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Cell-Type-Specific Activity of the Human Papillomavirus Type 18 Upstream Regulatory Region in Transgenic Mice and Its Modulation by Tetradecanoyl Phorbol Acetate and Glucocorticoids

ANGEL CID,[1]* PRASERT AUEWARAKUL,[1]† ALEJANDRO GARCIA-CARRANCA,[2] RONIT OVSEIOVICH,[2] HEIKE GAISSERT,[1] AND LUTZ GISSMANN[1]

Angewandte Tumorvirologie, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 242, D-69120 Heidelberg, Germany,[1] and Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, Ciudad Universitaria, 04510 México DF, México[2]

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The upstream regulatory region (URR) of human papillomavirus type 18 (HPV-18) harbors transcriptional promoter and enhancer elements which are thought to determine the cell-type specificity of the virus. In order to study the regulation of HPV-18 expression in vivo, we constructed transgenic mice carrying the bacterial *lacZ* gene under the control of the HPV-18 URR. Analysis of β-galactosidase activity by histochemical staining of tissue sections of four independent transgenic mice showed that the viral promoter was specifically active in epithelial cells within a variety of organs (e.g., tongue, ovary, uterus, testis, and small intestine). Very strong staining was observed in newborn transgenic mice in contrast to a weak activity found during fetal life. Determination of β-galactosidase activity in crude extracts from tissues of three lines of transgenic mice proved to be a useful tool for a quantitative analysis of transgene expression. In mice from two different transgenic lines treated with dexamethasone such measurements revealed a biphasic effect of the hormone on the activity of the enzyme in the stratified epithelium of the tongue (transient increase followed by a decrease). Northern (RNA) blot analysis showed similar changes in β-galactosidase mRNA in that tissue. Treatment with tetradecanoyl phorbol acetate (TPA) led to a twofold increase in both enzymatic activity and mRNA levels. Finally, combined treatments with dexamethasone and TPA showed that both factors interfered with each other in their respective effects on transgene expression, suggesting that a cross-talk mechanism between transcription factors could be involved in the regulation of the HPV-18 URR.

The human papillomaviruses (HPVs) are small DNA viruses responsible for a variety of epithelial neoplasias. About 70 types of HPVs have been identified (20), of which approximately one-third are associated with anogenital carcinomas. HPV types 16 and 18 (HPV-16 and HPV-18) are the viruses most commonly found in cervical cancers and in cervical carcinoma cell lines (e.g., HeLa) in which the viral oncogenes are consistently expressed, thus suggesting that these viruses play a causative role in the development of such malignancies (for reviews, see references 59 and 60). HPV-18 is the most prevalent virus associated with adenocarcinomas and small cell carcinomas of the uterine cervix, which have a particularly poor prognosis (see reference 49 and references therein). The HPV genome harbors a noncoding sequence, the so-called upstream regulatory region (URR), which contains the promoter and enhancer elements that regulate transcription of the E6 and E7 viral oncogenes. The stringent epithelial specificity of HPVs is thought to depend on their URRs. The HPV-18 URR contains binding sites for cellular and viral proteins which have been identified by DNase I footprint and binding competition experiments (5, 8, 13, 23, 35). The HPV-18 E2 gene product is a major regulatory protein which represses the transcriptional activity of its URR in human keratinocytes (8). However, in HPV-18-associated carcinomas the viral genome is usually integrated and the E2 gene is often found to have been deleted (45). Therefore, in malignant lesions, viral gene expression is likely to be regulated exclusively by cellular factors.

The HPV-18 URR contains two glucocorticoid receptor-binding sites that mediate transcriptional response to hormones (12) and two recognition sites for activator protein AP-1 which have been found to be functional (5, 23, 35, 53). Studies of the regulation mediated by these elements are facilitated by the fact that they can be externally modulated by glucocorticoid hormone and phorbol ester treatments, respectively. Because of the lack of a system permissive for HPV infection, most of such studies have been performed in immortalized or transformed cell lines. Therefore, the regulation of viral gene expression in normal epithelial cells, in which the virus is thought to be present in a long latency period before the onset of malignant transformation (48, 58), is poorly understood.

In this study we have attempted to characterize the in vivo activity of the promoter-enhancer region of HPV-18 by using a reporter gene construct for the generation of transgenic mice. The *Escherichia coli* β-galactosidase gene was chosen because the enzyme it encodes is innocuous, stable, and readily detectable (34). In four independent transgenic mice the HPV-18 URR exhibited a strict cell-type specificity, and the transgene was exclusively expressed in epithelial cells of different organs. Furthermore, a quantitative analysis based on reporter activity and mRNA levels in transgenic mouse tissues showed that the HPV-18 URR was responsive to glucocorticoid hormone and phorbol ester treatments and suggested that the glucocorticoid receptor and the activator protein AP-1 could negatively

* Corresponding author.
† Present address: Microbiology Department, Faculty of Medicine, Mahidol University, 10700 Bangkok, Thailand.

interfere with each other in their regulation of the HPV-18 URR in normal epithelial cells. To our knowledge, the transgenic mice described here are the first animal model for the study in vivo of the regulation of HPV gene expression.

MATERIALS AND METHODS

Plasmids. The plasmid pURR18*lacZ* was obtained by insertion of the whole URR of HPV-18 (9) into a promoterless *lacZ* expression vector (pC4AUG-β-Gal) (54). The small *Bam*HI fragment (1,050 bp) of HPV-18 containing the entire promoter-enhancer region of the viral early genes was introduced as a blunt-ended fragment into the *Sma*I site of pC4AUG-β-Gal. A clone was selected (named pURR18*lacZ*) which had the viral promoter in frame with the *lacZ* coding sequence, as confirmed by sequence analysis. The complete insert (5.4 kb) was excised from the plasmid with *Eco*RI and prepared for microinjection into fertilized eggs. Plasmid pURR-CAT has been described previously (52). A plasmid carrying a Rous sarcoma virus long terminal repeat chloramphenicol acetyltransferase (CAT) construct was used as a positive control for CAT activity in HeLa and mouse keratinocytes transfections.

Pronuclear injections. Purified insert from plasmid pURR18*lacZ* was injected into fertilized eggs as described previously (27). The DNA fragment used for microinjection was separated from plasmid DNA by digestion with *Eco*RI, purificated by agarose gel electrophoresis using glass powder (Geneclean kit; Dianova, Hamburg, Germany), and eluted with 10 mM Tris-HCl (pH 7.6)–0.25 M EDTA. DNA was diluted to 2 ng/μl for microinjection. Fertilized eggs were obtained following superovulation of (C57BL/6 × C3H)F_1 females followed by mating to (C57BL/6 × C3H)F_1 males. Of about 350 eggs injected, 240 survived and were transferred into the oviducts of pseudopregnant NMRI recipient females on day 1 of gravidity. Eighteen live newborn animals (10 females and 8 males) were obtained, of which 5 (4 females and 1 male) carried the transgene. To establish permanent lines, the founder animals were crossed to (C57BL/6 × C3H)F_1 nontransgenic mice. Further generations were obtained by crossing back the transgenic progeny to nontransgenic F_1 hybrids.

Extraction and analysis of DNA and RNA. Genomic DNA was extracted from mouse tail biopsies as described previously (27). Aliquots of 5 to 10 μg of DNA were digested with the appropriate restriction enzymes and run on 0.7% agarose gels. Transference to nylon filters and hybridization were performed by standard protocols (43). The presence of the transgene was detected by hybridization with the same *Eco*RI fragment from plasmid pURR18*lacZ* as was used for pronuclear injections.

