The EMBO Journal vol.10 no.6 pp.1337 - 1345, 1991

Extinction of the HPV18 upstream regulatory region in cervical carcinoma cells after fusion with non-tumorigenic human keratinocytes under non-selective conditions

Frank Rösl, Thomas Achtstätter[2], Tobias Bauknecht, Karl-Josef Hutter[1], Gerald Futterman[1] and Harald zur Hausen

Institut für Angewandte Tumorvirologie and [1]Institut für Experimentelle Pathologie, Deutsches Krebsforschungszentrum, 6900 Heidelberg, FRG

[2]Present address: Biotest Pharma, 6072 Dreieich, FRG

Communicated by H.zur Hausen

'Universal fuser' clones of a human papillomavirus type 16 positive cervical carcinoma cell line (SiHa) were established to study the effect of a non-tumorigenic fusion partner on the regulation of a stably integrated chloramphenicol acetyltransferase (CAT) gene controlled by the HPV18 upstream regulatory region under non-selective conditions. The CAT expressing cells were fused with both non-tumorigenic, spontaneously immortalized human keratinocytes (HaCaT) and non-modified SiHa cells. The resulting hybrids were characterized by restriction enzyme fragment length polymorphism analysis and flow cytometry. While the non-selectable, HPV18-driven indicator gene is constitutively expressed in SiHa cells, the CAT activity is extinguished in SiHa × HaCaT cells, but still present in SiHa × SiHa hybrids. Examination of the cytokeratin expression pattern reveals that the keratinocyte phenotype seems not only to be dominant in terms of the extinction of the HPV18 regulatory region but also by the conservation of most of the differentiation markers of the non-tumorigenic fusion partner. Cycloheximide treatment and intracellular competition experiments using the transient COS7 fusion–amplification technique are accompanied by the reactivation of the marker gene in previously CAT⁻ SiHa × HaCaT hybrids. These data strongly suggest that *trans*-acting negative regulatory factors derived from the non-malignant human keratinocytes are responsible for the extinction phenomenon.

Key words: cervical carcinoma cells/human papillomavirus/negative regulation/somatic cell hybrids

Introduction

Although specific types of human papillomaviruses (e.g. HPV16, 18) are etiologically involved in the development of anogenital cancer (reviewed in zur Hausen and Schneider, 1987), HPV infection and persistence *per se* seem not to be sufficient to induce malignant transformation. HPV16 or HPV18 DNA transfections on primary human keratinocytes only result in immortalized cells which are non-tumorigenic upon heterotransplantation into nude mice at least in early passages after immortalization (Dürst *et al.*, 1987; Pirisi *et al.*, 1987). The long latency period between primary infection and the final progression to cervical cancer argue for a requirement of additional cellular damaging events (zur Hausen, 1977a). Indeed, there is increasing evidence that the loss of genetic information or function is also an important step in the multi-hit concept of carcinogenesis of many human tumors (reviewed in Ponder, 1988; Sager, 1989).

In the case of HPV16/18 positive cervical carcinoma cells, chromosome 11 seems to play a role as one key factor in malignant transformation, since re-introduction of the corresponding normal allele via microcell transfer is sufficient to suppress the tumorigenic phenotype (Saxon *et al.*, 1986; Koi *et al.*, 1989).

Speculating along the working hypothesis that a chromosome 11 linked cellular control mechanism not only negatively interferes with tumorigenicity but also with HPV transcription (zur Hausen, 1977b, 1986) we initially used cellular hybrids between the HPV18 positive cervical carcinoma cell line HeLa and normal human fibroblasts or keratinocytes (Stanbridge, 1984) as a model to study viral gene regulation within cells of different genetic background.

In these cell systems, as well as in HPV16 immortalized, non-tumorigenic keratinocytes (Dürst *et al.*, 1987) it was demonstrated that 5-azacytidine (5-AzaC), a substance which causes both cell differentiation and gene induction by demethylation of formerly silent genes (Jones, 1985), is capable of selectively suppressing HPV16/18 transcription exclusively in non-tumorigenic cells at the level of initiation of transcription. The HPV18 gene expression, however, is not affected in either the tumorigenic segregants, which have lost one normal copy of chromosome 11, or in the parental HeLa cells. Heterografting of non-tumorigenic HeLa hybrids or HPV16 immortalized human keratinocytes into nude mice results in an arrest of the mitotic activity of the inoculated cells (Stanbridge, 1984; Dürst *et al.*, 1987). Interestingly, parallel examinations of the tissue grafts by *in situ* hybridization also reveal a selective down-regulation of HPV expression in non-malignant cells which precedes the termination of cell proliferation (Bosch *et al.*, 1990; Dürst *et al.*, 1991).

The absence of functional HPV16/18 transcripts in these experimental systems could support the concept that the viral control region might be a potential target for *trans*-acting negative regulatory proteins (Rösl *et al.*, 1988; zur Hausen, 1989). Further evidence which might point in this direction originates from observations that the upstream regulatory region (URR) of HPV16 and HPV18 leads to low levels of transcriptional activity in transient transfection assays on primary human fibroblasts and keratinocytes (Bernard *et al.*, 1989; Romanczuk *et al.*, 1990). A possible causal relationship between the loss of genetic function on chromosome 11 and HPV16 gene expression is provided by another set of studies. It has been demonstrated that the efficiency of HPV16 transcription in primary human fibroblasts is directly correlated with a small deletion on one copy of chromosome 11 (Smits *et al.*, 1988, 1990).

