs are indicated by boxes. Nucleotides in boldface are from the pBLCAT3 sequence.

determined by primer extension analysis. The pST4CAT construct was linearized at the *Sma*I site (nt 2094 in the pBLCAT3 sequence) and used as template for an in vitro transcription reaction. The RNA transcripts were hybridized to a ^{32}P-end-labeled 22-nt-long synthetic oligonucleotide complementary to the *cat* coding sequence (nt 523 of the noncoding strand of pBLCAT3). The hybrids were extended by reverse transcriptase and were analyzed on a sequencing gel along with a dideoxy sequencing ladder generated by the same oligonucleotide and double-stranded pST4CAT plasmid DNA. Several closely spaced primer extension products were observed (Fig. 4A, lane 5), and the 5′ end of the most prominent one was mapped at nt 3036. This result confirms the presence of a transcriptional promoter with an initiation site located in the small *Dra*III (nt 2999)-*Alu*I (nt 3042) fragment close to the ATG 3057.

Analysis of the DNA sequence upstream of P_{3036} (Fig. 4B) revealed a potential TATA box (TATAA) at nt 3012 to 3016, 24 nt upstream from the RNA start site, and a CAAT box (GCAAT) present at nt 2949 to 2953. A potential Spl-binding sequence, AGGTGG, was found close to the TATA box. Although this sequence is not a perfect consensus sequence for Spl (GGGCGG [18, 19]), Spl-binding sites matching the 5′-NGGNGN-3′ consensus sequence (24) have been found to be functionally active in BPV-1 (41, 61) and in HPV-16 (24). Several papillomavirus promoters are regulated by the viral E2 protein. We looked for the presence of E2-responsive elements ($ACCN_6GGT$ [1]) in the P_{3036} region. No evidence for such a regulatory element was found.

The AGGTGG sequence binds Spl and is essential for P_{3036} activity. We were interested to know whether or not the Spl-related binding sequence AGGTGG was necessary for expression from P_{3036}. First, DNA mobility shift experiments were performed to demonstrate that Spl binds to the AGGTGG sequence (Fig. 5). A 20-bp-long oligonucleotide, which covers the HPV-18 sequence from nt 2991 to 3010 and includes the AGGTGG Spl-like sequence, was used in the assay with an extract from HeLa cells (Fig. 5, lanes 1 to 4) or with purified Spl protein (Promega) (Fig. 5, lanes 5 to 8).

Three DNA-binding complexes were observed when proteins in HeLa extract were used in the assay (Fig. 5, lane 2),

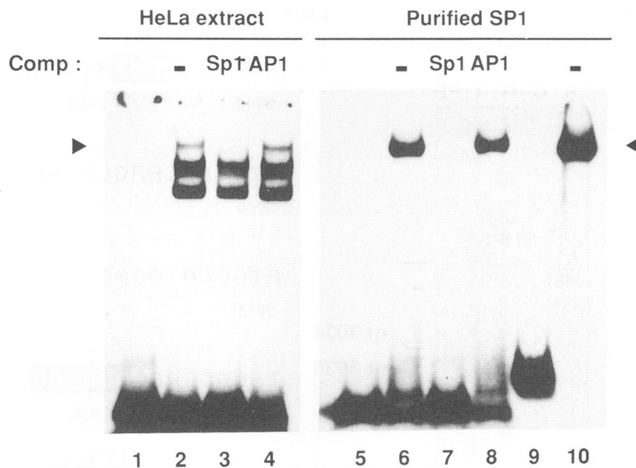

FIG. 5. Binding of Sp1 to a site proximal to P_{3036}. DNA mobility gel shift experiments were done with a whole-cell extract of HeLa cells (lanes 2 to 4) or purified Sp1 protein (lanes 6 to 8) and a 20-mer end-labeled oligonucleotide (nt 2991 to 3010) containing the Sp1-like sequence upstream of P_{3036}. Lanes 1 and 5 show the labeled oligonucleotide alone. Lanes 2 and 6 show the complexes formed in the absence of competing oligonucleotide. Lanes 3 and 7 show inhibition by a 20-fold excess of an oligonucleotide containing the Sp1 site of the HSV *tk* promoter (7), and lanes 4 and 8 show inhibition by the same excess of an unrelated-Sp1 oligonucleotide containing the AP1 binding site of HPV-18 (nt 7785 to 7804). Lanes 9 and 10 show the result of a binding experiment with end-labeled HSV *tk* Sp1 oligonucleotide in the absence (lane 9) or presence (lane 10) of Sp1 protein.