Total-cell RNA was extracted from tissues homogenized with a Polytron homogenizer (Brinkmann) by using the guanidinium thiocyanate method (43). RNA aliquots of 10 μg were electrophoresed through 1% agarose gels in the presence of 1.1% formaldehyde (32). *lacZ* transcripts were detected by using the same *Eco*RI fragment as for DNA blots. Hybridization and washing protocols were performed as previously described (1). The hybridization probes were generated by primer extension labelling (22).

Transfections, cell cultures, and animal treatments. Transfections were performed by electroporation essentially as described previously (15). HELP-I cells were grown in Dulbecco modified Eagle medium (DMEM) with 10% fetal calf serum. Subconfluent dishes were trypsinized, washed, and incubated in 1-ml sterile cuvettes for 15 min in cold HEBS buffer (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.5], 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM dextrose) containing the indicated amounts of plasmid DNA plus salmon sperm DNA as a carrier to yield a final DNA concentration of 20 μg/ml. Cells were exposed to a pulse of 300 V and 250 μF in a Bio-Rad gene pulser apparatus and incubated for 48 h. Cellular extracts were prepared, and their protein contents were determined by the Bradford assay (10). Sixty micrograms of protein of the respective extracts was assayed for CAT activity as described previously (26).

Primary explants were obtained from skin of the tails of several transgenic mice. For the culture of fibroblasts, tissues were minced and placed into culture dishes containing a small amount (to facilitate attachment) of DMEM with 10% fetal calf serum. For selection of keratinocytes, the dermis was first removed by scraping with a scalpel, and then tissues were maintained in keratinocytes-SFM selective medium (GIBCO, Life Technologies, Berlin, Germany).

To determine the effect of glucocorticoids on transgene expression, dexamethasone (Sigma, Munich, Germany) was administered intraperitoneally as a phosphate salt at a final dose of 1 mg/kg of body weight or orally as pure (crystalline) hormone dissolved in ethanol at 10 mg/ml and then diluted in drinking water at a final concentration of 4 μg/ml (10^{-5} M). TPA (tetradecanoyl phorbol acetate; Sigma) was dissolved at a concentration of 1 mg/ml in acetone and administered in drinking water at a final concentration of 0.26 μg/ml (400 nM).

β-Galactosidase histochemistry and immunofluorescence. Cultured cells were rinsed twice with phosphate-buffered saline (PBS) (150 mM NaCl, 15 mM sodium phosphate [pH 7.3]) and then fixed and stained according to the method of MacGregor et al. (34). After 5 min of incubation at 4°C in fixation medium (2% paraformaldehyde, 0.2% glutaraldehyde, 2 mM $MgCl_2$ in PBS), cells were washed with PBS–2 mM $MgCl_2$ and overlaid with a histochemical reaction mixture containing 1 mg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Sigma) per ml, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$, 0.01% sodium desoxycholate, and 0.02% Nonidet P-40. Incubation was done at 37°C for 12 to 20 h.

Fixation and staining of whole organs and embryos was performed as described previously (38, 44) with slight modifications. In brief, fixation was extended for 2 h (organs) or 12 h (embryos), and desoxycholate and Nonidet P-40 were added. Subsequently, tissues were saturated in 30% sucrose in PBS–2 mM $MgCl_2$ for 3 to 12 h at 4°C and then frozen in isopentane-liquid N_2. Frozen sections of tissues were air dried, refixed, washed, and stained as described above.

For immunofluorescence, tissue sections of organs from transgenic mice and nontransgenic controls were fixed 5 min in methanol and 2 min in acetone at −20°C. After rehydration for 15 min in PBS, sections were stained by incubating 45 min at room temperature with monoclonal anti-*E. coli* β-galactosidase (Boehringer Mannheim) diluted in PBS, followed by a second incubation with fluorescein isothiocyanate (FITC)-conjugated anti-mouse antibody.

Living embryos cultured in vitro after injection with the URR18*lacZ* construct or the plasmid pC4AUG-β-Gal as a negative control were stained with fluorescein di-β-D-galactopyranoside (ImaGene LacZ Expression Kit; Molecular Probes Inc., Eugene, Oreg.) following the manufacturer's instructions.

Quantitation of β-galactosidase activity in mouse tissues. Determinations of β-galactosidase activity were performed by standard methods (reviewed in reference 34) adapted for crude extracts of mouse tissues. For most experiments littermates 2 to 4 months old belonging to the second and third transgenic generations were used. All animals analyzed were hemizygous. Males were preferred to females to avoid hor-

