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Characterization of a Transcriptional Promoter of Human Papillomavirus 18 and Modulation of Its Expression by Simian Virus 40 and Adenovirus Early Antigens

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RNA present in cells derived from cervical carcinoma that contained human papillomavirus 18 genomes was initiated in the 1.053-kilobase *Bam*HI fragment that covered the complete noncoding region of this virus. When cloned upstream of the chloramphenicol acetyltransferase gene, this viral fragment directed the expression of the bacterial enzyme only in the sense orientation. Initiation sites were mapped around the ATG of open reading frame E6. This promoter was active in some human and simian cell lines, and its expression was modulated positively by simian virus 40 large T antigen and negatively by adenovirus type 5 E1a antigen.

There is increasing evidence that papillomaviruses are involved in the etiology of cervical carcinoma. A very high percentage, about 80%, of tumor biopsies studied so far have been demonstrated to contain one of three human papillomaviruses (HPV), HPV16, HPV18 or HPV33, in an exclusively integrated or integrated and free state (2a, 4, 6, 27, 31). Cell lines, like HeLa, derived from such tumors maintain part of the viral genetic material. The HPV18 genome integrated in these cells is expressed at the RNA level as at least two specific RNAs that are initiated in the viral sequences and terminated in the adjacent cellular sequences (31). Expression of the viral genome in many tumor biopsies and in cell lines derived from cervical cancer cells points to an eventual active role of the viral sequences in maintaining the transformed state of the cells. Also, it indicates that these cultured cell lines should represent a good permissive system to study the expression of genital HPVs.

The noncoding region of the bovine papillomavirus type 1 (BPV1) harbors important regulatory elements, such as an origin of replication (35), a plasmid maintenance sequence (23), a promoter, and a transcriptional enhancer (32, 37). The noncoding region of the cottontail rabbit papillomavirus (CRPV) can also act as a promoter in certain cell lines (8). It was of interest to search for sequence elements that regulate transcription of the genital HPVs. Are they restricted to the noncoding sequences of the virus? Are they responsible for the tissue tropism observed with most papillomaviruses? Since papillomaviruses do not grow in tissue culture, we undertook to dissect the genome and insert the noncoding region in recombinant plasmids coding for a reporter enzyme of bacterial origin, chloramphenicol acetyltransferase (CAT), described by Gorman et al. (11). Such constructions can be introduced into different cell lines by calcium phosphate coprecipitation and permit a simple and accurate assay of the activity of promoters. We demonstrate that the noncoding region of HPV18, when placed in the correct orientation relative to the *cat* gene, can function as a promoter in a limited number of cell lines, including HeLa. Its activity is modulated by immortalizing viral oncogenes: it is increased by the large T antigen of simian virus 40 (SV40) and decreased by the Ela gene product of adenovirus type 5.

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MATERIALS AND METHODS

Plasmids. The basic construction of all CAT expression plasmids used in this study was similar. They contained the *cat* coding region of 773 base pairs (bp) with its own ATG located about 30 nucleotides (nt) from the 5′ end of the *cat* fragment devoid of any promoter sequence. They contained, in addition, the intron of the small t antigen and the early polyadenylation site of SV40 situated 3′ to the CAT transcription unit, as well as the origin and the ampicillin resistance sequences of pBR322. The basic construction first described by Gorman et al. (11) was slightly modified by Herbomel et al. (17) to generate several unique cloning sites 5′ to the CAT coding region that make it possible to insert any sequence to be tested for promoter activity. The plasmids used in this study are detailed in Table 1.

Cell growth and transfection. The cell lines used in this study are described in Table 2. All cells were grown in Dulbecco modified Eagle medium supplemented with 7% fetal calf serum in 9-cm petri dishes. The day before transfection, cells were seeded at a density of 10^6 cells per dish. For each plate, 10 μg of plasmid DNA purified by two cesium chloride gradient centrifugations was precipitated with calcium phosphate by standard procedures (36) and added to the medium covering the cells. The following day, the calcium phosphate-DNA precipitate was washed away and fresh medium was added for an additional 24 h.

RNase mapping analysis. RNAs were extracted as described previously (5). Samples (30 μg) were treated with 20 U of RNase-free DNase I (Genofit) for 15 min at 37°C and then phenol extracted and ethanol precipitated. An SP6 RNA probe was synthesized by transcription of a linearized plasmid containing a 375-bp *Rsa*I fragment from plasmid p18/SB1/42 inserted in the antisense orientation adjacent to the SP6 promoter (24). The 375-bp start in the CAT coding sequences includes the junction between *cat* and HPV18 (137 nt) and 238 nt of the HPV18 genome. Transcription products were fractionated on 4% acrylamide–7 M urea

TABLE 1. Plasmids used in this study

Plasmid	Promoter sequence		Reference(s)
	Origin	Coordinates relative to cap site	
cat-containing			
pSB1	No promoter		11, 17
pRSV	RSV LTR	−183 to +41	12
pSVE	*Pvu*II-*Hin*dIII fragment of the SV40 early promoter	−280 to +60	18
pSVL	*Hin*dIII-*Pvu*II fragment of the SV40 late promoter		
pE4	*Taq*I-*Eco*RI subfragment of *Eco*RI fragment C of adenovirus type 2	−329 to +32	7
Other			
pMK16-SV40	Complete sequence of SV40 with a short deletion in the origin of replication		10
pBR-SVORI	*Hin*dIII-*Pvu*II fragment including the enhancer and origin of replication of SV40 cloned in pBR322		
pE1a	Complete transcription unit of adenovirus type 5 E1a		3, 29
pE1aΔ	Deletion of the coding sequence from the E1a transcription unit		3, 29

sequencing gels, and full-length transcripts were eluted from the acrylamide for 90 min at 45°C. Hybridization with 10^6 cpm of purified probe per sample was done at 45°C overnight as described by Melton et al. (24). RNase digestion was done at 37°C for 30 min with 60 μg of RNase A per ml and 3 μg of RNase T_1 (Boehringer) per ml. The digests were treated with 5 μg of proteinase K per ml, phenol extracted, and analyzed on an 8% acrylamide–7 M urea sequencing gel.