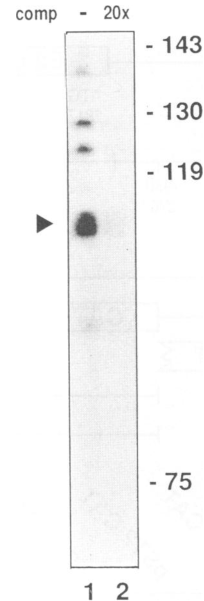

FIG. 6. Inhibition of P_{3036} activity by an oligonucleotide containing the HSV *tk* Sp1-binding motif. The pST4CAT plasmid (0.2 μg) was transcribed in a whole-cell extract of HeLa cells without (lane 1) or with (lane 2) a 20-fold excess of a 32-mer oligonucleotide containing the Sp1 site of the HSV *tk* promoter. RNA transcripts were analyzed by primer extension as described in Fig. 4. The arrowhead shows the main extended product mapped at nt 3036. Size markers (in nucleotides) are indicated on the right.

but only the top complex appeared to be specific for Sp1, because it was the only one inhibited with an excess of Sp1 binding sequence (lane 3) but not with a similar excess of AP-1 binding sequence (lane 4). Binding was also inhibited when an excess of unlabeled HPV-18 oligonucleotide was added to the reaction (data not shown). Similar results were obtained when we used purified Sp1 protein rather than a cell extract (lanes 5 to 8). These results demonstrated that Sp1 binds the HPV-18 sequence AGGTGG.

To investigate whether the Sp1-like sequence was important for transcription from the P_{3036} promoter, the Sp1 herpes simplex virus (HSV) *tk* oligonucleotide was used as a competitor in an in vitro transcription experiment (Fig. 6). The pST4CAT construct linearized at the *Sma*I site was transcribed with nuclear extract of HeLa cells which had been incubated with or without a 20-fold excess of competing oligonucleotide. The in vitro transcripts were analyzed by primer extension as described above. Transcription in vitro from the P_{3036} promoter (lane 1) was specifically blocked by competing Sp1 sequence (lane 2), whereas transcription was not reduced by competing oligonucleotide containing the AP-1 binding site (data not shown). This preliminary experiment suggested that binding of Sp1 to the Sp1-like binding sequence is important for transcription from the P_{3036} promoter in vitro.

The importance of the Sp1-like binding sequence AG GTGG for promoter activity was further studied with a transient CAT expression assay. Part of the AGGTGG sequence upstream of P_{3036} promoter was deleted in the plasmid pST4CAT to generate pST4-DraIII (Fig. 7). These two constructs were transfected into HeLa cells, and CAT activity was measured 40 h after infection. Expression from the P_{3036} promoter (pST4CAT, lane 1, Fig. 7) was reduced by a factor of 90 after deletion of the Sp1-like binding sequence (pST4-DraIII, lane 2, Fig. 7). These results indicate that Sp1 is a critical element in the P_{3036} promoter and is necessary for transcriptional activity.

The P_{3036} promoter is activated by E2. Several papillomavirus promoters are stimulated by the viral E2 *trans*-activator protein (62), which acts on viral *cis* elements containing the $ACCN_6GGT$ palindromic sequence (25, 31, 33, 65, 72). E2 was also shown to activate heterologous promoters without E2-responsive elements (26, 29). In this case, the N-terminal domain of the protein, which contains the activation domain of E2 and no DNA-binding domain, is sufficient for stimulation of promoter activity (26). It has been reported that E2 can stimulate transcription from a synthetic promoter even in the absence of E2-binding motifs if Sp1 sites are placed proximal to the start site (41). We demonstrated above that an Sp1 site is located close to the start site at nt 3036. We wanted to test whether E2 was able to activate transcription from the P_{3036} promoter in the absence of E2-binding motifs with the aid of Sp1.