On the other hand, fusions of primary human fibroblasts or keratinocytes with the cervical carcinoma cell line HeLa (Stanbridge, 1976) did not result in a significant reduction of the steady state level of HPV18 mRNA in stable hybrids kept in tissue culture (Schwarz *et al.*, 1987; Rösl *et al.*, 1988). A possible explanation of this obviously inconsistent observation could be an epigenetic modification, such as DNA hypermethylation (Holliday, 1987), of putative negatively regulating gene(s) permitting HPV18 transcription after fusion of cells *in vitro*. This interpretation is also supported by our experiments using a demethylating substance (5-AzaC) which might lead to the activation of such hypermethylated genes followed by a down-regulation of HPV16/18 gene expression in non-tumorigenic cells (Rösl *et al.*, 1988). Based on the finding that antisense RNA directed against the transforming ORFs E6/E7 results in a decrease of cellular growth (von Knebel Doeberitz *et al.*, 1988), it seems reasonable to assume that an appropriate level of HPV expression is essential for the maintenance of the proliferative phenotype of cervical carcinoma cells. Therefore, the selection for growing cells *in vitro* could uniformly represent a selection for functional HPV transcription.

To circumvent this apparent complication and suspecting that the viral control region is the target for putative negative regulatory proteins, we devised a strategy which allows the investigation of the influence of a non-tumorigenic fusion partner on the transcriptional regulation of the HPV18 URR under non-selective conditions. In the following report we demonstrate that an HPV18-controlled CAT gene, which was stably introduced into an HPV16 positive cervical carcinoma cell line (SiHa) is constitutively expressed, but extinguished after fusion with non-tumorigenic, spontaneously immortalized human keratinocytes. We describe some differentiation properties of these somatic cell hybrids and provide evidence that the extinction mechanism is mediated by the involvement of *trans*-acting negative regulatory factors derived from the non-tumorigenic keratinocytes as fusion partner.

Results

Transient transfection versus stable integration: formation of SiHa × HaCaT hybrids

Transient transfection studies of CAT plasmid ($pA_{10}CAT_2$) under the control of the HPV18 URR (pH18CAT) exhibit substantial transcriptional activity in HPV16 positive cervical carcinoma cells (Figure 1, lane d). In parallel experiments, however, using standardized conditions to correct the transfection efficiencies, the same construct is nearly transcriptionally silent in non-tumorigenic spontaneously immortalized human keratinocytes (HaCaT cells, Boukamp *et al.*, 1988; see Figure 1, lane b). One possible explanation for the transcriptional block of HPV18 URR driven plasmids in human keratinocytes might be the presence of negatively regulating factors. Alternatively, the inactivity of the CAT gene could also be attributed to the presence of only limiting amounts or the complete absence of positive regulatory proteins, which may be indispensable to support initiation of transcription.

To discriminate between these two possibilities, the pH18CAT plasmid was first stably introduced into the cellular environment of an HPV16 positive cervical carcinoma cell line (SiHa) under non-selective conditions,

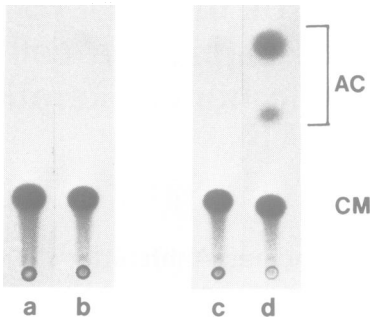

Fig. 1. Transient transfection assays of pH18CAT on cervical carcinoma cells and non-tumorigenic human keratinocytes. The CAT activity of pH18CAT plasmids on non-tumorigenic, spontaneously immortalized human keratinocytes (HaCaT, lane b) and HPV16 positive cervical carcinoma cells SiHa (lane d). 1×10^6 cells were transfected with 16 μg pH18CAT along with 4 μg of a β-actin promoter-driven luciferase gene. Cell extracts were prepared 48 h after transfection and assayed for luciferase activity to correct the transfection efficiencies. Extracts corresponding to 6000 luciferase counts were incubated for 2 h at 37°C to perform the CAT reaction. The acetylation products were finally separated by TLC. Lane a: negative control, transfection of a promoter/enhancerless CAT plasmid (pBLCAT3) on HaCaT. Lane c: pBLCAT3 on SiHa cells. The locations of the [^{14}C]chloramphenicol substrate (CM) and the acetylated form (AC) are indicated.

which should ascertain the presence of specific positive regulatory factors necessary for constitutive CAT expression. Subsequently, stable somatic cell hybrids between SiHa and HaCaT keratinocytes were established in order to investigate the influence of the non-tumorigenic fusion partner on the regulation of the marker gene under non-selective conditions.