CAT assays. At 40 h after the addition of calcium phosphate-DNA precipitate, the cells were washed twice with phosphate-buffered saline and then scraped with a rubber policeman, pelleted, and suspended in 150 μl of 0.25 M Tris (pH 7.5)–15% glycerol. The cells were sonicated, and the extract was cleared by centrifugation. CAT assays were done on various volumes of extract, depending on the level of activity expected. To each assay, 0.5 μCi of [^{14}C]chloramphenicol and 16 μl of 10 mM acetyl coenzyme A were added, and the reaction was carried out at 37°C for various lengths of time. For incubations longer than 45 min, fresh acetyl coenzyme A was added. The reaction was stopped, and chloramphenicol was extracted with ethyl acetate. The solvent was dried, and the pellet, suspended in 10 μl of ethyl acetate, was spotted on silica gel thin-layer plates. Chromatography was performed for 1 to 2 h in chloroform-methanol (19:1). After autoradiography overnight, the spots of chloramphenicol and its 3′-acetylated derivative were cut out and counted in scintillation liquid to quantitate the conversion of chloramphenicol to its acetylated derivatives. CAT activity was expressed as picomoles of chloramphenicol converted into its 3′-acetylated form by 1/10 of the extract obtained from one plate in 60 min at 37°C.

RESULTS

Nucleotide sequence of noncoding region of HPV18. The DNA of HPV18 was isolated from a genital tumor and cloned in a bacteriophage vector. Its characterization by partial sequence analysis and by comparison with the known sequence of the related genital HPV6b allowed rough assignment of the various open reading frames and the noncoding region (31). The noncoding region was contained within a small *Bam*HI fragment of 1.053 kilobases (kb). The complete nucleotide sequence of this fragment (Fig. 1) revealed that the 208 nt at its 5′ end corresponded to the end of open reading frame L1, while 20 nt at its 3′ end were located within the beginning of open reading frame E6. Hence, the noncoding region of this virus is composed of 825 bp. It contains typical TATA and CAAT boxes and several other sequences conserved among papillomaviruses.

Construction of CAT expression plasmids. The small *Bam*HI fragment was excised from plasmid pBR/HPV18 containing the entire genome of the virus cloned into the *Eco*RI site of pBR322 (4). The purified fragment was inserted in both orientations in the *Bgl*II site just before the CAT coding sequence in a derivative of the pSB1 vector (18), a modified version of pSV0CAT of Gorman et al. (11). The orientation was determined by digestion with *Dra*I restriction endonuclease, which cleaves the insert at a single site near the end of open reading frame L1. Plasmid p18/SB1/42 contains the HPV18 fragment in the sense orientation, whereas plasmid p18/SB1/S2 contains the same fragment in the reverse orientation (Fig. 2). The sequence of the junction between the viral and *cat* sequences in p18/SB1/42 is shown at the bottom of Fig. 2. The first putative AUG of open reading frame E6 was not in phase with the AUG of the *cat* open reading frame. This situation, already encountered in other *cat* constructions, reduces but does not totally block translation of the bacterial enzyme in a chimeric mRNA (8; unpublished observations). Furthermore, as shown below, a fraction of the viral RNA was initiated inside the E6 initiator AUG.

TABLE 2. Cell lines used in this study

Cells	Origin	Viral functions expressed	Reference
Human			
HeLa	Genital carcinoma	HPV18	31
SW13	Adrenocortical carcinoma		22
HepG2	Liver carcinoma		1
293	Embryonic fibroblasts transformed with adenovirus type 5	Adenovirus E1a	2, 14
Monkey			
CV1	Kidney		
COS1	Kidney (CV1)	SV40 T antigens	9
Vero	Kidney		
Mouse			
3T6			
C127	Fibroblasts		
COP5	C127	Polyomavirus T antigens	33
ID13	C127	BPV1 early	21