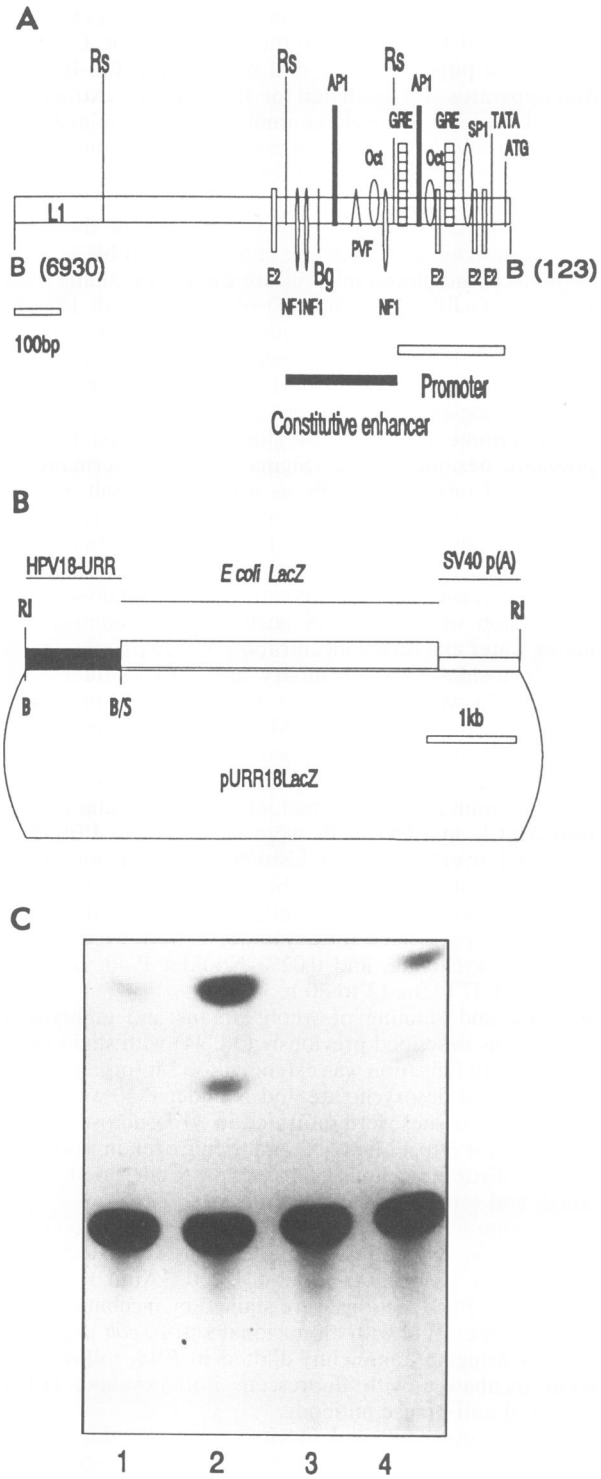

FIG. 1. Schematic representations of the HPV-18 URR and the pURR18*lacZ* plasmid and transcriptional activity of this construct in mouse keratinocytes. (A) A *Bam*HI fragment with a size of 1,050 bp (from nucleotide nt 6930 to 123; numbering according to Cole and Danos [18]) comprises the complete HPV-18 URR, which is located between the truncated L1 and E6 open reading frames. The proximal promoter and the constitutive enhancer are indicated by open and closed bars, respectively. The TATA box and ATG of E6 as well as sites of interaction with viral (E2) and cellular (NF1, AP1, PVF, Oct, glucocorticoid-responsive element [GRE] and SP1) factors are indicated (4, 12, 13, 23, 24, 29, 41). (B) Structure of the pURR18*lacZ* plasmid. The *Bam*HI fragment harboring the HPV-18 URR was ligated to a pUC plasmid containing an *E. coli lacZ* gene with simian virus 40 poly(A) sequences. The insert was excised with *Eco*RI for injection into fertilized eggs. (C) shows the result of a representative CAT assay after transient transfection of HELP-I mouse keratinocytes (11) with the plasmids pURR18-CAT (lanes 1 and 3) and pRSV-CAT (lanes 2 and 4) as a positive control. HELP-I cells were transfected with 3 and 6 μg of pURR-CAT and 1 and 2 μg of pRSV-CAT (lanes 3, 1, 4 and 2, respectively). CAT assays were performed for 60 min at 37°C with equal amounts of protein. Abbreviated restriction sites: B, *Bam*HI; Bg, *Bgl*I; RI, *Eco*RI; S, *Sma*I; Rs, *Rsa*I.

monal cycles that could lead to uncontrolled variability in transgene expression from animal to animal (see Results). The synthetic derivative ONPG (*o*-nitrophenyl-β-D-galactopyranoside; Sigma) was chosen as the substrate. ONPG is converted by the enzyme to galactose and the chromophore *o*-nitrophenol, which can be quantified spectrophotometrically by measuring A_{420}. Briefly, organs were homogenized with the Polytron in PM2 buffer (33 mM NaH_2PO_4, 66 mM Na_2HPO_4, 0.1 mM $MnCl_2$, 2 mM $MgSO_4$, 40 mM β-mercaptoethanol [pH 7.3]) and centrifuged at 12,000 × *g* three times in a Microfuge to remove cellular debris and supernatant of lipids. The soluble protein concentration in extracts was estimated by the method of Bradford (10). Reactions were carried out at 37°C in a final volume of 1 ml containing 100 to 400 μg of protein and 800 μg of substrate per ml. Control reaction mixtures with nontransgenic crude extracts and a blank without protein were run in parallel. Immediately after adding ONPG, the initial A_{420} of the samples was read. After exactly the same time of incubation for each sample (60 to 180 min, depending on the tissue, so that readings ranged from 0.2 to 1.5) the A_{420} of the samples was read again. The β-galactosidase activity was calculated, after subtracting the initial values, as follows: units = (380 × A_{420})/time (in minutes), where 380 is a constant such that 1 unit is equivalent to the conversion of 1 nanomole of ONPG per minute at 37°C (36).

RESULTS

Transcriptional activity of the HPV-18 URR in mouse keratinocytes. The HPV-18 URR contains three functional segments separated by *Rsa*I restriction sites (Fig. 1A): the distal enhancer with unknown function, a central fragment also called constitutive or cell-type specific enhancer (50), and the proximal fragment containing the promoter. The isolated proximal promoter has been shown to have no enhancer activity in transient transfection assays in HeLa cells (5, 52). In agreement with previous reports (52), we found that a *Bgl*I-*Bam*HI fragment containing the proximal promoter and most of the constitutive enhancer had only part of the activity of the complete URR in the same cells (data not shown). Moreover, the whole HPV-18 URR was able to drive expression of a CAT gene when transfected into HELP-I keratinocytes (11), a spontaneously transformed line derived from primary cultures of mouse epidermal cells (Fig. 1C). Therefore, we used the complete URR for our experiments with transgenic mice.

The structure of the plasmid pURR18*lacZ* used for these experiments is summarized in Fig. 1B. It consists of the whole URR of HPV-18 (9) as a 1,050-bp *Bam*HI fragment (from nucleotide 6930 to 123, numbered according to Cole and Danos [18]), cloned in front of the *E. coli* β-galactosidase gene in the plasmid pC4AUG-β-Gal (54). This plasmid contains an *E. coli lacZ* open reading frame (3,359 bp) missing the first seven codons and an additional fragment with a size of 846 bp

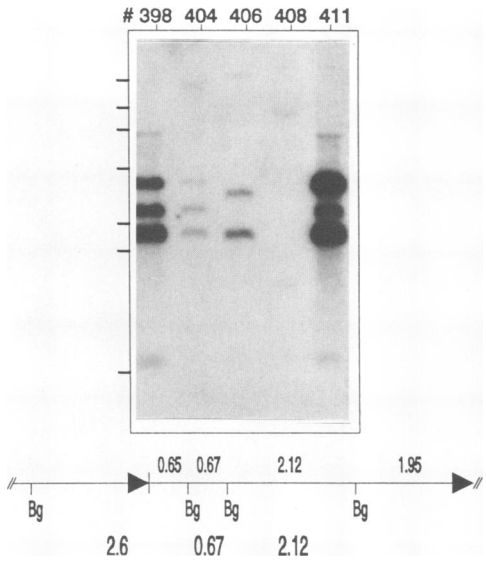

FIG. 2. Genomic organization of the transgenes in five transgenic mice analyzed by Southern blot hybridization. Mouse tail DNA (5 μg), digested with *Bgl*I, was electrophoresed on a 0.7% agarose gel, transferred to a nylon filter, and hybridized with a radiolabelled *Eco*RI insert from plasmid pURR18*lacZ* shown in Fig. 1. The filter was exposed overnight to allow the upper fragments to be recognized, and therefore the small 0.67-kb fragment gives a very weak signal in mice 404, 406, and 408. After longer exposure, however, this band was clearly seen also in these mice. The 0.67- and 2.1-kb bands are derived from the internal *Bgl*I fragments of the transgene. The 2.6-kb band observed in mice 398, 404, and 411 is indicative of a tandem head-to-tail array as shown in the scheme at the bottom in which the main arrow represents the entire *Eco*RI fragment of plasmid pURR18*lacZ* (see Fig. 1B), the restriction sites for *Bgl*I (Bg) are shown, the size of the expected fragments are given in kilobases. The 3.3-kb fragment observed in mice 398, 404, and 411 was consistently found in all Southern analyses of these mice using *Bgl*I and results probably from incomplete digestion due to methylation of the *Bgl*I site in the HPV-18 URR (the first in the main arrow) (see the text). Bacteriophage lambda *Hin*dIII markers corresponding to 23.1, 9.4, 6.6, 4.4, 2.3, 2.0 and 0.6 kb (from top to bottom) are shown on the left of the autorradiograph.

located downstream, which contains the intron and polyadenylation signal of the simian virus 40 small-t gene.