The two plasmids pST4CAT and pST4-DraIII were transfected into HeLa cells with or without plasmids expressing either the full-length (E2) or N-terminal (N-E2) domain of BPV-1 E2. The results are shown in Fig. 7. The activity of the P_{3036} promoter (Fig. 7, lanes 1, 5, and 7) was abolished when the Sp1 site was deleted (lane 2), and neither E2 (lane 3) nor N-E2 (lane 4) was able to restore activity from P_{3036}. In contrast, when the Sp1 site was present, a fivefold stimulation of promoter activity was observed with both E2 (lane 6) and N-E2 (lane 8). Assuming that the N-E2 expression vector containing a translational termination linker did indeed produce only the N-terminal domain of E2, these results confirm that this domain is sufficient for *trans*-

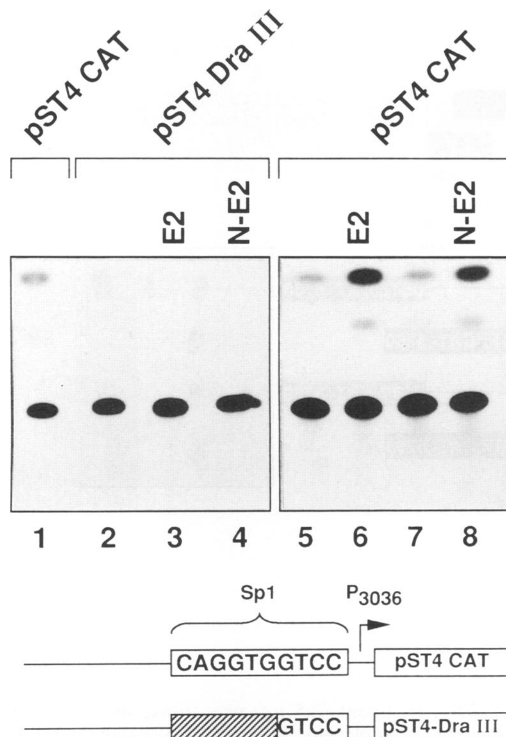

FIG. 7. Role of the Sp1-binding site and E2 in P_{3036} activity. The Sp1-binding site in pST4CAT was partially deleted (hatched box) to generate pST4-DraIII. HeLa cells were transfected with 20 μg of pST4CAT (lane 1 and lanes 5 to 8) or pST4-DraIII (lanes 2 to 4) and 5 μg of C59 (E2) or C59KpnTTL (N-E2), where indicated.