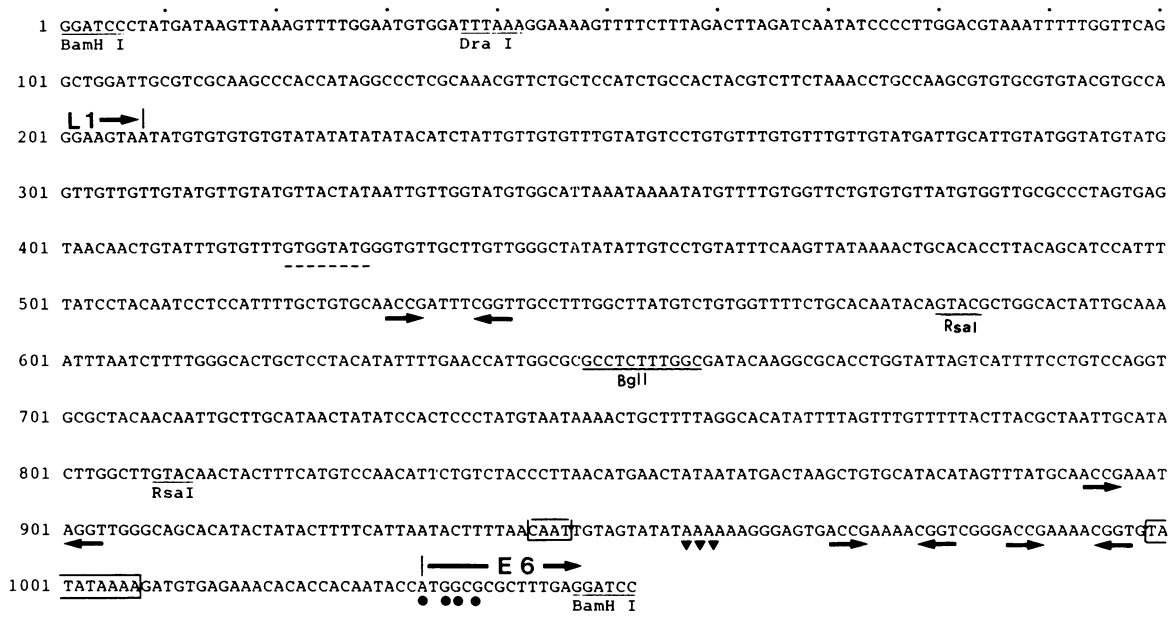

FIG. 1. Nucleotide sequence of the *Bam*HI fragment of HPV18. The end of the L1 and the beginning of the E6 open reading frames are indicated. The TATA and CAAT consensus sequences are boxed, and the *Dra*I, *Rsa*I, and *Bgl*I restriction sites and the homology of the SV40 enhancer and inverted repeats conserved among papillomaviruses are underlined. Initiation sites are indicated for the 155-nt protected fragment (●) and the 230-nt protected fragment (▼), as determined in mapping experiments.

Promoter activity in human cells. To determine the activity of the HPV18 constructions in different cell lines, we included in our experiments two reference plasmids: pRSV, containing the long terminal repeat (LTR) of Rous sarcoma virus (RSV) as a promoter, a construction that gives high CAT enzymatic activity in every cell line we tested; and pSB1, a promoterless plasmid, to assess the background level of this system caused by cryptic initiations in the pBR322 sequences (see Table 1 for plasmid structures). Usually, 10^6 cells in 9-cm dishes were transfected with 10 μg of each plasmid preparation by the calcium phosphate coprecipitation technique described in Materials and Methods. Forty hours later, cells were harvested, extracts were prepared by sonication, and CAT activity was assayed by conversion of labeled chloramphenicol to its monoacetylated forms under conditions that ensure proportionality between the enzymatic activity and the percentage of chloramphenicol conversion. The cell lines used in this study are described in Table 2. We assayed first the human cell lines HeLa, SW13, HepG2, and 293. As already mentioned, HeLa cells are derived from a carcinoma of the cervix and have been shown to contain several copies of HPV18 DNA integrated in the cellular genome (31). This DNA is transcribed into two specific RNAs of 1.6 and 3.5 kb which are initiated from their own promoter, lying in the small *Bam*HI fragment. SW13 is derived from a human adrenocortical carcinoma (22). No HPV18 sequences have been detected in the total DNA or RNA of the nontransfected cells (results not shown). A third cell type used is the HepG2 cell line, derived from a human hepatocarcinoma (1). Finally, we transfected 293 cells, which are human embryonic fibroblasts transformed by adenovirus type 5 and which express constitutively the E1a antigens (2, 14). The CAT activities measured after transfection into these four cell lines are presented in Table 3. The values given are the mean of three to four independent transfection experiments done with different plasmid preparations. In two of the four cell lines, the CAT activity directed by the plasmid with the insert in the sense orientation (p18/SB1/42) was 40 and 100 times above the activity measured with the opposite orientation (p18/SB1/S2), which remained close to the background level (plasmid pSB1). The CAT activity obtained with the sense HPV18 fragment was about 10% of that of the RSV LTR in SW13 cells but only about 1% in HeLa cells. These values indicate that the *Bam*HI fragment of HPV18 acts as a low to moderate promoter in HeLa and SW13 cells, respectively. Very low activity relative to the RSV promoter was obtained in HepG2 and 293 cells.

Transfection of nonhuman cells. Papillomaviruses are usually restricted to a single species. Only fibropapillomaviruses such as BPV1 partially overcome the species barrier, as they induce tumors in rodents and transform rodent cells in culture. In agreement with these observations, the noncoding region of this virus is active (as a transcriptional enhancer) in rodent cells (32). On the other hand, the CRPV noncoding region is active in chimeric *cat* plasmids only in keratinocytes derived from a carcinoma induced by this virus (8).

To define more precisely the range of expression of the HPV18 promoter, we transfected p18/SB2/42 or p18/SB1/S2 and our test plasmids pRSV and pSB1 into a number of other nonhuman mammalian cell lines. These include established lines such as simian CV1 and Vero and mouse C127 and 3T6 and virus-transformed cell lines such as simian COS1 cells, mouse COP5 cells, and mouse ID13 cells (C127 cells transformed by BPV1). The CAT activity observed in all these cells was rather poor; only COS1 showed activity exceeding 1% of that of pRSV. These cells had the highest ratio in CAT activity between the sense and antisense constructions, a good indication for genuine promoter activity (Table 4). The COS1 cells, which are derived from CV1 cells by transformation with SV40 (9), became more permissive for the HPV18 promoter than the parental cells. On the contrary, in COP5 cells, which are C127 mouse cells transformed by