Generation of transgenic mice carrying the *lacZ* gene under the control of the HPV-18 URR. An *Eco*RI fragment with a size of about 5.4 kb was isolated from the pURR18*lacZ* plasmid that excluded vector sequences which have been frequently found to inhibit transgene expression (27). This fragment was injected into the pronuclei of eggs of F_2 (C57/BL6 × C3H) hybrids. Five independent transgenic animals were identified by Southern blot analysis (Fig. 2). One of these (mouse 408) had lost part of the transgene because the internal 2.1-kb *Bgl*I fragment within the *lacZ* open reading frame was absent. The remaining four animals showed the expected pattern for a single copy integrated per cell genome (mouse 406) or several copies, integrated as a single head-to-tail concatemer, which were estimated as 10 to 15 (for mouse 398) and 20 to 30 copies (mouse 411) by slot blot hybridization. Restriction enzyme analysis revealed that mouse 404 carried more than one copy per cell genome since a 2.6-kb *Bgl*I fragment compatible with a head-to-tail integrated concatemer was obtained. In all transgenic mice analyzed using *Bgl*I, a 3.3-kb band was consistently found, suggestive of partial digestion at the *Bgl*I site in the URR (Fig. 2). *Bgl*I recognizes the following underlined sequence within the HPV-18 URR, 5′-GCGCGCCTCTT′TG GCGC-3′, and is sensitive to methylation in the last cytosine, which is possible here because it is in a CpG sequence. Further analysis of the different transgenic mice and their progeny using the methylation-sensitive restriction enzyme *Cfo*I, which recognizes the sequence 5′-GCG′C-3′ present three times in the above fragment, revealed a restriction pattern compatible with methylation within this sequence of the URR (data not shown).

Three transgenic mice (no. 398, 406, and 411) transmitted the transgene to offspring whereas mouse 404 did not, probably because of mosaicism. Therefore, we used this animal as well as the three transgenic lines established from founders 398, 406, and 411 for further characterization. Founders 406 and 411 transmitted the transgene efficiently (to 38 and 43% of the descendants, respectively), whereas the female founder 398 was also mosaic and less than 20% of its offspring carried the transgene. Moreover, in this line the transgene was carried by the X chromosome as deduced from the fact that all the female progeny of F_2 transgenic males carried the transgene, whereas the male progeny did not.

Tissue-specific expression of the URR18*lacZ* construct. The pattern of expression of β-galactosidase was determined by in situ staining of fixed tissue sections from adult mice by using X-Gal. X-Gal is cleaved by β-galactosidase to yield insoluble indigo, which is blue. In 10 to 15 mice analyzed from lines 406 and 411 as well as mouse 404, specific staining was detected in a variety of tissues (Fig. 3), namely the tongue, gonads, uterus, stomach, small intestine, kidney, hair follicles, respiratory epithelium, and choroid plexus. Only weak specific staining could be detected in the tongues and uteri of eight mice of line 398. In all these organs, the enzymatic activity was restricted to epithelial cells. Certain variability in the β-galactosidase activity, especially in the genital tract, observed in females was attributed to hormonal changes during the estrous cycle (see below) and was not further studied.

The dorsal surface of the tongues of the transgenic mice exhibited the strongest activity (Fig. 3A, B and C). In this region the mouse tongue is covered with stratified squamous epithelium from which extend pointed filiform papillae. In mouse 404 and in line 411 animals, the staining was located in the filiform papillae and the underlying stratum granulosum (Fig. 3C), whereas mice of line 398 showed only faint staining in the filiform papillae. In contrast, the mice of line 406 displayed an intense β-galactosidase activity throughout the epithelium (Fig. 3A and B).

In the ovary, intense enzymatic activity was observed in follicular cells of growing follicles and in theca cells (Fig. 3D), but faint staining was also seen in primordial cells and corpora lutea (data not shown). In the uterus, the strongest activity was found in the uterine glands and the simple columnar epithelium of the endometrium (Fig. 3E). Expression was also observed in the differenciated layers of the squamous epithelium of the vagina and, more weakly, of the cervix (data not shown). In the testis, the bacterial β-galactosidase activity detected by X-Gal staining in the interstitial Leydig cells (Fig. 3F), was confirmed by immunofluorescence using monoclonal antibodies (data not shown) because some staining was also observed in the interstitial cells of nontransgenic controls. The Leydig cells of the testis originate from the mesenchyme but are considered gland cells (28). Interestingly, the epithelial cells of the nephrons and the choroid plexus, which were also positive for X-Gal staining (see below), develop from mesenchymal cells.

In the skin no expression was found in the dermis or in any kind of epidermis (ventral or plantar from the ear or the tail), except for the hair follicles, 50 to 80% of which were found to be stained in a specific area of the outer root sheath near the