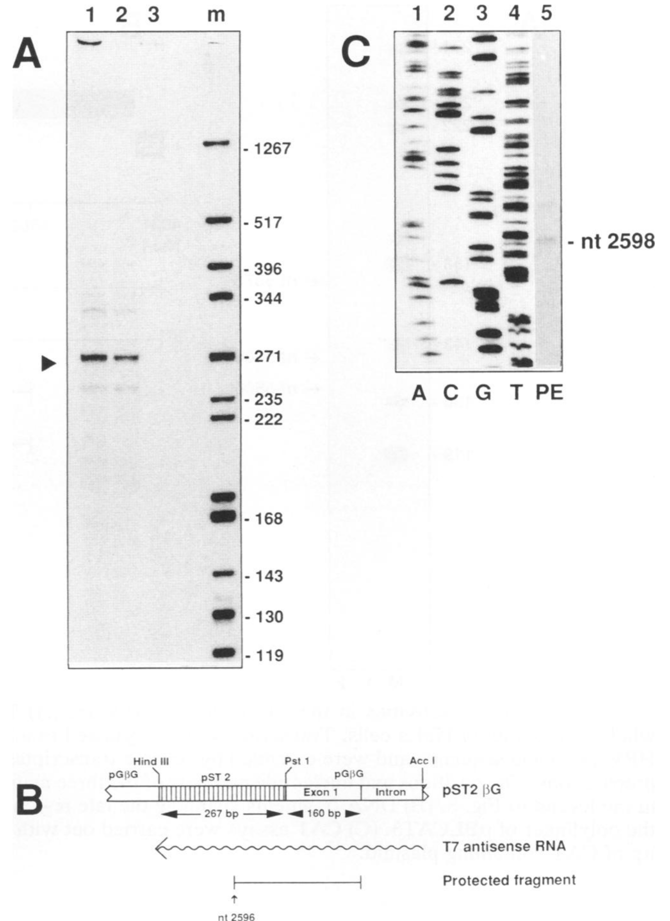

FIG. 8. RNase protection and primer extension analysis of pST2βG RNA. (A) Total cytoplasmic RNA (10 μg) from HeLa cells transfected with 20 μg of pST2βG DNA was hybridized to a T7 antisense RNA probe as described in Materials and Methods. Protected fragments were analyzed on a sequencing gel. HeLa cells in lanes 1 and 2 were transfected with DNA from two different plasmid preparations. Lane 3 is a control with RNA from untransfected cells. The arrowhead shows the main protected fragment of about 270 nt. Size markers were radiolabeled fragments of pBR322 digested with *Hin*fI and *Ava*I restriction enzymes. (B) Schematic representation of the pST2βG construct. The HPV-18 DNA fragment (hatched box) from pST2CAT was inserted between the *Hin*dIII and *Pst*I sites of pGβG(−) to generate pST2βG. The wavy line represents the T7 antisense RNA probe, which is complementary to pST2βG from the *Acc*I to the *Hin*dIII sites (see Materials and Methods). The protected fragment of about 270 nt is shown with the initiation site mapped around nt 2596. (C) Thirty micrograms of total cytoplasmic RNA from pST2βG-transfected cells was hybridized to a 20-mer oligonucleotide complementary to the β-globin sequence (see text). The extended product mapped at nt 2598 is indicated. The marker is a dideoxy sequencing ladder with the same oligonucleotide as primer and pST2βG DNA as template.

activation in the absence of E2-responsive elements. They also indicate that, in the absence of Sp1 motifs, E2 is not able to activate transcription from P_{3036}. This is consistent with the notion (41) that Sp1 is the target protein of E2. Alternatively, the Sp1 motifs may be essential for any P_{3036} promoter activity.

Analysis of the promoter located upstream of the E2 ORF: mapping of P_{2598}. Because the promoter located upstream of E2 was detected only in vivo (Fig. 3, lane 3), we used an RNase protection method to map the RNA start site of transcripts produced in transfected cells. We inserted the 267-bp DNA fragment containing the promoter activity from pST2CAT upstream of the β-globin coding sequences in the plasmid pGβG(−). This new plasmid, called pST2βG, was transfected into HeLa cells. RNA was extracted and analyzed by RNase protection with a T7 antisense RNA probe as indicated in the legend to Fig. 8B. A major protected fragment of approximately 270 bp was observed (Fig. 8A, lanes 1 and 2), with a 5′ end mapping around nt 2596. No protected fragment was observed with RNA isolated from untransfected cells (lane 3).

To confirm the mapping determined by RNase protection, the same RNA was used in a primer extension assay with a 20-mer oligonucleotide complementary to the β-globin sequence [nt 1680 to 1699 in the pGβG(−) sequence]. The extended products were analyzed on a sequencing gel alongside a dideoxy sequencing ladder generated by the same oligonucleotide and pST2βG DNA (Fig. 8C). The RNA start site was mapped to position 2598 (P_{2598}; lane 5) in agreement with the RNase protection results.

Analysis of the sequence around 30 bp upstream of nt 2598 did not show the presence of a TATA box, but a sequence matching a CAAT box (CCAAT, nt 2564 to 2568) was found. Interestingly, a potential Sp1 motif AGGTGG (nt 2484 to 2489) was identified 104 bp upstream of the initiation site. DNA mobility shift experiments were performed with a 20-bp-long oligonucleotide (nt 2477 to 2496) covering the AGGTGG site and protein extract from HeLa cells or