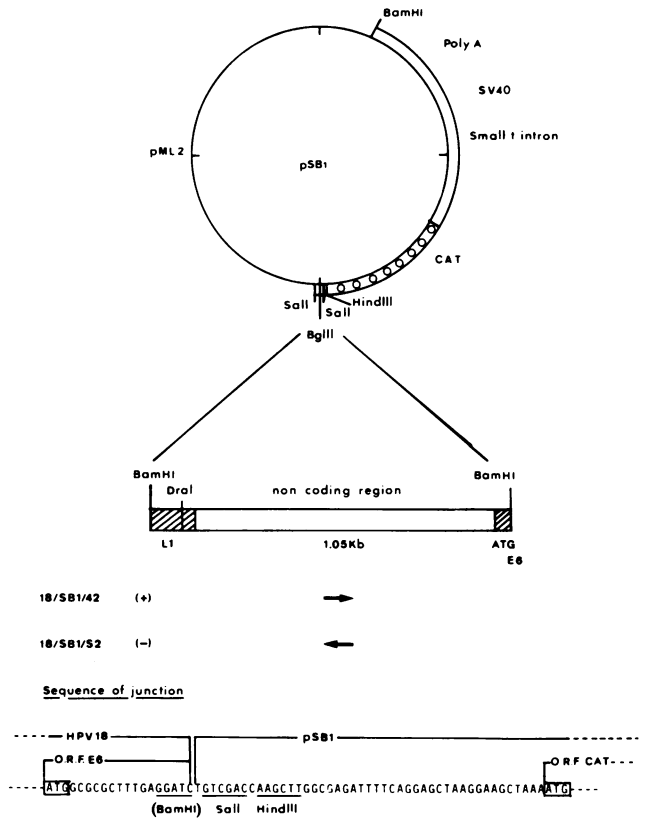

FIG. 2. Structure of CAT expression plasmids. The structures of the promoterless pSB1 plasmid and the HPV18 *Bam*HI fragment are diagrammed. The viral fragment was inserted in the sense orientation (+) to give plasmid p18/SB1/42 and in the antisense orientation (−) to give plasmid p18/SB1/S2. The sequence of the junction and the positions of the ATG translation initiation codons of the E6 and CAT coding sequences are indicated. O.R.F., Open reading frame.

polyomavirus T antigen, the promoter activity remained at the background level (results not shown). It appears from these results that the HPV18 sequence is a very poor promoter in the nonhuman mammalian cells that we assayed with the exception of simian COS1 cells. The transfection efficiencies were highly variable between cell lines. This was reflected in the variations in CAT activity obtained with pRSV and with pSVE (results not shown). However, the overall conclusion of the weakness of the HPV18 promoter in these cell lines, except maybe COS1 cells, remains true. Our conclusions on the strength of the HPV18 promoter rely on comparison with pRSV activities. Strong modulation of this promoter in different cells could affect the values we obtain. However, except for 293 cells, we did not observe marked variations in the activity ratio between pRSV and SV40 early *cat* constructs after transfection (unpublished observations).

Mapping of RNA start sites. To determine the initiation site(s) of transcription, total cytoplasmic RNAs from transfected HeLa or SW13 cells were extracted and subjected to SP6 RNA mapping (see Materials and Methods). A typical mapping experiment is shown in Fig. 3. Three predominant protected fragments of 375, 230, and 155 nt were detected. The upper band corresponds to full protection of the sequences in the SP6 probe corresponding to the 375-bp *Rsa*I fragment from the HPV18-*cat* construction that was subcloned in the SP6 plasmid. Its protection corresponded to transcripts initiated upstream of the *Rsa*I site in the noncoding region or in plasmid sequences preceding the HPV18 DNA. To verify whether the 230-bp or 155-bp protected fragments were colinear with the probe, we used a second probe which included sequences from the *Pvu*II site in the CAT coding sequences to the *Bgl*I site in the HPV18 sequence. This new probe contained 21 additional residues at its 5′ end and 159 additional residues at the 3′ end relative to the previous probe. Two protected bands of 251 and 176 nt were found, suggesting that these bands correspond to initiation sites located 75 residues upstream from the E6 ATG and around this triplet, respectively. The variations in CAT activity in all our experiments corresponded to variations in the intensity of the 155-bp protected fragment. The CAT activities determined for a sample of the transfected HeLa and SW13 cells used for the present RNA preparations are presented in the inset of Fig. 3. CAT activity was roughly 30 times higher in SW13 than in HeLa cells. The relative intensities of the protected 155-bp fragment deduced by visual inspection were in good agreement with these enzymatic activities. It is probable that the translation of the CAT RNA corresponding to the protected 230-bp and 375-bp fragments is very inefficient since these RNAs will include two and four or more AUGs before the CAT translation initiation AUG, respectively. Furthermore, at least a fraction of the RNA initiated around the E6 ATG codon will lack an intact codon, increasing the efficiency of CAT translation. The results indicating that a major start site is located around the ATG of open reading frame E6 are in agreement with primer extension experiments performed to map the endogenous HPV18-specific RNA start sites in HeLa cells (A. Schneider-Gaedicke and E. Schwarz, EMBO J., in press) and with the SP6 mapping experiment presented in Fig. 4.

SP6 mapping was performed with total RNA extracted from HeLa cells and a probe covering the junction between the noncoding region and the E6 open reading frame of HPV18; it clearly showed protected bands of 128, 214, and 216 nt (Fig. 4). The second two correspond to heterogeneous initiation sites around the ATG of E6, while the first corre-

TABLE 3. CAT activity in human cell lines

Plasmid (orientation)	CAT activity (pmol/h)[a]			
	HeLa	SW13	HepG2	293
p18/SB1/42 (+)	30 (0.8)	1,200 (11)	0.75 (0.0007)	75 (0.1)
p18/SB1/S2 (−)	0.75	10	0.75	75
pRSV	3,450	10,500	105,000	75,000
pSB1	1	3	ND[b]	75

[a] Expressed as picomoles of 3-monoacetyl form of chlorampenical obtained in 1 h of incubation with 1/10 of the cell extract. Numbers in parentheses for p18/SB1/42 represent percentage of pRSV activity.
[b] ND, Not determined.