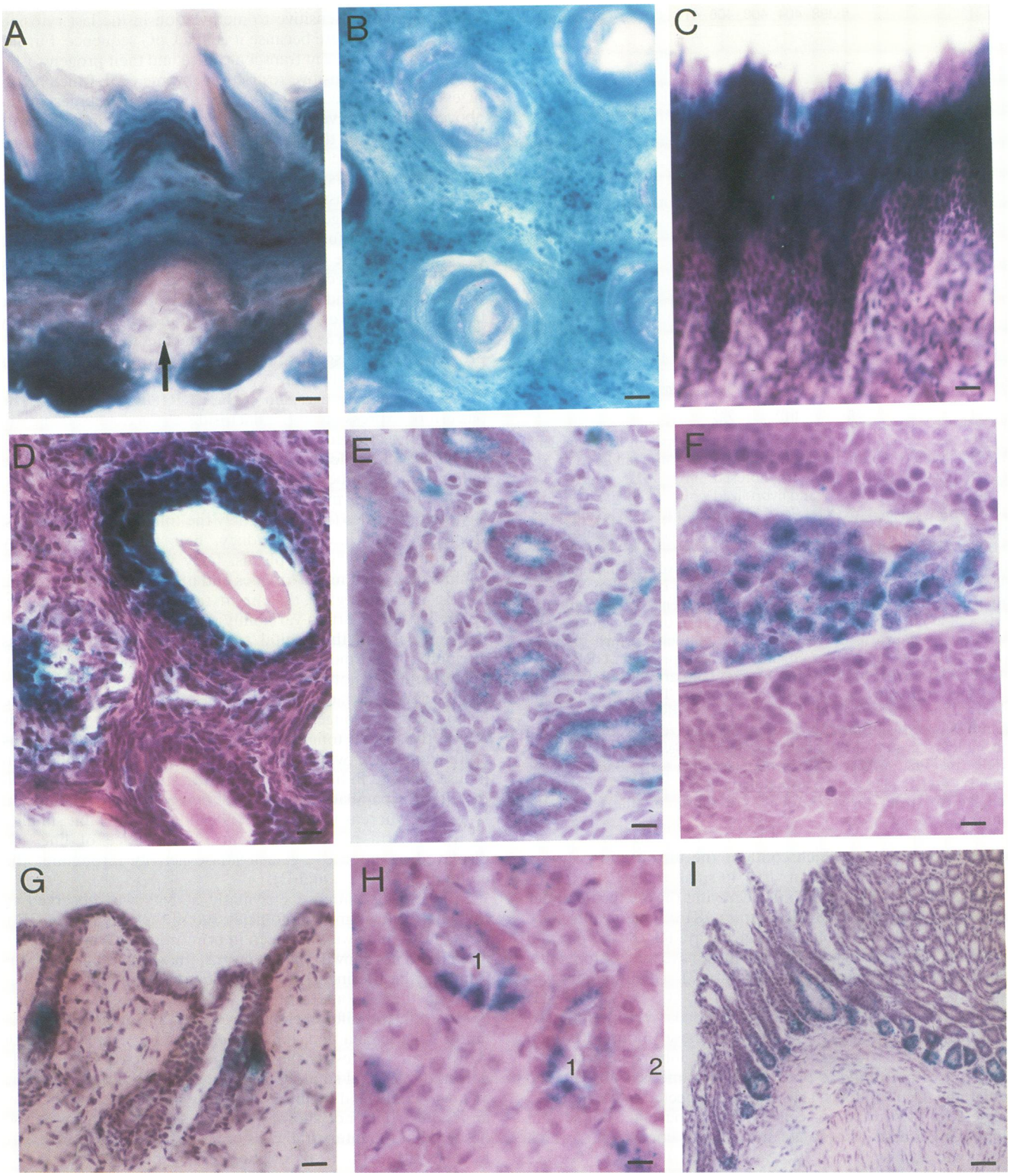

FIG. 3. Histochemical detection of *E. coli* β-galactosidase in tissues of adult URR18*lacZ* transgenic mice by using X-Gal stain, which is cleaved by the enzyme to yield blue indigo. Following staining, the tissue sections were counterstained with hematoxylin and eosin. Photographs show sections representative of the β-galactosidase activity found in three independent transgenic mice and their progenies (see the text). (A) Sagital section of the tongue of a mouse of line 406. The β-galactosidase activity is observed throughout the stratified epithelium. The arrow points to a dermal papilla. (B) Tongue of a mouse of line 406 cut transversally through the granular layer. Homogeneous β-galactosidase activity is observed throughout this layer. The white holes correspond to dermal papillae. (C) Frontal section of the tongue of a mouse of line 411. The enzymatic activity is located in the upper layers of the epithelium. (D) β-Galactosidase activity is seen in a mature follicle (upper right) but not in growing follicles (lower right) of the ovary. Staining on the left may correspond to a parasagital section of a mature follicle. (E) Transversal section of a

opening of the sebaceous gland (Fig. 3G). In contrast, no expression was detected in tail hair follicles. In the kidney, staining was observed in the cortex in the epithelium lining the proximal but not the distal convoluted tubules (Fig. 3H). In the digestive tract, activity was found at the base of the villi of the grandular stomach (Fig. 3I) and the cripts of Lieberkühn of the small intestine. In the brain, β-galactosidase activity was observed in the ependymal cells, which form a specialized epithelium lining the choroid plexus in the brain ventricles. Finally, in the respiratory system, the epithelium of the trachea and big bronchi showed some staining (data not shown).

Expression of the transgene in fetal tissues and newborn mice. HPV-18 infects the genital tract and thus likely can be transmitted perinatally as has been found for some HPVs (48). It is unknown, however, whether viral gene expression depends on the age of the individual at the time of infection. Therefore, we examined by X-Gal staining the β-galactosidase activity during early development, in fetuses and in newborn URR18*lacZ* transgenic mice of the 406 and 411 lines. The HPV-18 URR was described previously to be inactive in undifferentiated mouse F9 cells (53). In agreement with this observation we did not detect the X-Gal color reaction in preimplantation embryos (morula and blastocysts) developed in vitro from one-cell embryos into which the URR18*lacZ* construct had been injected (data not shown). Nevertheless, β-galactosidase activity was detected latter in development on days 13.5 (data not shown) and, more remarkably, 17.5 of gestation (Fig. 4A). Interestingly, from day 13 to the end of pregnancy the circulating levels of progesterone increase gradually (42) (see below). The staining was most prominent in the epithelia of the tongue and oral mucosae (Fig. 4A); however, some activity was also detected in the intestine, gonads, and choroid plexus (not shown).

The relatively low level of transgene expression found in fetal tissues contrasted with a strong β-galactosidase activity detected 1 day later, immediately after birth (19 days of gravidity) in nasopharyngeal, oral, and lingual epithelia (Fig. 4B, C, and D), the expression being particularly intense in the stratified epithelium of the soft palate. Such a strong staining was not observed in the oral cavities of adult animals.

β-Galactosidase activity in primary explants of the skin of URR18*lacZ* transgenic mice. In our study, the pattern of expression of the URR18*lacZ* transgene excluded both skin fibroblasts and keratinocytes although, the HPV-18 URR has been shown previously to function in mouse NIH 3T3 fibroblasts (7), and we have shown expression from the HPV-18 URR in a mouse keratinocyte cell line (Fig. 1C). Therefore, we tried to see whether the cell type specificity of the URR18*lacZ* transgene could be altered when a transgenic mouse tissue was cultured in vitro. To this end, we established primary explants from the skin of founders 404, 406, and 411 and two offspring of the latter two. The skin of the tail was chosen because no expression at all was detected in it by X-Gal staining (see above). Primary explants were done from skin biopsies of intact dermis from which, under the conditions described, after 2 to 3 weeks an outgrowth of cells, which consisted almost exclusively of dermal fibroblasts was observed. X-Gal staining of these cells in the two transgenic lines and the founder 404 showed that they expressed the transgene efficiently (as shown in Fig. 4E for line 411), whereas no activity was detected in control explants from nontransgenic mice (data not shown), thus suggesting a different transcription regulation of the transgene in fibroblasts in vivo and in vitro. On the contrary, when dermal tissues were removed and the epidermis was grown in appropriate selective medium, a culture of keratinocytes was obtained in which no β-galactosidase activity was detected (data not shown), in agreement with the absence of expression observed in vivo in these cells, indicating a more stringent control of the HPV-18 URR in epidermal keratinocytes than in fibroblasts.

Quantitation of β-galactosidase activity in transgenic mouse tissues. In order to attempt a quantitative characterization of reporter activity in the URR18*lacZ* transgenic mice, we adapted standard methods for β-galactosidase detection (33, 34) to develop a protocol that allowed reliable determinations of β-galactosidase activity in crude extracts from organs. For these experiments, ONPG was chosen as the substrate because it proved to be of sufficient sensitivity for the purpose. In agreement with previous studies on characterization of the *E. coli* β-galactosidase (reviewed in Wallenfels and Weil [56]), we found that the enzyme was very stable and resistant to inactivation when crude extracts from different organs (e.g., tongue, testis, and uterus) were prepared and assayed at 37°C for 1 to 5 h. However, after extended periods of incubation (15 to 20 h) the specific activity of the enzyme decreased to about one-third of the initial activity (data not shown), probably as a consequence of partial degradation of enzyme and/or substrate and some degree of protein precipitation. Furthermore, the β-galactosidase activity was found to depend linearly upon the amount of protein used, as shown in Fig. 5B for the tongue and small intestine. Therefore, in the following experiments reactions were carried out for 1 to 3 h with 100 to 400 μg of protein. Under the conditions described, we did not observe significant variability among measurements in animals of the same line. Hydrolysis of ONPG due to endogenous β-galactosidase activity was monitored by simultaneous analysis of nontransgenic littermates. However, it should be noted that in all tissues tested except for the tongue the endogenous activity measured was higher than expected from the X-Gal staining of nontransgenic controls. This is likely to be due to the different affinities of the endogenous β-galactosidases for the substrate used in each case (56).