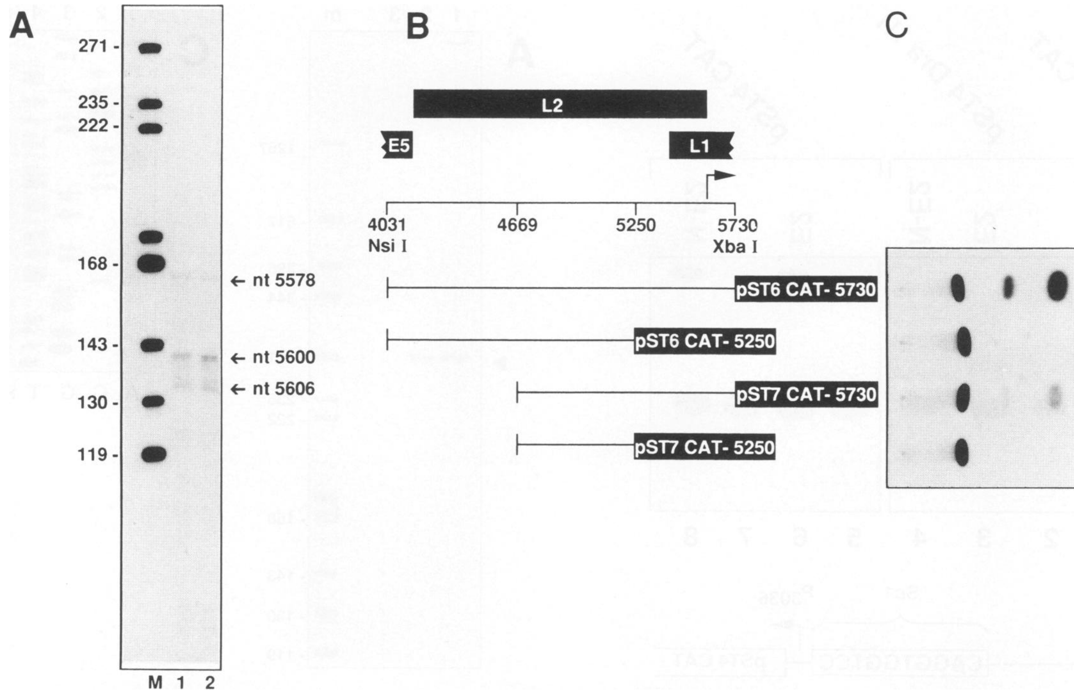

FIG. 9. Promoter activities in the late region of HPV-18. (A) The *Nsi*I-*Xba*I DNA fragment (0.5 μg) was transcribed in vitro with a whole-cell extract of HeLa cells. Transcripts were hybridized to a 20-mer end-labeled oligonucleotide (5′ at nt 5743) complementary to the HPV-18 coding sequence and were extended by reverse transcriptase. Lanes 1 and 2 are results of transcription with two different template preparations. The positions and nucleotide numbers of the three main extended products are indicated on the right. The markers are described in the legend to Fig. 8. (B) DNA fragments spanning the late region from the *Nsi*I site (nt 4031) to the *Xba*I site (nt 5730) were inserted into the polylinker of pBLCAT3. (C) CAT assays were carried out with a reaction time of 3 h with extracts from HeLa cells transfected with 20 μg of CAT-containing plasmid.

purified Sp1 protein. No binding of Sp1 was observed (data not shown). In contrast, the AGGTGG sequence located at position 2998 close to P_{3036} has been demonstrated to bind Sp1 (Fig. 5). These results point to the fact that the nucleotides flanking the core sequence of an Sp1 motif play a role in the specificity of DNA binding. A weight matrix analysis (10) of the sequence covering the potential Sp1 site at position 2484 did not define this sequence as a binding site for Sp1, whereas the motif found upstream of P_{3036} was recognized as an Sp1-binding site, consistent with the results of the gel mobility shift experiments. In addition, cotransfection experiments with both E2 and N-E2 did not result in the stimulation of the P_{2598} promoter (data not shown). Taken together, these results are in agreement with the model in which *trans* activation by E2 in the absence of E2-binding motifs is mediated by the Sp1 protein which binds to elements close to the initiation site.

Identification of promoter activity in the late region of HPV-18. The *Nsi*I (nt 4031)-*Bam*HI (nt 6929) fragment (*Nsi-Bam*), which essentially contains the late region of the HPV-18 genome (Fig. 1), was isolated and used as template in an in vitro runoff transcription assay. A specific transcript of approximately 1,320 nt was observed (data not shown), suggesting the presence of an RNA start site around nt 5600. To map this promoter and to determine the polarity of the transcription, RNA transcripts synthesized in vitro were hybridized to an oligonucleotide (5′ end at nt 5743) complementary to the HPV-18 sequence and were analyzed by primer extension (Fig. 9A). Three main extended products were observed, with 5′ termini mapping at nt 5578, 5600, and 5606, close to the end of the L2 ORF. To confirm the presence and to test the in vivo activity of this promoter (P_{5600}), the *Nsi*I (nt 4031)-*Xba*I (nt 5730) fragment was cloned into pBLCAT3 (Fig. 9B). The resulting construct (pST6CAT-5730) was found to be active in HeLa cells (Fig. 9C). The removal of the viral sequences which overlap the L2-L1 region (pST6CAT-5250) reduced the activity of the promoter to the background level. Interestingly, when a fragment of 638 nt was deleted at the 5′ end of the viral sequences (pST7CAT-5730), an 8.5-fold reduction of the promoter activity was observed, indicating the presence of a *cis*-acting enhancer element. Additional deletion at the 3′ end of the promoter element (pST7CAT-5250) completely abolished the ability of the L2-L1 region to drive expression of the reporter gene. These results confirmed the presence of an enhancer-promoter element in the late region of the HPV-18 genome with RNA initiation sites located close to the end of the L2 ORF.