The CAT^+ SiHa cells were modified to be used as 'universal fuser' by introducing a dominant selection marker conferring resistance to G418 (Geneticin) and selecting for a deficient hypoxanthine phosphoribosyl transferase (HPRT) phenotype, rendering them sensitive in hypoxanthine–aminopterine–thymidine (HAT) medium (Pereira-Smith and Smith, 1988). Fusion with HaCaT keratinocytes and selection in HAT/G418 medium therefore eliminates the parental SiHa cells due to their HPRT deficiency and the HaCaT cells due to their sensitivity to G418. Only those cell hybrids survive that are resistant to both G418 and HAT.

In total, 30 individual hybrids were obtained from three independent fusion experiments, from which several clones were investigated in greater detail. Figure 2 depicts the cell morphology of two representative somatic cell hybrids which are morphologically different from the two parental fusion partners but show more similarities to HaCaT than to SiHa cells. Stable hybrid formation was further confirmed by flow cytometry (unpublished data) and by restriction enzyme fragment length polymorphism (RFLP) analysis (Figure 3A). Using the *Msp*I RFLP of the Harvey *ras* proto-oncogene (Krontiris *et al.*, 1985) and the 6.6 kb Ha-*ras* fragment as a chromosome 11-specific hybridization probe, two alleles of 1.4 and 1.0 kb in size can be visualized in HaCaT cells (Chandler *et al.*, 1987). In the cervical carcinoma cell line SiHa, however, only one 1.0 kb band is detectable, indicating the presence of either a homo- or a hemizygous condition of the Ha-*ras* region in these cells (Figure 3A). After examination of the hybrid DNA, each clone showed the occurrence of both alleles at a ratio of band intensities either equal to or greater than those present in HaCaT DNA.

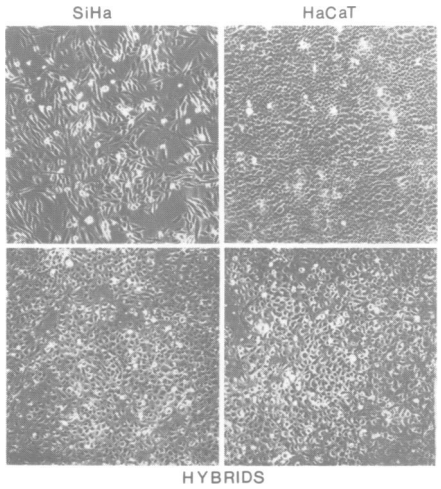

Fig. 2. Cell morphology of the parental SiHa and HaCaT cells in comparison with two representative somatic cell hybrids. Magnification: 200×.

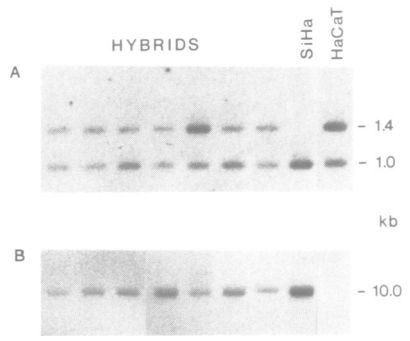

Fig. 3. RFLP analysis to confirm the hybrid nature of the HAT/G418 selected SiHa × HaCaT clones. Panel **A**: 10 μg genomic DNA of each hybrid clone and the parental SiHa/HaCaT cells were digested with *Msp*I. After Southern transfer, the filter was hybridized with the gel-purified 6.6 kb *Bam*HI fragment of the Ha-ras proto-oncogene. Panel **B**: the same DNA, but cleaved with the HPV16 no-cut enzyme *Hin*dIII. Autoradiography is given after hybridization with unit-length viral DNA. The band sizes are indicated in kilobases (kb).

To demonstrate unambiguously the hybrid nature of the selected clones, the same DNA was digested with *Hin*dIII (which does not cut the viral DNA) and hybridized with ^{32}P-labeled unit-length HPV16 DNA. As clearly seen in Figure 3B, all somatic cell hybrids carrying two Ha-*ras* alleles also retained the unrearranged HPV16 integration locus derived from the parental SiHa cells (El Awady *et al.*, 1987; Baker *et al.*, 1987).

Extinction of the constitutive HPV18 URR-controlled CAT expression in SiHa cells after fusion with human keratinocytes

Having verified the hybrid nature of the HAT/G418 selected fusion clones, we examined the expression of the HPV18 URR-driven marker gene in the cervical carcinoma cell line SiHa in comparison with the CAT expression in cellular fusions made between SiHa and HaCaT cells. If the keratinocytes lacked positive transcription factors, it would be expected that the regulatory proteins would become diluted in the hybrid environment which should only result in a reduction of the CAT signal. In considering the involvement of putative negative regulators, however, the marker gene should be suppressed in stable hybrids, a situation already proposed due to the absence of transcriptional activity of pH18CAT plasmids in HaCaT cells under transient transfection conditions (see Figure 1, lane b).