TABLE 4. CAT activity in other mammalian cells

Plasmid (orientation)	CAT activity (pmol/h)[a]			
	Simian			Murine 3T6
	COS1	CV1	Vero	
p18/SB1/42 (+)	225 (1.25)	2 (0.2)	48 (0.01)	22.5 (0.5)
p18/SB1/S2 (−)	4.5	2	2.2	9
pRSV	18,000	900	450,000	4,050
pSB1	3	2	ND	7.5

[a] See Table 3, footnotes *a* and *b*.

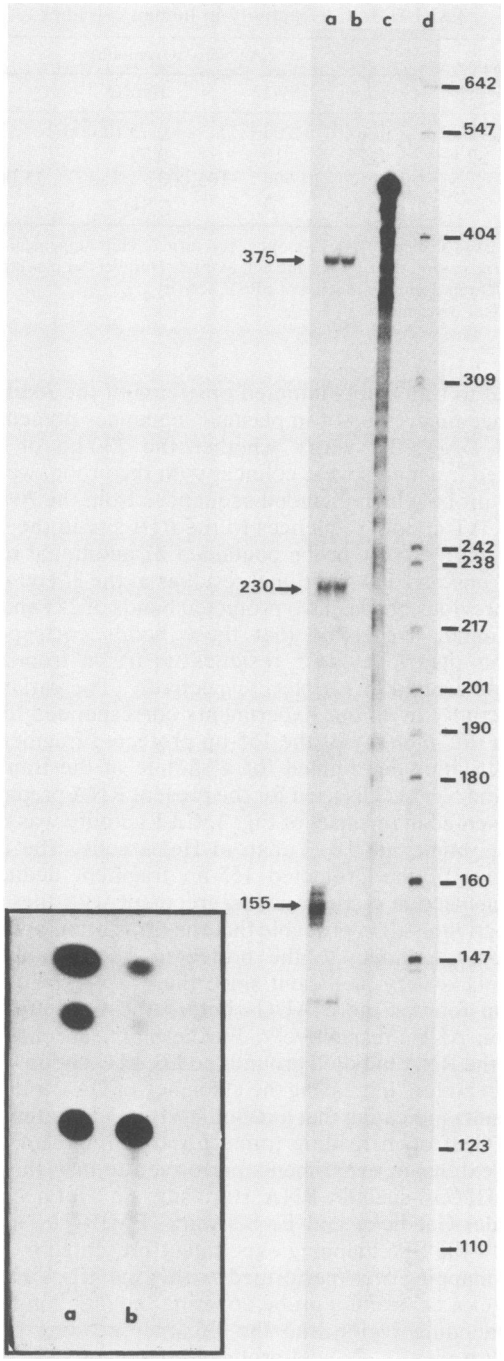

FIG. 3. RNase mapping analysis of the HPV18 CAT RNA. Total cytoplasmic RNA from SW13 (lanes a) and HeLa (lanes b) cells transfected with 10 μg of the p18/SB1/42 plasmid was hybridized to a complementary 375-nt *Rsa*I single-stranded SP6 RNA probe as described in Materials and Methods. About 30 μg of total RNA was hybridized to the probe. Three protected fragments of 375, 230, and 155 nt were detected in both HeLa and SW13 transfected cells. CAT activities were assayed with an extract obtained from 1/10 of the cells used for RNA extraction, and the resulting chromatograms are shown in the inset. In this assay, the CAT activities in SW13 and HeLa cells were 2,000 and 75 pmol of monoacetyl chloramphenicol produced in 1 h, respectively. These activities correlate well with the relative abundance of the 155-nt protected species. In contrast, the two additional protected fragments were present in similar amounts in both cell lines. Undigested probe (lane c) and *Hpa*II-restricted pBR322 DNA (lane d) served as molecular size markers. Sizes are shown in nucleotides.

sponds to a spliced version of the E6 RNA initiated in the same site, as demonstrated and discussed by Schneider-Gaedicke and Schwarz (in press). The high background or intermediate bands seen in this experiment are partially due to overexposure of the film. In addition, the presence of large amounts of viral RNA in HeLa cells (31) may result in the accumulation of some degradation products. This het-