Analysis of the early region by transcription in vitro. The *Bam*HI (nt 119)-*Eco*RI fragment (Fig. 1), which contains roughly the coding sequences of HPV-18 which are integrated in carcinoma cell lines (57), was also analyzed by using the in vitro runoff transcription assay. We found no evidence for promoter activity within this template. These results are in agreement with the observation that all of the viral transcripts detected in HPV-18-derived tumor cell lines are initiated in the vicinity of the start codon for the E6 ORF, just at the end of the LCR (56).

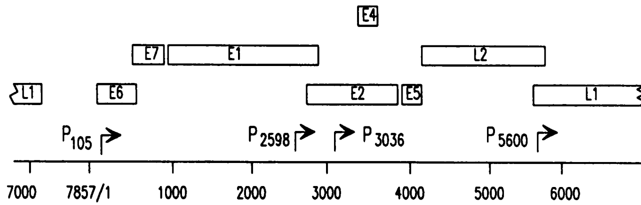

FIG. 10. Map of the promoters identified in the genome of HPV-18. The promoter P_{105} identified by Thierry and Howley (70), as well as the promoters P_{2598}, P_{3036}, and P_{5600}, identified in this study, are indicated by arrows.

DISCUSSION

In this work, three novel promoters were detected within the genome of HPV-18. They were all active in HeLa cells. The initiation sites of the first two were mapped in the early region at nt 2598 (P_{2598}) and at nt 3036 (P_{3036}), whereas the start of the third one was located around nt 5600 (P_{5600}) in the late region (Fig. 10). P_{2598} is found upstream of the initiation codon (ATG 2817) used for the synthesis of the full-length E2 protein, and P_{3036} is located within the coding sequences of E2 close to an internal methionine (ATG 3057) (12). In BPV-1, two promoters (P_{2443} and P_{3080}) have been identified in the E2 region (3, 63, 77), and transcription from these two promoters results in mRNAs encoding two forms of the E2 protein (38). The first species of mRNA codes for the full-length protein, which represents the transactivator form of E2 (62), whereas the second species of mRNA codes for a truncated form of E2 initiated at an internal methionine. The short E2 can act as a transcriptional repressor (38). In BPV-1 a second species of mRNA is produced from P_{2443} and is spliced to encode the E5 gene product (53, 63, 77). By analogy, it is possible that the two promoters identified in the E2 region of HPV-18 may be involved in producing two different forms of the E2 protein and the E5 gene product, but this remains to be proved.

The promoter P_{2598} has several interesting properties. It has strong activity in vivo (as shown by a CAT assay), but it is not active in vitro. It does not contain a TATA-like sequence, but the sequence CCAAT is present 34 bp upstream of the initiation site. Transcription has been observed from promoters without a TATA box but having an initiator sequence (59) at the start site. The P_{2598} promoter contains a sequence (CCATT, nt 2591 to 2595) which matches the central core of the initiator sequence. Interestingly, transcription from the adenovirus major late start site, in the absence of a TATA box but in the presence of the initiator sequence, was observed in vivo but not in vitro (30).

The AGGTGG sequence, which binds the transcription factor Spl, was demonstrated to be essential for the activity of the P_{3036} promoter. Spl is involved in transcription from several papillomavirus promoters (24, 41, 61, 67). In these promoters, the classical 5′-GGGCGG-3′ motif (GC box) of Spl (18, 19) is not well conserved, and Gloss and Bernard (24) proposed that the sequence required for binding matches the motif 5′-NGGNGN-3′. In HPV-18, an AG GTGG motif is present upstream of both P_{2598} and P_{3036}. The AGGTGG sequence upstream of P_{3036} binds Spl in a gel retardation assay and is necessary for promoter activity. In contrast, the AGGTGG motif upstream of P_{2598} does not bind Spl. It has been proposed that the 5′- and 3′-flanking sequences of the Spl core sequence are also important for binding, and an extended consensus sequence (G/T)(G/A)G GCGG(G/A)(G/A)(C/T) for Spl-binding sites has been defined (9, 10). Two other studies further demonstrated that the C in the center of the GC motif could be replaced by either a T (40) or an A (37). The Spl motif C**AGGTGG**T**GC** of the P_{3036} promoter is similar to the extended consensus sequence for Spl, and results of a weight matrix analysis showed that this sequence is an Spl site with an affinity comparable to the SV40 (I) GC motif (10). On the other hand, the homology of the A**AGGTGG**CCA sequence upstream of P_{2598} with the Spl extended consensus binding motif is low, and the weight matrix analysis did not define this sequence as an Spl motif. These observations confirmed that the nucleotides flanking the 3′ end of the Spl core sequence are particularly important for binding of Spl.