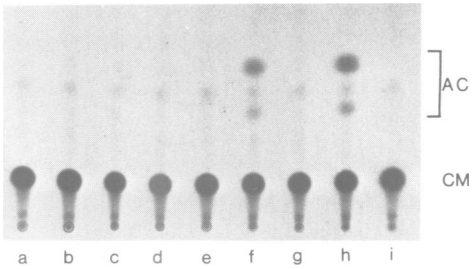

Fig. 4. Extinction of the constitutive CAT expression in SiHa cells after fusion with HaCaT keratinocytes. Lane f: constitutive expression of the integrated pH18CAT gene in SiHa cells. The CAT assay was performed for 2 h at 37°C by using 20 μg of total cellular protein. Lane h: same as in lane f, but 40 μg cellular extact was used for incubation. Lanes a–e: extinction of the CAT activity in stable SiHa × HaCaT hybrids, employing the same assay condition described for lane f. Lane g: same as in lane h, using the double amount of protein of one representative hybrid clone. Lane i: HaCaT cell extract, supplemented with [^{14}C]chloramphenicol (CM) as a negative control. The positions of the acetylated forms (AC) are indicated.

The results given in Figure 4 indeed favor the latter interpretation. Lanes f and h show the constitutive CAT signal in SiHa cells before the fusion event. As already anticipated, SiHa × HaCaT hybrids fail to express the HPV18 URR-driven indicator gene (lanes a–e). Even incubation after a 2-fold increase of the protein concentration to compensate for dilution of the CAT gene product in hybrid cell extracts (40 μg protein of hybrid extract in lane g versus 20 μg of SiHa cellular extract, lane f) does not lead to the detection of CAT activity above the level of the negative control, using a HaCaT extract supplemented with [^{14}C]chloramphenicol (lane i). Stable and complete extinction in all 22 individually tested hybrid clones was noted, clearly demonstrating the dominance of the keratinocyte phenotype over cervical carcinoma cells on the basis of the regulation of the CAT gene as marker. Furthermore, to exclude the possibility that the extinction mechanism is exceptional only for the integration site of pH18CAT in this particular SiHa clone, we also established stable hybrids with another 'universal fuser' clone having a different integration site for the indicator gene. Parallel experiments performed with these cells again revealed complete extinction in eight out of eight hybrid clones excluding a position effect of *cis* regulatory sequences at the integration locus (Rösl *et al.*, 1989; and unpublished data).

To gain further insight into the extinction phenomenon and to confirm that the absence of the CAT signal in SiHa × HaCaT hybrids is due to a suppression of CAT transcription, poly(A)$^+$ selected RNA derived from the parental and hybrid cells was examined by Northern blot analysis. As shown in Figure 5 (panel A), CAT specific transcripts were only detectable in non-fused SiHa cells (lane a) whereas the corresponding mRNA is down-regulated in somatic cell hybrids (lanes c–f). Subsequent hybridization of the same filter with a β-actin specific cDNA probe confirms that equal amounts of RNA were loaded in each lane (panel B).

To investigate whether there is an involvement of labile repressor molecules, treatment of hybrid cells with the protein synthesis inhibitor cycloheximide (CHX) should lead

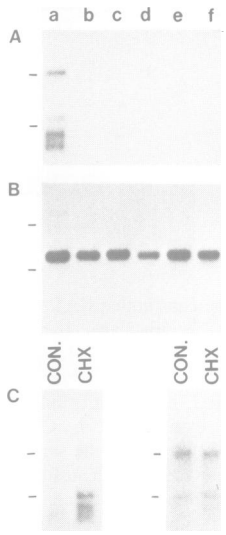

Fig. 5. Northern blot analysis of CAT transcription in SiHa cells and in somatic cell hybrids. Approximately 1.5 μg poly(A)$^+$ selected mRNA was size-fractionated on a 1% agarose gel and transferred to GeneScreen plus filter. Panel ***A***: hybridization to a ^{32}P-labeled CAT probe. Panel **B**: the same filter was used for consecutive hybridization to a ^{32}P-labeled β-actin probe. Lane a: RNA from SiHa cells. Lane b: RNA from HaCaT keratinocytes. Lanes c–f: RNAs from four representative hybrid clones made between SiHa and HaCaT cells. Panel **C**: reactivation of CAT transcription in SiHa × HaCaT cells after treatment with 75 μg/ml cycloheximide for 4 h. Approximately 3 μg of poly(A)$^+$ selected RNA from control (CON.) and cycloheximide (CHX) treated cells were loaded in each lane. Left: hybridization with a CAT-specific probe. Right: control hybridization of the same filter using a genomic probe for the ribosomal RNA.

to a reactivation of the corresponding CAT transcripts. To prove this assumption, SiHa × HaCaT clones were treated with 75 μg/ml CHX for 4 h and the RNA was subjected to Northern blot analysis. Figure 5 (panel C, left side) shows the result of a representative hybrid clone, demonstrating a strong reactivation of CAT transcription after CHX treatment. Control hybridization with a genomic DNA probe homologous to ribosomal RNA (right side of panel C) reveals the application of equal amount of mRNA. These data suggest that inhibition of protein synthesis leads to a removal of labile proteins which negatively interfere with pH18CAT expression in SiHa × HaCaT hybrids. Due to the tight linkage between HPV expression and cell proliferation (von Knebel Doeberitz *et al.*, 1988) there is no significant reduction of the SiHa HPV16 transcription by comparing viral gene expression in hybrid clones and in non-fused cervical carcinoma cells (unpublished data). This result is in good agreement with observations made in other hybrid systems (Stanbridge, 1984) also demonstrating the maintenance of the initial HPV18 mRNA steady state level in HeLa × fibroblast or keratinocyte hybrid clones under *in vitro* cultivation conditions (Schwarz *et al.*, 1987; Rösl *et al.*, 1988).