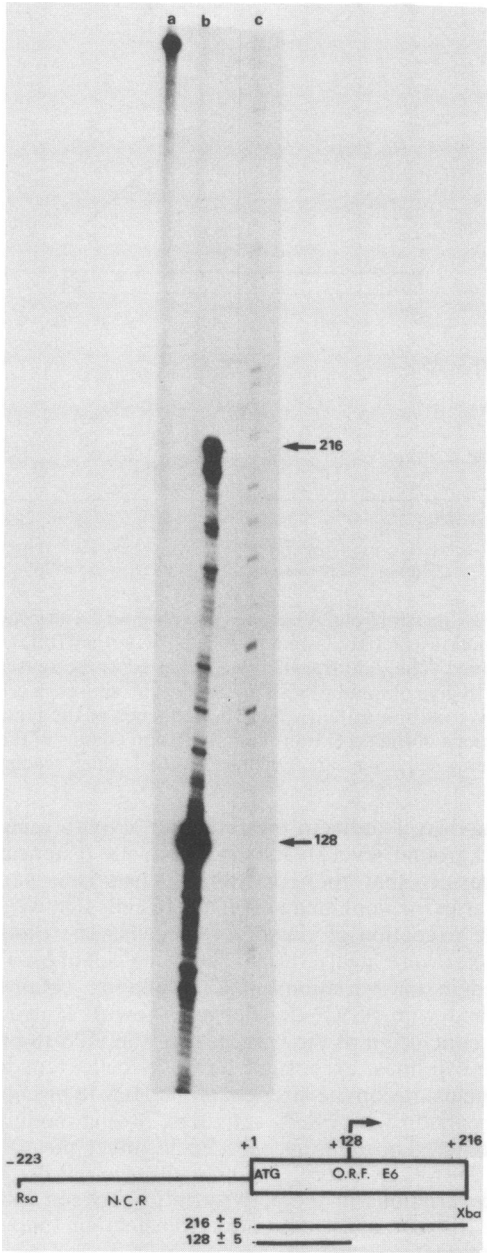

FIG. 4. RNase mapping analysis of endogenous HPV18 RNA in HeLa cells. RNase mapping experiments were performed with 30 μg of total cytoplasmic RNA extracted from nontransfected HeLa cells. The probe used is shown at the bottom. Two protected fragments of about 216 and 128 nt were detected, which correspond to initiation at the ATG of open reading frame E6, followed by a colinear (216 nt) and a spliced (128 nt) version of open reading frame E6, as described in Schneider-Gaedike and Schwarz (in press). Lane b, Undigested probe (lane a) and *Hpa*II-restricted pBR322 DNA (lane c) served as size markers (as indicated in Fig. 3). N.C.R., Noncoding region; O.R.F., open reading frame.

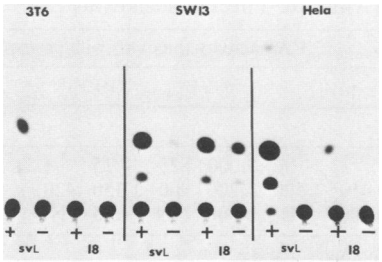

FIG. 5. Modulation of promoter activity by SV40 large T antigen. Autoradiograms of the chromatograms of CAT assays performed with 1/4 of the cell extracts incubated for 45 min. Cotransfection of 10^6 mouse 3T6 or human SW13 or HeLa cells with 5 μg of either pSVL or p18/SB1/42 together with 10 μg of pMK16-SV40 (lanes +) or pBR-SVORI (lanes −). CAT activity values are given in Table 5.

erogeneous initiation site is located 28 to 30 nt downstream of a TATA box indicated in Fig. 1. The fact that it maps in the first ATG of the E6 open reading frame is reminiscent of the results obtained with CRPV transcripts mapped in VX2 rabbit carcinoma cells (5) and could preclude the use of this ATG as the translational start site.

Activation of transcription by SV40 large T antigen. The results with COS1 cells described above led us to speculate about a possible role of the SV40 large T antigen in the recognition of the HPV18 promoter in monkey cells. To clarify this point, we cotransfected different cell lines with p18/SB1/42 and pMK16-SV40, a plasmid which carries the entire coding sequence for SV40 large T antigen (see Materials and Methods). Plasmids containing enhancer sequences can affect transcription of cotransfected plasmids by sequestering either positive (30) or negative (3, 13) *trans*-acting cellular factors. To exclude such complications, we uscd, as a negative control in cotransfection experiments, the enhancer-origin region of SV40 cloned in pBR322 (pBR-SVORI). CV1, 3T6, HeLa, and SW13 cells were transfected with 5 μg of the test plasmid and 10 μg of pMK16-SV40 or pBR-SVORI. To confirm that cotransfected SV40 T antigen-containing plasmids were active in our experiments, we cotransfected in parallel experiments a plasmid containing the late promoter of SV40 before *cat*. It has been recently shown that SV40 T antigen activates transcription from the viral late promoter even in the absence of viral DNA replication (19).

The CAT activities obtained after cotransfection with either the negative control or the plasmid which expresses the SV40 T antigen (pMK16-SV40) are presented in Fig. 5 and summarized in Table 5. In the four cell lines CAT activities directed by the SV40 late promoter were greatly enhanced in the presence of the SV40 T antigen. This led to a 70- to 100-fold increase in activity in CV1 and HeLa cells, in which the origin-containing plasmids can replicate, while only a 10-fold increase was observed in mouse 3T6 cells, in which they do not replicate. We found that expression of the HPV18 sequence was enhanced by SV40 T antigen in human HeLa and SW13 cells and in mouse 3T6 cells. This activation was high in HeLa cells (10-fold) and moderate in SW13 and 3T6 cells (3-fold) (Table 5). In SW13 cells it resulted in a very high final activity which approached almost half of pRSV activity, while in 3T6 cells, despite the presence of T antigen, the HPV18 promoter activity remained very weak.

TABLE 5. Effect of SV40 T antigen

Cells	CAT activity (pmol/h)[a] with promoter:					
	pSVL[b]		HPV18[b]		None (pSB1)	pRSV
	−	+	−	+		
HeLa	150	14,550	30	390	1	3,450
SW13	240	3,900	1,200	3,400	3	10,500
3T6	90	900	15	45	7.5	4,050
CV1	4.5	420	3	3	3	900
COS1	3,000	ND	225	ND	3	18,000

[a] See Table 3, footnotes *a* and *b*.
[b] Cotransfection experiments were done with plasmids containing (+, pMK16-SV40) or lacking (−, pBR-SVORI) SV40 T antigen sequences.