In good correlation with the intensity of the staining obtained by histochemistry (Fig. 3), significant levels above the background of endogenous β-galactosidase activity were found in animals of the lines 406 and 411, namely in the tongue, testis, small intestine, kidney, and uterus (Fig. 5A) but not in the ovary or skin (data not shown). The highest activities measured corresponded to the tongue, in contrast to the activities in the other tissues in which values were one order of magnitude lower. Interestingly, line 411 showed higher values in all tissues except for the tongue in which line 406 exhibited activities three- to fourfold higher, despite the fact that this line carries only one copy of the transgene in contrast to more than 20 copies integrated in line 411. This difference is probably due to the generalized expression throughout the epithelium of the tongue observed in line 406 by histochemical staining in con-

uterine horn. Staining is observed in the uterine glands and the simple columnar epithelium. Disperse staining in the endometrium corresponds to endogenous activity also observed in nontransgenic mice. (F) Section of the testis showing part of two seminiferous tubules separated by interstitial Leydig cells (in the middle), which show β-galactosidase activity. (G) Section of the skin. Three hair follicles are shown in which the staining is located in a specific area of the outer root sheath. (H) In the kidney the β-galactosidase activity is observed in the cuboidal cells lining the proximal (1) but not the distal (2) convoluted tubules. (I) Grandular portion of the stomach. At the base of the mucosa are zymogenic cells which express the bacterial β-galactosidase. Calibration bars 32 μm (A, B, C, and G), 16 μm (E, F, and H), and 64 μm (I).

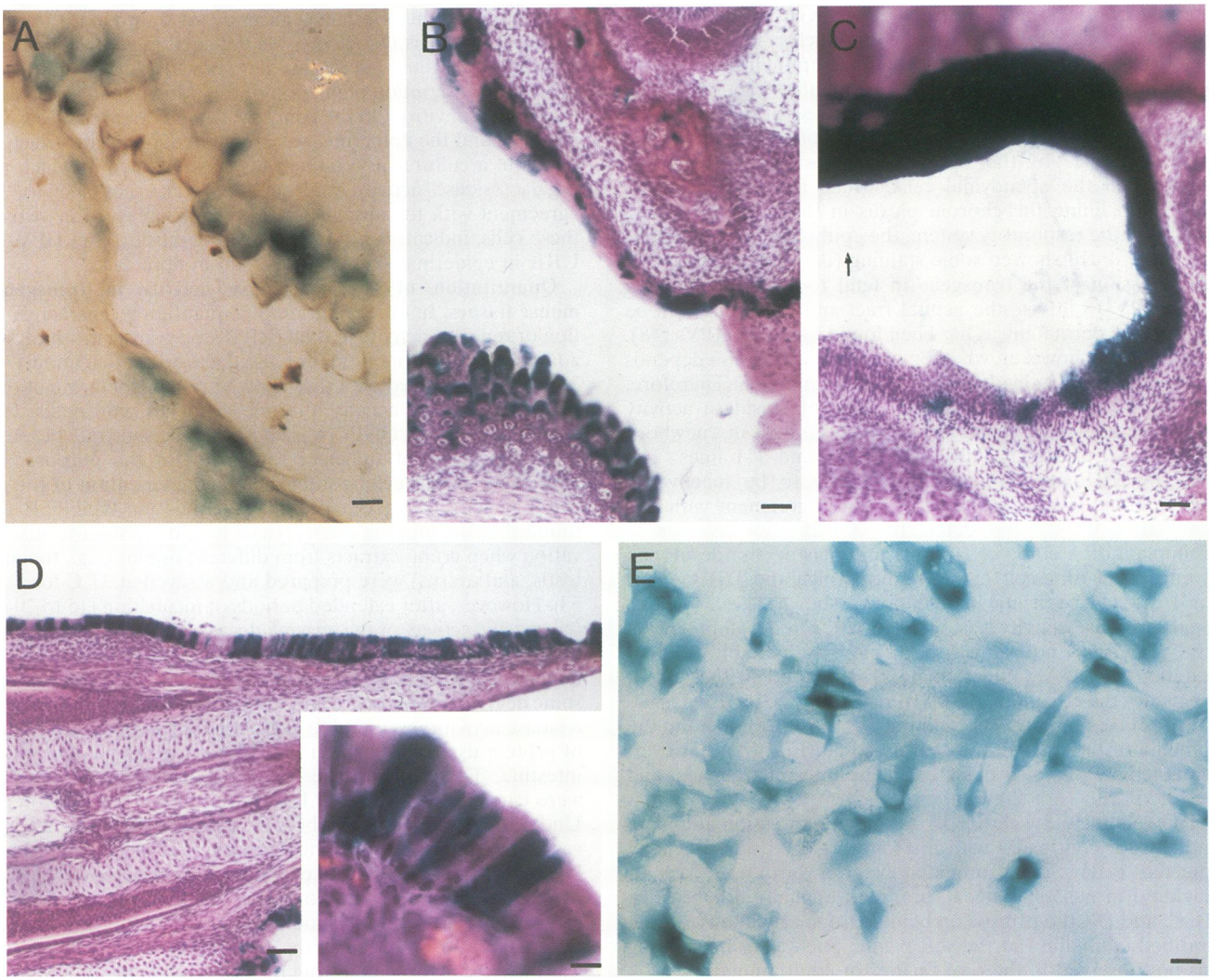

FIG. 4. Histochemical detection with X-Gal of *E. coli* β-galactosidase in sections of fetal and newborn transgenic mice and in cultured cells from the tail of a transgenic mouse. Sections correspond to transversal cuts. Except for panels A and E, tissues were counterstained with hematoxylin and eosin. No staining was observed in the same tissues or cultures from nontransgenic controls. (A) Oral cavity of a fetus of line 411 at day 17.5 of gravidity. Staining is seen in the tongue (papillae) and oral mucosae. (B) Section of the oral cavity of a newborn mouse of line 411. The tongue and pharynx show intense β-galactosidase activity. (C) Strong activity of the bacterial enzyme in the soft palate of newborn mice. The arrow points towards the oral cavity. (D) Enzymatic activity observed in the pseudostratified ciliated columnar epithelium (seen at a higher magnification in the inset [lower right]) of the nasal mucosae of newborn animals. (E) β-Galactosidase activity in cultured fibroblasts from the skin of the tail of a transgenic mouse (founder 411). The primary explant was performed as described in Materials and Methods. After 4 weeks of culture, the cells were fixed and stained with X-Gal. Calibration bars, 32 μm (A), 64 μm (B, C, and D), and 16 μm (inset in panel D and E).

trast to a more restricted expression in line 411, in which only the differentiated upper layers showed the X-Gal staining (Fig. 3A and C). Finally, very low but still significant activities were measured in the tongues and uteri of mice of line 398.

Effect of glucocorticoids and TPA on transgene expression. The proximal promoter of the HPV-18 URR contains two glucocorticoid-responsive elements (Fig. 1B) which have been shown to mediate transcriptional activation by these hormones in transient-expression experiments (12, 55). On the other hand, the two AP-1 binding elements contained in the HPV-18 URR (Fig. 1B) were found to be essential for its transcriptional activity in several human keratinocyte cell lines (53).