In order to ascertain that CAT extinction is mediated by non-tumorigenic keratinocytes as fusion partners rather than by the fusion event itself, SiHa × SiHa hybrids were established. Utilizing the same strategy, 'universal fuser' clones were PEG treated with non-modified SiHa cells and subsequently selected in HAT/G418 medium. In contrast to SiHa × HaCaT clones (see Figure 2 for comparison, the

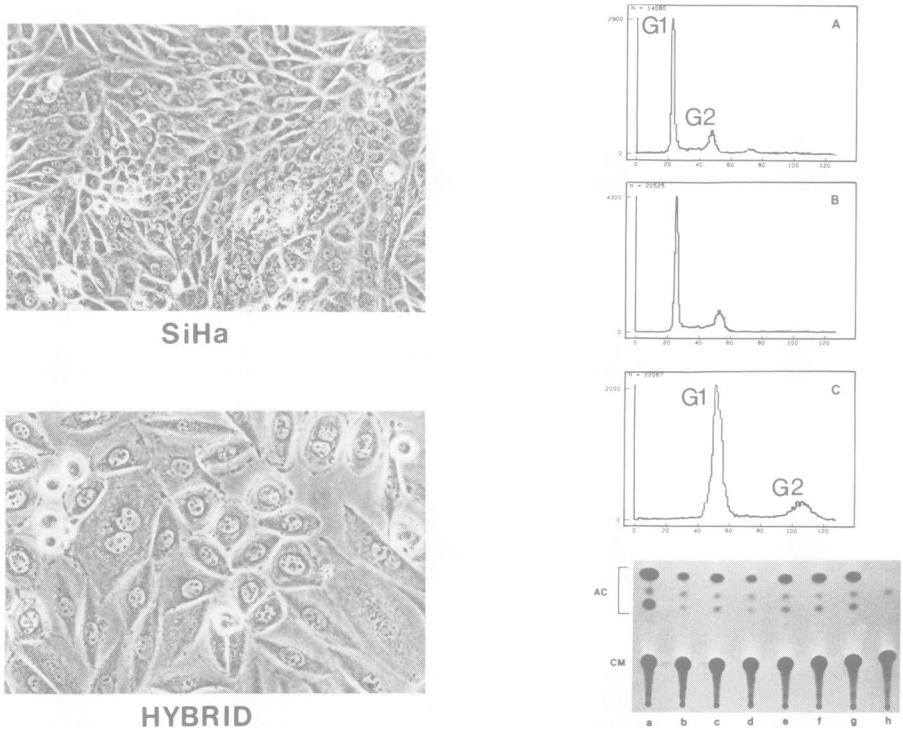

Fig. 6. CAT expression is not extinguished in SiHa × SiHa hybrids. **Left:** microscopic examinations of the cell morphology of the parental SiHa cells in comparison with one representative SiHa × SiHa hybrid clone. Magnification 250×. **Right**: flow cytometric DNA frequency distributions and cell cycle analysis of the parental and hybrid cell lines. Abscissa: relative fluorescence intensities of DAPI stained cell nuclei, directly corresponding to the DNA content per cell. Ordinate: number of counted cells. Panel **A**: 'universal fuser' clone. Panel **B**: non-modified SiHa cells. Panel **C**: SiHa × SiHa hybrid cells. The positions of the G_1 and G_2 peaks of the cell cycle are indicated. Lower panel; monitoring the CAT activity of the 'universal fuser' cells before the fusion event (lane a) in comparison with six SiHa × SiHa hybrid clones (lanes b–g) using 50 μg of total cellular protein. Lane h: negative control, HaCaT cell extract supplemented with [^{14}C]chloramphenicol (CM). The reactions were performed for 2 h at 37°C. The positions of the acetylated forms of [^{14}C]chloramphenicol are indicated (AC).

parental SiHa cells and the resulting SiHa × SiHa hybrids reveal essentially the same cell morphology. The hybrid nature of the selected clones already becomes apparent due to the higher nucleus/cytoplasm ratio, being considerably larger than the non-fused parental cells (Figure 6, left side). To confirm the hybrid state in this case, RFLP analysis is inherently more difficult, since both fusion partners have the same chromosomal complement. This can be circumvented by measuring the DNA content of the hybrid clones in comparison with the parental SiHa cells by flow cytometry. Monitoring the distribution of the DNA amount relative to the cell cycle, the flow cytometric profiles of the 'universal fuser' clones (Figure 6, right, panel A) and of the non-modified SiHa cells (panel B) are nearly identical. As illustrated in panel C, the G_1 peak (n number of chromosomes) of one representative SiHa × SiHa clone exactly coincides with the G_2 peak ($2n$ number of chromosomes) of the non-fused parental cells, confirming the tetraploid state and the hybrid nature of the HAT/G418 selected cells. Testing for CAT activity demonstrates that in contrast to the fusions made with HaCaT keratinocytes, the expression of the marker gene is not significantly affected in SiHa × SiHa hybrids (lower panel, lanes b–g). The slight reduction of the CAT signal in comparison to the non-fused positive control (lane a) probably results from a dilution effect of the CAT gene product in cellular extracts of tetraploid hybrids using equal amounts of protein for incubation (see Figure 4).