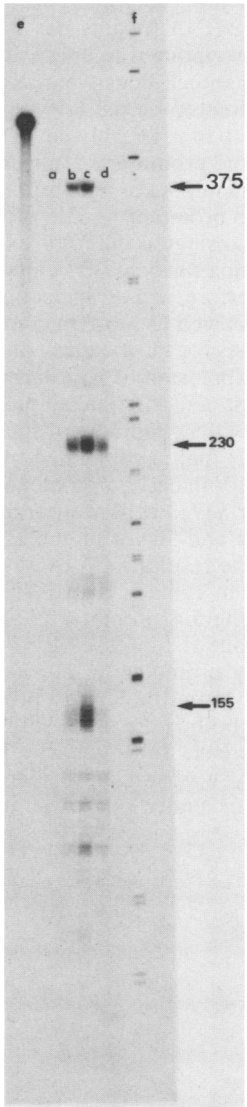

FIG. 6. RNase mapping analysis of the HPV18 CAT RNA in the presence of SV40 T or adenovirus E1 antigen. RNase mapping experiments were performed as described in the legend to Fig. 3 with the same probe. Total cytoplasmic RNA from SW13 cells either not transfected (lane a), transfected with 5 μg of p18/SB1/42 (lane b), cotransfected with 5 μg of p18/SB1/42 and 10 μg of pMK16-SV40 (lane c) or 5 μg of p18/SB1/42 and 10 μg of pE1a (lane d) were hybridized to the probe. The same three protected fragments of 375, 230, and 155 nt were observed as in the experiments presented in Fig. 3. The corresponding CAT activities were 350 (lane b), 900 (lane c), and 175 (lane d) pmol/h. Undigested probe (lane c) and *Hpa*II-restricted pBR22 DNA (lane f) as molecular size markers.

Surprisingly, the SV40 T antigen did not activate transcription from the HPV18 promoter in monkey CV1 cells, while it strongly increased (70- to 100-fold) transcription from the late promoter of SV40. RNase mapping experiments performed with RNA extracted from cotransfected SW13 cells strongly suggest that activation by SV40 T antigens occurs at the transcriptional level, since a marked increase in the 155-bp protected band was seen in the presence of SV40 T antigen (Fig. 6). However, we cannot totally exclude at this stage that T antigen stabilizes the chimeric mRNA. In a separate series of experiments we excluded that transcription stimulation was caused by an increase in the copy number of intracellular HPV18-containing plasmids. The SV40 T antigen did not promote replication of the papillomavirus-containing DNA (results not shown).

Repression of transcription with adenovirus E1a gene product. Among the human cell lines we assayed was 293 cells. The CAT activity directed by the HPV18 noncoding region in these cells was very low, roughly equal to the background level obtained with the promoterless plasmid (Table 3). This suprising result pointed to a possible repression of transcription from the HPV18 promoter by E1a proteins. To test this hypothesis, we cotransfected different expression plasmids with an E1a-containing plasmid that expresses this protein. As a control, we performed cotransfections with the same series of plasmids, as well as a plasmid containing a deleted form of E1a (3, 29). As for the experiments described above, we monitored the expression of E1a in the transfected cells by the use of two test constructions in parallel experiments: pE4, which contains the promoter of the early E4 gene of adenovirus type 5 (7) that is stimulated by E1a, and pSVE, which contains the SV40 early promoter which is repressed by E1a (3). HeLa or SW13 cells were transfected with 5 μg of the test plasmids and 10 μg of the E1a or E1a-deleted plasmids. Some of the resulting CAT activities are shown in Fig. 7 and presented in Table 6. Expression of E1a enhanced transcription from the E4 promoter (30-fold in HeLa and 3-fold in SW13), while it repressed by 3-fold transcription from the SV40 early promoter (in our experimental conditions). Transcription from the HPV18 promoter was clearly repressed by E1a in both HeLa and SW13 cells by about the same threefold decrease in efficiency. Furthermore, this reduction in activity was similar to that observed for the SV40 early promoter under our experimental conditions.

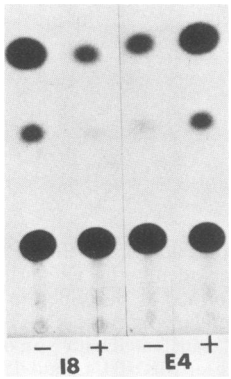

FIG. 7. Modulation of promoter activity by adenovirus E1a. Autoradiograms of CAT assays of SW13 cells cotransfected with 5 μg of either p18/SB1/42, pE4, or pSVE together with 10 μg of E1a-coding plasmid (+) or a control plasmid harboring a deletion in the E1a coding sequences (−). CAT activity values are given in Table 6.

TABLE 6. Effect of adenovirus E1a

Cells	CAT activity (pmol/h)[a] with promoter:							
	pE4[b]		pSVE[b]		HPV18[b]		None	pRSV
	−	+	−	+	−	+		
HeLa	3	90	3,000	825	15	4.5	1	3,450
SW13	450	1,500	4,200	900	1,150	420	3	10,000
293 (E1a$^+$)	32,300	ND	600	ND	75	ND	75	75,000

[a] See Table 3, footnotes *a* and *b*.

[b] Cotransfection experiments were done with plasmids containing a complete (+, pE1a) or deleted (−, pE1aΔ) adenovirus type 5 E1a transcription unit.

These results could explain the very low activity observed with the HPV18 promoter in 293 cells: the repression achieved in these cells may be greater than that in cotransfection experiments, due perhaps to the presence in them of higher concentrations of E1a proteins. An alternative explanation for the background activity in 293 cells is that normal or transformed human fibroblasts do not contain factors that are required for the expression of HPV18 DNA. An example of SP6 RNA mapping experiments performed with RNA extracted from SW13 cells cotransfected with an E1a-coding plasmid (Fig. 6) suggests that the repression of CAT activity is correlated with a decrease in the yield of the 155-bp protected fragment.