The activity of the P_{3036} promoter could be further increased by coexpression of the BPV-1 E2 protein. E2 is a sequence-specific DNA-binding activator protein (1, 45). It can also stimulate transcription in the absence of E2-binding motifs (26, 29). This general *trans*-activation process requires only the N-terminal domain of E2 (N-E2), which contains the functional transcription activator domain (26). Consistent with the absence of any E2-binding motifs, the P_{3036} promoter was stimulated by both E2 and N-E2. The level of activation (5-fold) was comparable to that obtained by Haugen et al. (26) in the absence of E2-binding sites, but was significantly lower than the stimulation of transcription (20- to 50-fold) generated by sequence-specific binding of E2 (68). Li et al. (41) observed activation of transcription by E2 in the absence of E2-binding motifs when Spl sites were placed proximal to the start site, and a model was proposed in which E2 and Spl synergistically interact to stimulate transcription. The Spl site located upstream of P_{3036} appears to be implicated in E2 *trans* activation because, after deletion of this site, the promoter did not respond to E2. A similar observation was made for the P_{3080} promoter of BPV-1 (41). Finally, we showed that P_{2598} in HPV-18, which lacks an Spl-binding motif, was not stimulated by E2. These results are in agreement with the model in which the E2 protein is targeted to the promoter region by physical interaction with Spl.

A promoter (P_{5600}) was identified close to the end of the L2 ORF in the late region of the HPV-18 genome. Although this promoter is located in the late region, it seems unlikely to be involved in the expression of the late genes. L1 mRNAs from several human and animal papillomaviruses have been characterized, and all have a similar structure, with a small second exon considerably upstream of nt 5600 (in the E2/E4 region). Thus, the biological significance of P_{5600} is uncertain. Promoters for the late genes have been characterized in BPV-1 (3), in HPV-1 (51), in the cottontail rabbit papillomavirus (76), and in HPV-8 (65). They are located in the L1 proximal part of the LCR. The activity of these promoters is generally restricted to differentiating keratinocytes and, with the exception of HPV-8 (65), is not detected in cell culture. We did not find evidence for a promoter at the beginning of the HPV-18 LCR (50 and unpublished results).

We found no evidence for initiation sites during an in vitro transcription analysis of the E6-E7-E1 early region of HPV-18. The 5′ ends of transcripts encoding an E1/E4 fusion protein have been mapped within E7 in HPV-6, HPV-11 (11, 49), HPV-16 (32), and HPV-31b (36). The transcripts starting within E7 in HPV-16 and in HPV-31b are increased during keratinocyte differentiation. As a consequence, a promoter playing a similar role in HPV-18 would not be detected during transcription by a HeLa cell extract because such an extract would lack factors expressed during late stages of

epithelial differentiation. The development of a productive system for HPV-18, perhaps analogous to those developed for HPV-11 and HPV-31b (15, 47), may make possible the mapping of promoters which are induced during epithelial differentiation. Interestingly, the mRNA coding for the E1/E4 protein of HPV-6 and HPV-11 is synthesized very early during differentiation of epithelial cells (64), and we observed that the promoter for this mRNA is active in the in vitro transcription assay (unpublished results). These observations suggest that a major difference between oncogenic (HPV-16, HPV-18, and HPV-31b) and nononcogenic (HPV-6 and HPV-11) viruses is in the way in which the production of the E1/E4 mRNA is regulated.

In HPV-18, only the promoter for E6 has been previously characterized. RNA mapping studies with other papillomaviruses have suggested that additional promoters may exist in other parts of the genome. Here, we provided evidence for three novel promoters in the genome of HPV-18. Further work will be necessary to test whether the promoters located in the E2 region and close to the end of the L2 ORF are active in infected tissues and to identify the transcripts produced.

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