The cytokeratin expression pattern of HaCaT cells is preferentially conserved in somatic SiHa × HaCaT hybrids

The morphological resemblance of the SiHa × HaCaT clones to the parental HaCaT cells (see Figure 2) and the extinction of the HPV18 URR-controlled reporter gene suggest that the keratinocyte phenotype is dominant in somatic cell hybrids. To investigate whether this is also true for keratinocyte specific differentiation traits, we examined the expression of the cytokeratins (CKs) being regarded as useful markers for epithelial cell differentiation (Franke and Moll, 1988). Two-dimensional polyacrylamide gel electrophoresis reveals that the tumorigenic SiHa cells have a simple CK profile, producing only CKs 7, 8, 18 and 19, respectively (Figure 7, left, panel A). This expression pattern is very similar to that found in the HPV18 positive cervical carcinoma cell line HeLa (Franke *et al.*, 1981) and in SV40 transformed human keratinocytes (Banks-Schlegel and Howley, 1983). In contrast, the parental HaCaT cells display a much more complex composition of cytoskeletal proteins (panel B). In addition to the keratins present in SiHa cells, CKs 4, 5, 6, 13, 14 and 16 respectively can be discerned, which is consistent with data published by Boukamp *et al.* (1988). The CK production in somatic cell hybrids (see Table I for summary), despite slight variations in their extent of synthesis, essentially resembles the protein profile detected in HaCaT cells (panel C).

CK expression was further analyzed within individual

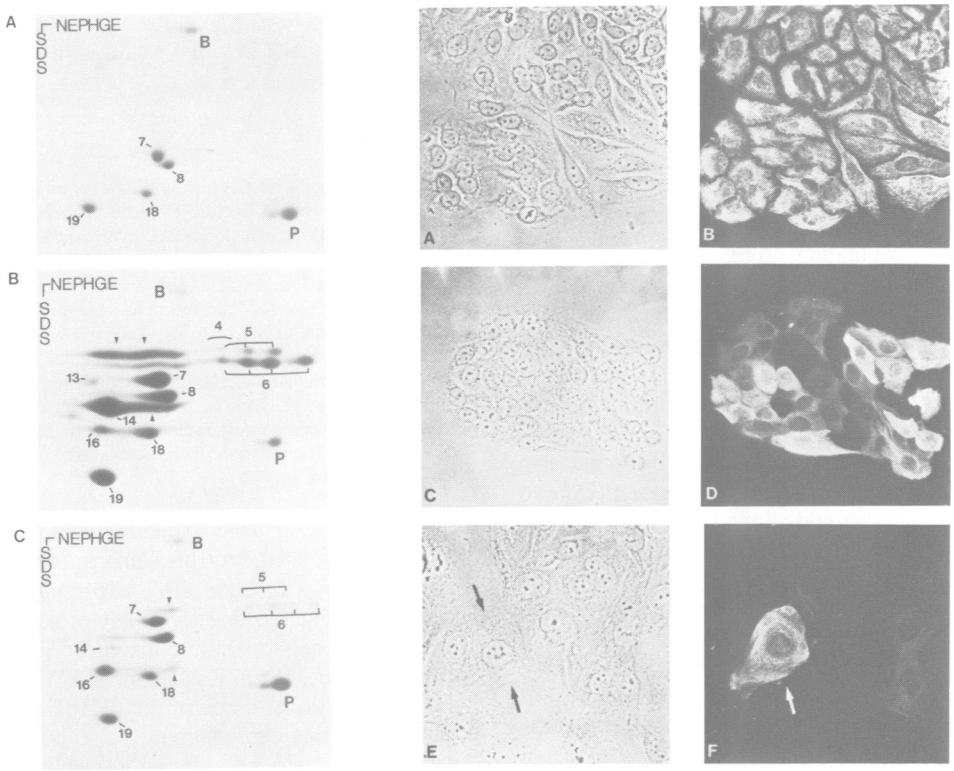

Fig. 7. Expression of cytokeratins in parental SiHa/HaCaT cells in comparison with the hybrid clones. **Left**: two-dimensional polyacrylamide gel electrophoresis analysis of cytoskeletal polypeptide composition obtained from SiHa (A), HaCaT (B) and one representative hybrid clone, F11 (C). First dimension: non-equilibrium pH gradient gel electrophoresis (NEPHGE). Second dimension: SDS–PAGE. Marker proteins used: bovine serum albumin (B); 2-phosphoglycerolkinase from yeast (P). The cytokeratins are numbered according to the Moll nomenclature (Moll *et al.*, 1988). The arrowheads indicate cytokeratin complexes which were not completely separated. **Right**: immunofluorescence and phase contrast microscopy of SiHa (A, B), HaCaT (C, D) and one hybrid clone (F11, E, F). Panel **B**: SiHa cells homogeneously stained for CK 8 using the antibody Ks8.1.42 which demonstrates their genuine epithelial character. Panel **D**: HaCaT cells showed a heterogeneous expression pattern for CK 4 as detected by the monoclonal antibody 6 B10. Panel **F**: a single hybrid cell (F11) reveals bright staining with the CK 4 specific antibody (indicated by arrows).