In order to investigate these two aspects of the HPV-18 URR regulation in vivo in the context of normal mouse tissues, we determined the effects of dexamethasone and TPA on β-galactosidase activity and mRNA levels in mice of lines 406 and 411 by quantitation with ONPG and Northern blot analyses. The tongue was chosen as the ideal tissue for these experiments because it exhibited the strongest activity in both transgenic lines (Fig. 3 and 6A) and the lowest endogenous activity. To avoid variability in transgene expression due to changes in the endogenous progesterone level during the estrous cycle of the females, male instead of female transgenic offspring were used as well as control nontransgenic littermates. It was reported previously that glucocorticoid-responsive elements of several HPVs, including HPV-18, function also as progesterone-responsive elements and are activated by this hormone (12). Moreover, we obtained indirect evidence of a stimulating effect of progesterone in URR18*lacZ* transgenic females by inducing them to superovulate. This consists in a hormonal treatment which results in the release of a high number of oocytes and the formation of the corresponding corpora lutea, which contain

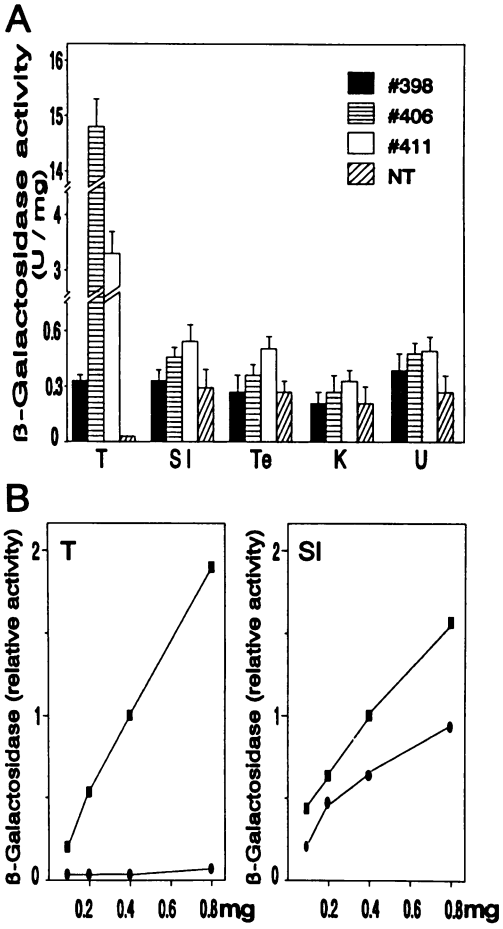

FIG. 5. Quantitative analysis of β-galactosidase activity in crude extracts from tissues of the URR18*lacZ* transgenic mice. Quantitation was performed as described in Materials and Methods, with the synthetic derivative ONPG as the substrate. (A) Specific β-galactosidase activities determined in different tissues of animals of the lines 398, 406, and 411 and nontransgenic (NT) controls. The data are presented as means ± standard errors. Results for four to six animals contributed to each point. Abbreviations: T, tongue; SI, small intestine; Te, testis; K, kidney; U, uterus. (B) Correlation between β-galactosidase activity and protein concentration determined for tongues (T) and small intestines (SI) of transgenic mice of line 411 (squares) and nontransgenic controls (circles). Activities are given as relative values to the activity obtained with 0.4 mg, set arbitrarily at 1, and are the means of three independent experiments.

progesterone-secreting cells that help to maintain pregnancy. A stronger β-galactosidase activity was found in the epithelium of the tongue of superovulated females than in untreated females (data not shown). In contrast, previous reports attributed no effect to the hormone dihydrotestosterone (released by the testis) on HPV expression (25).

Treatment of the URR18*lacZ* mice with dexamethasone revealed a decrease in β-galactosidase activity after 24 h of hormone administration by either intraperitoneal injection of a single dose or addition to drinking water. Since this result contrasted with the stimulation of transcription in vitro by the hormone reported previously (see above), we studied in more detail the time course of enzymatic activity in comparison to the β-galactosidase mRNA levels in the same tissue determined by Northern blot hybridizations following hormone administration (orally). It was found (Fig. 6A) that in both

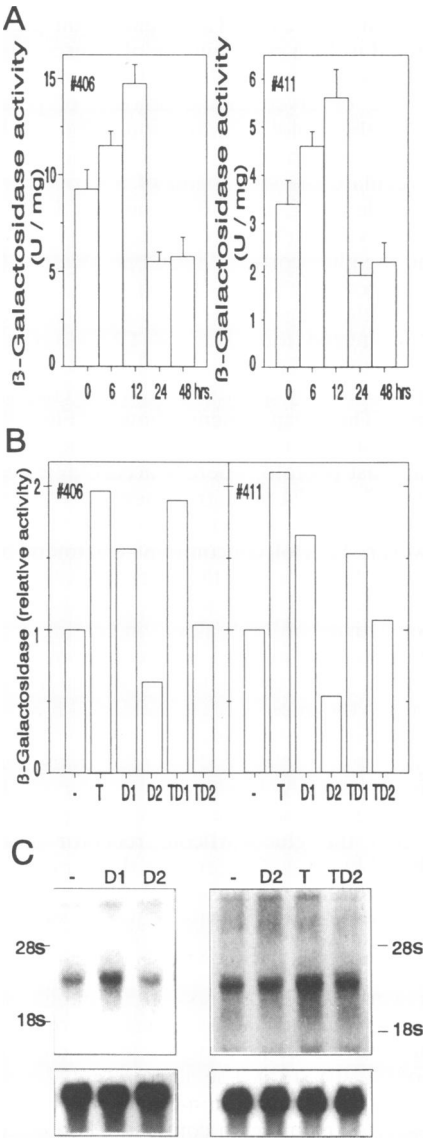

FIG. 6. Effect of dexamethasone and TPA on transgene expression. (A) Time course of the specific β-galactosidase activity after dexamethasone administration, determined in crude extracts of the tongues of transgenic mice from lines 406 and 411. Mice were treated orally with the hormone and at the indicated times were sacrified for quantitative analysis of crude extracts obtained from the tongue. (B) Quantitation of β-galactosidase activity in the tongues of transgenic progeny of lines 406 and 411 following treatments with dexamethasone (for 12 h, D1; for 24 h, D2), TPA (for 12 h, T) and dexamethasone plus TPA (for 12 h with TPA and for either 12 or 24 h with dexamethasone, TD1 or TD2, respectively). Relative values refer to the activity in untreated (−) animals set as 1. Activity data are the means of three independent experiments. (C) Northern blot analyses performed with total RNA extracts from biopsies of the tongue obtained in the experiments described for panel A. Abbreviations are the same as for panel B. The arrow points to the β-galactosidase mRNA (3.5 kb). The positions of the 28S (4.7-kb) and 18S (1.9-kb) RNAs are indicated. GADPH (glyceraldehyde-3-phosphate dehydrogenase), shown in the lower panels, was used as a control.

transgenic lines there was a transient increase in activity (50 to 80%) 12 h after hormone administration followed by a decrease to about 50% of the initial activity after 24 h, which persisted for another 24 h. These effects on β-galactosidase activity seemed to be a consequence of transcriptional regulation of the transgene induced by the hormone because similar variations were observed in the amount of β-galactosidase mRNA by Northern blot hybridizations (Fig. 6C, left panel).

Previous studies of the mechanisms by which glucocorticoids negatively regulate the expression of a variety of genes (reviewed in Schüle and Evans [46]) showed a down-modulation of the AP-1 activity by the hormone due to a direct interaction of AP-1 and the glucocorticoid hormone receptor (30, 47, 57). In order to investigate whether such a mechanism could be implicated in the negative regulation of the URR18*lacZ* transgene, we tested the effect of TPA, known to rapidly induce AP-1 activity (2), in mice of lines 406 and 411 treated with dexamethasone by quantitation of the enzymatic activity in the tongue. These experiments showed (Fig. 6B) that after 12 h of treatment with TPA the β-galactosidase activity increased at least twofold, whereas according to our previous experiments, 12 and 24 h of treatment with dexamethasone were followed by increased and decreased activities, respectively. However, 12 h of concomitant treatment of TPA and dexamethasone led to a slightly lower activation in lines 406 and to a 50% less activation in line 411 compared with the induction of activity of TPA alone, indicating that the transcription factors involved do not cooperate but interfere instead in their respective induction of the HPV-18 URR. Moreover, when the mice were treated for 24 h with dexamethasone and TPA administered during the last 12 h, no effect on enzymatic activity was observed in either transgenic line. The β-galactosidase mRNA levels detected by Northern blots underwent changes in the same direction (Fig. 6C, right panel), suggesting that the glucocorticoid receptor and the AP-1 activity induced by TPA interfered in their transcription regulation of the URR18*lacZ* transgene.