DISCUSSION

Cervical carcinoma is intimately associated with infection by certain HPVs (4, 6, 27, 31). One of these viruses is HPV18, and its genome is found in about 20% of cervical carcinoma biopsies and in cell lines, such as HeLa, derived from such tumors. In many of these cell lines the viral genome is transcribed as mRNA encoding the two putative viral early proteins E6 and E7. In the present study, we have undertaken the characterization of the transcription control elements of this virus. A DNA fragment containing the entire noncoding region was cloned in the two possible orientations in a CAT expression plasmid. These constructions were introduced by calcium phosphate coprecipitation into several cell lines in parallel with a plasmid lacking or containing a strong promoter, the RSV LTR. The plasmid containing the HPV18 fragment in the sense orientation showed significant CAT activity in several cell lines. No or very low activity was obtained with the HPV18 sequence in the reverse orientation. The activity of the HPV18 promoter sequence depended greatly on the cell type used. Relative to the very strong RSV promoter, it varied between 1% of RSV in HeLa and monkey COS1 cells to 10% of RSV in human SW13 adrenocortical carcinoma cells. It was inactive in human hepatocarcinoma HepG2 and 293 and monkey CV1 cells and barely active in mouse 3T6 fibroblasts and monkey Vero cells. It is difficult to draw a simple scheme of narrow cell specificity from these results. It is possible that the HPV18 promoter is active mainly in primate cells of epithelial type. However, it is clear that this promoter is not exclusive to the target cell of the virus, the cervical epithelial cell. It does not seem to require a cell that is engaged in the pathway of keratinocyte differentiation. The HPV18 promoter does not exhibit the very stringent tissue specificity observed with the virus in vivo. A rather simple possibility to explain this fact would be that the stringency is mediated by another element within the viral DNA not present in the HPV18 sequences in the CAT plasmid. In this context, it is

worth mentioning that the homologous region of another HPV, HPV1, the agent of deep plantar wart, cloned in plasmid pSB1 did not lead to any CAT expression in the cell lines used in this study (8; F. Thierry, unpublished results). It is possible that the transcriptional control of genital HPVs infecting simple epithelia, such as that of the cervix, is less stringent than that of viruses infecting keratinizing epithelia, such as skin.

Mapping of the 5′ ends of the RNA showed that in both transfected HeLa and SW13 cells, at least two initiation sites upstream and around the 5′ ATG of open reading frame E6 are used. The heterogeneous site around this ATG is identical to that mapped for the endogenous HPV18 RNA in HeLa cells. The site preceding this ATG by 75 bp did not have a counterpart in endogenous HeLa RNA. The transcription studies performed with only a few of the transfected cell lines measured steady-state levels and not the rate of primary transcription. Nevertheless, there is little experimental evidence for differential stabilities of CAT RNA in different cell lines. The HPV18 promoter is more active in SW13 cells, which do not contain viral sequences, than in HeLa cells, which actively transcribe their endogenous HPV18 genomes. It is clear that this promoter can function in the absence of *trans*-acting viral proteins. We are presently attempting to test whether the lower activity observed in HeLa cells is due to competition by endogenous viral DNA for limiting transcription factors or to repression by a viral gene product.

The results of the cotransfection experiments reported here show that SV40 large T antigen activates transcription from the HPV18 noncoding region in HeLa and SW13 cells. We have verified that SV40 large T antigen does not induce replication of the plasmids containing the noncoding region of HPV18 in cotransfection experiments, and thus its effect, either direct or indirect, is at the transcriptional level (results not shown). The fact that SV40 T antigen does not activate transcription in CV1 cells indicates that the activity observed in COS1 cells is due not to a direct interaction with SV40 T antigen but rather to a factor(s) associated with their transformed phenotype. We can tentatively deduce from these results that transcription from the HPV18 promoter depends on at least three different parameters: the state of transformation of the cells, their species origin, and their epithelial nature.

Transcription from the HPV18 promoter is also sensitive to the adenovirus type 5 E1a antigen. In cotransfection experiments, transcription from the HPV18 noncoding region was reduced roughly threefold in the presence of E1a coding sequences. Repression of transcription by the adenovirus E1a protein is well documented (3, 34). It is presumed to occur by inhibition of enhancer function. While we have not been able in this study to demonstrate the existence of such an enhancer in the HPV18 noncoding region, this result and other experiments in progress suggest the existence of such an enhancer. Moreover, the consensus sequence GTG GTATG, present in enhancer elements repressed by the E1a-like SV40 72-bp repeat, is found 600 nt upstream of the RNA start site (Fig. 1). An enhancer element has been characterized for BPV1, and its activity is greatly stimulated by the product of open reading frame E2 (32). The homologous open reading frame of HPV18 is interrupted by integration of the viral DNA in the genome of HeLa cells. Therefore, the putative product of E2 cannot be expressed in these cells. Many other cells derived from genital carcinomas exhibit the analogous integration pattern of genital HPVs HPV16 and HPV18, interrupting E2. However, we cannot exclude the possibility that expression of the viral E2 protein increases the relative activity of the HPV18 promoter.

What are the open reading frames actually transcribed in vivo from the HPV18 promoter characterized in this study? Both open reading frames E6 and E7 adjacent to this promoter are transcribed in HeLa cells (31) as well as in CRPV- and BPV1-transformed cells (5, 16, 26). We can tentatively deduce that the regulation of expression from the HPV18 promoter by the SV40 T or adenovirus E1a antigen, demonstrated in this study, should concern the expression of open reading frames E6 and E7 in its natural host cell. The products of these open reading frames play an important role in cell transformation with BPV1 (37). Transcription data for cells derived from cancer biopsies indicate that they may play such a role in tumors associated with genital papillomaviruses. We have shown here that expression from the HPV18 sequence is modulated by viral early antigens which are associated with regulation of viral transcription as well as with cellular immortalization (20, 25, 28). Superinfection of HPV18-infected cells with other genital viruses, such as herpes simplex virus type 2, which carry a function similar to E1a, can modulate the expression of the papillomavirus genome and decrease its lytic activity. This could result in a long-term latent infection by HPV18 in some cells, which may induce some cellular mutations or gene amplification, changing the growth properties of the cell (39).

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