Table I. Expression of CK polypeptides in somatic cell hybrids after fusion of HaCaT and SiHa cells[a]

Cells	Cytokeratin polypeptide no.																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Parental cell lines																			
HaCaT				(+)	+	+	+	+					(+)	+		+		+	+
SiHa							+	+										+	+
Cell hybrid clones																			
F-1					+	+	+	+						+		+		+	+
F-2					+	+	+	+						+		+		+	+
F-5					+	+	+	+						+		+		+	+
F-7					+	+	+	+						+		+		+	+
F-11				$+^{tr}$	+	+	+	+						+		+		+	+

[a]As determined by two-dimensional PAGE and immunofluorescence microscopy. +, main components; (+), minor componets; $+^{tr}$, trace amount.

cells using immunofluorescence microscopy. While HaCaT keratinocytes (see Table I) and SiHa cells (Figure 7, right, panels A and B) were uniformly stained with a CK 8 monoclonal antibody specific for a simple epithelial phenotype (Franke *et al.*, 1981), CK 4, a protein commonly used as marker for stratification (van Muijen *et al.*, 1986), is not expressed in cervical carcinoma cells (see Table I), but is expressed with reduced frequency in non-tumorigenic HaCaT cells (panels C and D). Interestingly, one hybrid clone even maintains the expression of CK 4, indicating that some cells seem to be committed to local stratification (panels E and F).

Due to the conservation of these differentiation markers of the non-tumorigenic fusion partners, it can be concluded that the keratinocyte phenotype is also dominant in terms of cytoskeletal protein expression in SiHa × HaCaT fusion clones.

The extinguished pH18CAT marker gene can be reactivated after transient fusion with COS7 cells

To further substantiate that the extinction mechanism is mediated by negative regulating proteins, transient fusion experiments with SV40 large T antigen-expressing COS7 cells (Gluzman, 1981) were carried out. The rationale for this approach is based on the fact that our pH18CAT reporter construct was initially derived from $pA_{10}CAT_2$, a plasmid harboring the SV40 origin of replication (Laimins *et al.*, 1984). Since several reports claimed that integrated SV40 origin-containing plasmids were not only excised but also amplified in the presence of SV40 large T antigen (Conrad *et al.*, 1982), this strategy should allow a reactivation of the extinguished marker gene by intracellular competition for repressor molecules after high copy amplification of the template. The results of the transient fusion experiments are summarized in Figure 8 (panel A). Lanes a and d show that the mere co-cultivation of two independent, CAT extinguished hybrid clones with COS7 cells did not lead to any detectable CAT activity. However, the marker gene is reactivated in a time dependent manner 3 (lanes b and e) and 4 days (lanes c and f) after the fusion event. To demonstrate that the reactivation process is accompanied by DNA amplification, low mol. wt DNA was prepared 4 days after hybrid formation and transfected into competent bacteria. The appearance of resistant clones, in contrast to transfections of DNA extracted from the mixed controls, clearly confirms the excision and amplification process. Remarkably, testing individual colonies for expression of

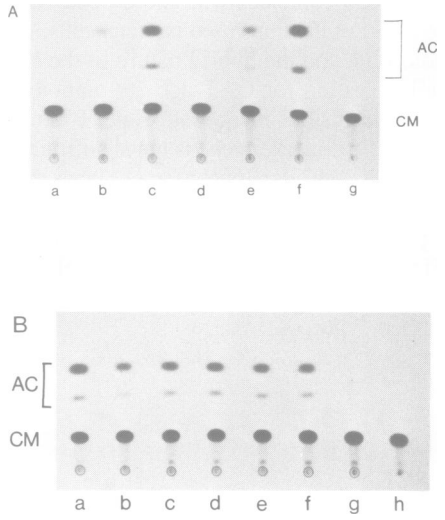

Fig. 8. Reactivation of CAT activity after transient fusion with COS7 cells and rescue of the excised DNA in bacteria. Panel **A**: CAT assay of two independent SiHa × HaCaT hybrids after co-cultivation for 4 days with COS7 cells (mixed controls, lanes a and d). CAT activity of the same clones 3 (lanes b, e) and 4 days (lanes c, f) after transient fusion with COS7 cells. The reactions were performed for 2 h at 37°C using 100 μg total cellular extract. Lane g: negative control, HaCaT extract supplemented with [^{14}C]chloramphenicol (CM). Panel **B**: CAT activity of individual bacterial clones harboring the rescued pH18CAT plasmid (lanes b–f). Lane a: positive control, constitutive expression in SiHa cells. Lane g: untransfected bacteria. Lane h: negative control (see panel A). The position of the acetylated [^{14}C]chloramphenicol (AC) is indicated.

a functional CAT gene (panel B, lanes b–f) in comparison with untransfected controls (lane g) and CAT^+ SiHa cells (lane a) shows that the CAT expression is maintained in bacteria despite the absence of any prokaryotic transcription initiation signals, a finding also described in other studies (Jenkins *et al.*, 1983; Kashanchi and Wood, 1989). Hence, the transcriptional block of the pH18CAT gene in SiHa × HaCaT hybrids can apparently also be overcome by gene amplification paralleled by an intracellular *in vivo* titration of negative regulatory factors.