DISCUSSION

Previous work on the expression of papillomaviruses in transgenic mice revealed a tissue-specific expression of the bovine papillomavirus genome, which induced fibropapillomas in the skin of the mice (31). The data presented here show that the HPV-18 URR is transcriptionally active in mouse tissues in a cell-type-specific manner. In contrast, Choo et al. (14) have reported that the same promoter-enhancer element failed to drive transcription in transgenic mice. However, this is likely to be a consequence of the lethality caused by the reporter used in that work (the simian virus 40 large-T antigen gene) and the subsequent selection of those embryos in which expression was low enough so as not to be lethal. We have obtained results compatible with this explanation also with transgenic mice carrying HPV-16 early genes under the control of strong heterologous promoters (15a).

The experiments presented here resulted in the following major findings. (i) The HPV-18 URR is active exclusively in epithelial cells of a variety of mouse tissues (Fig. 3 and 4). (ii) Expression of the transgene is very weak during embryogenesis but is strongly activated at birth (Fig. 4A to D). (iii) Ectopic expression in fibroblasts can be achieved when these cells are cultured from explants of the skin (Fig. 4E). (iv) Determinations of β-galactosidase activity in crude extracts from organs made possible a quantitative characterization of transgene expression, as shown for the stratified epithelium of the tongue (Fig. 5 and 6). (v) The transcriptional activity of the HPV-18 URR is regulated in vivo by glucocorticoids in a complex manner including positive and negative effects (Fig. 6). (vi) TPA increased the transcriptional activity of the URR18*lacZ* transgene, and both dexamethasone and TPA mutually interfered in their respective effects on transgene expression (Fig. 6).

The expression of the regulatory region of a human virus in transgenic mice could be restricted by an inefficient interaction of mouse transcription factors and the viral sequences. Nevertheless, the studies on the expression driven by the HPV-18 URR in transgenic mice described here revealed an efficient transcription in a specific cell type that agrees well with the specificity of the virus. Therefore, the information obtained in this system may be of value to the understanding of papillomavirus gene expression in humans. Indeed, β-galactosidase activity was found in tissues which also under natural conditions are known to support viral gene expression. HPV-18 is most consistently associated with adenocarcinomas and small cell carcinomas than with squamous cancers of the uterine cervix (49). In agreement with that, in transgenic mice the HPV-18 URR was found to be active in the uterine glands from which adenocarcinomas originate (Fig. 3E). On the other hand, the URR18*lacZ* transgene was expressed in a variety of epithelial cells within several organs (most notably the tongue) (Fig. 3). Under natural conditions this virus has not been found in all these tissues; however, our results indicate that the transcription factors required for efficient expression from the viral URR are present in a variety of epithelial cell types and suggest that the narrow tissue specificity of the virus may be due to other parameters (e.g., the presence or absence of receptors for the virus).

An interesting finding was that the URR18*lacZ* transgene is expressed in fetal tissues, in particular in the epithelia of the tongue and oral cavity (Fig. 4B, C, and D), which are in contact with the amniotic fluid. Recently, HPV-16 DNA has been detected by means of polymerase chain reaction in the amniotic fluid of pregnant women who carried HPV-induced lesions (3). Although it is still unclear whether papillomavirus infections actually do occur in utero, perinatal infections have been well documented (48); however, particular information about HPV-18 is not available. Both intrauterine and perinatal transmission would be compatible with our data.

The β-galactosidase expression found in fibroblasts cultured in vitro from primary explants (Fig. 4E) raises the interesting possibility of a switch in the specificity of the viral promoter induced by changes in the conditions in which the cells are maintained. A possible explanation for this fact is that the fibroblasts, stimulated to proliferate by the biopsy itself and/or the presence of serum in the culture medium, could undergo changes in the expression of transcription factors like those described for the activator protein AP-1 when quiescent fibroblasts were stimulated by serum (16, 17, 37).

We have evidence that the HPV-18 URR is methylated in all transgenic mice analyzed. Since methylation of DNA has been implicated in regulation of gene expression (21, 39, 51) and in vitro methylation of the HPV-18 URR has been shown to down-regulate its activity in transient transfection assays (40), a more precise analysis will be required to discern whether such a modification within the URR could account for the differences in expression observed in the three transgenic lines described despite their differences in the copy number of the transgene.

The second part of our study focused on the transcription regulation of the HPV-18 URR in the URR18*lacZ* transgenic mice. In terms of regulation, the promoter/enhancer region of the URR18*lacZ* transgene resembles that of the integrated viral genome as it is found in cervical carcinomas, in which the

viral E2 *trans*-repressor is missing, in that it is regulated exclusively by cellular transcription factors; therefore, these mice provide a good model in which to analyze the regulation of the URR by those factors. In vitro, such analyses are facilitated by quantitation of the reporter activity in protein extracts from previously transfected cells. Our results demonstrate that a quantitative determination of β-galactosidase activity is also possible in crude extracts from mouse tissues and that they can provide reliable information about the level of expression of the transgene. We have been able to detect activity of the bacterial enzyme over the endogenous background in a variety of tissues (Fig. 5A), in good correlation with the expression previously detected by X-Gal staining. The highest specific activity was found in the epithelium of the tongue, in agreement with the strong signal obtained by X-Gal staining of this tissue (Fig. 3).

Using the stratified epithelium of the tongues of transgenic mice as a model to study in vivo the transcription regulation of the HPV-18 URR, we have found in time course experiments that glucocorticoids induce expression shortly after treatment (6 to 12 h) but down-regulate the HPV-18 URR later (24 to 48 h of treatment). The apparent contradiction of this negative regulation with the induction of the viral URR previously observed in vitro (19, 55) could be explained by the fact that our experiments reflect the behavior of normal epithelial cells, whereas the experiments in vitro were performed with carcinoma cell lines in which activating factors could be increased, as described for p92, an enhancer binding protein increased in many tumor cells (41). Differences in the doses of hormone and the amount of it affecting the target cells in vivo and in vitro as well as the period of treatment should also be considered. Furthermore, in the stratified epithelium of the tongue, we have found that TPA induces a significant increase of the expression from the HPV-18 URR (Fig. 6). In transient transfection assays, transcription from the HPV-18 URR is increased at least three- to fivefold by TPA in SiHa cells, an HPV-16-positive cervical carcinoma cell line (40). In contrast, in HeLa cells the transcriptional activity of the integrated HPV-18 URR is not affected by TPA, suggesting that it is already activated at the maximal level (5). This discrepancies might reflect the different composition of the panel of transcription factors available in normal mouse cells and in the different cancer cell lines. On the other hand, dexamethasone treatment almost abolished the induction by TPA, whereas TPA impaired the down-regulation by glucocorticoids (Fig. 6), indicating that a mechanism involving interference and cross talk of both types of transcription factors could be involved in the regulation of the HPV-18 URR in a manner similar to that described for the collagenase promoter (see reference 46). These striking results point to the importance of obtaining new lines of mice transgenic for mutated URRs with inactivated AP-1-binding sites and/or the YY1 silencer-binding element, recently shown to repress the viral promoter via interference with its AP-1-mediated induction (5). Such studies could contribute to a better understanding of the mechanisms by which HPV gene expression is regulated in vivo in the context of normal tissues.

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