Discussion

Previous experiments have shown that in contrast to the promoter/enhancer element of SV40, the URRs of HPV16 and HPV18 reveal rather low levels of transcriptional activity

in human fibroblasts or keratinocytes in transient transfection assays, whereas the same constructs were remarkably active in cervical carcinoma cells (Bernard *et al.*, 1989; Romanczuk *et al.*, 1990; see also Figure 1).

The absence of a significant signal using CAT reporter constructs might argue for the presence of negative regulating proteins directed against the HPV-specific control region, as already described for the promoter/enhancer element of polyoma virus (Sleigh, 1987) and the long terminal repeat of Moloney murine leukemia virus (Loh *et al.*, 1990) in rodent embryonal carcinoma cells. Alternatively, it may result from the absence of positive transcription factors which are required for the formation of a functional transcription complex, an observation which has been reported for the human papova JC virus in non-glial cells (Tada *et al.*, 1989).

To distinguish between these possibilities, we introduced an HPV18 URR-driven reporter plasmid (pH18CAT) into the HPV16 positive cervical carcinoma cell line SiHa (Baker *et al.*, 1987; El Awady *et al.*, 1987) to ensure constitutive gene expression of the construct. This approach allows direct examination of pH18CAT regulation before and after the fusion event and circumvents the problems encountered in transient transfection assays, such as titration effects for *trans*-acting effector molecules (Gorman *et al.*, 1985), variations in DNA topology or improper chromatinization of the template (Weintraub *et al.*, 1986). Moreover, since the CAT^+ SiHa clones can be used as 'universal fuser', stable hybrids can be isolated without further modification of the other fusion partner (Pereira-Smith and Smith, 1988).

Our results show that the HPV18 URR-controlled reporter gene, which is constitutively expressed in malignant SiHa cells, becomes extinguished after fusion with non-tumorigenic, spontaneously immortalized human keratinocytes (Figures 4 and 5), but remains unaltered using cervical carcinoma cells as fusion partners (Figure 6). It should be emphasized that CAT extinction was also noted after hybrid formation with primary human keratinocytes although the number of growing clones was extremely low (unpublished data). Here, most of the HAT/G418 selected clones senesced suggesting that immortality and transformation are recessive traits in these cellular hybrids (O'Brien *et al.*, 1986).

Further analysis of SiHa × HaCaT hybrids was performed by characterizing the expression pattern of the CKs, which are considered as useful markers for epithelial cell differentiation (Franke and Moll, 1988). Using two-dimensional gel electrophoresis and immunofluorescence microscopy, we noticed that the complex CK profile of HaCaT cells is mainly preserved in all investigated hybrids (Figure 7, see also Table I for summary). These observations indicate that the regulatory program of the non-malignant keratinocytes seems to be dominant over the phenotype of cervical carcinoma cells not only in terms of transcriptional down-regulation of the HPV18 URR-controlled reporter gene but also in the maintenance of CK expression in SiHa × HaCaT fusion clones. These results are in accord with comparative studies made in other hybrid systems also showing a conservation of most of the differentiation traits of the non-tumorigenic fusion partner (for review, see Harris, 1990).

Previous investigations have uncovered several potential mechanisms for the down-regulation of a constitutively expressed gene after somatic cell hybridization. Extinction can be mediated either by direct interaction of negatively regulating proteins with specific *cis*-acting DNA target sequences (Lenardo *et al.*, 1989; Junker *et al.*, 1990). Alternatively, this process can also operate indirectly via a down-regulation of positive transcription factors (Avvedimento *et al.*, 1988), or both mechanisms can even coincide in the same cellular environment (Tripputi *et al.*, 1988). Moreover, *de novo* methylation is also a regulatory way for transcriptional silencing of a particular marker gene (Zaller *et al.*, 1988).

Using the *MspI*−*HpaII* isoschizomer assay (McClelland, 1981), *de novo* methylation at least at 5′-CCGG-3′ sequence stretches can be excluded since there are no differences of the CAT gene *MspI*−*HpaII* digestion patterns in non-fused SiHa cells in comparison with stable hybrids (unpublished data). Furthermore, the absence of transcriptional activity of pH18CAT in HaCaT cells under transient transfection conditions (Figure 1) also argues against this mechanism.

The reappearance of CAT-specific gene expression after CHX treatment (Figure 5, panel C) rather favors the interpretation that negative *trans*-acting regulatory factors are directly involved in the extinction process. Moreover, taking advantage of the fact that the reporter construct harbors a functional SV40 origin of replication (Laimins *et al.*, 1984), transient fusion of the CAT^- hybrids with SV40 large T antigen-expressing COS7 cells (Gluzman, 1981) results in excision and episomal amplification of the template (Conrad *et al.*, 1982). This strategy allows an intracellular titration of putative negative regulators by DNA amplification (Nir *et al.*, 1986). On the other hand, it has also been reported that SV40 large T antigen can trans-activate the URR of HPV18 (Thierry *et al.*, 1987). However, such a process cannot solely account for the strong reactivation of the reporter construct after transient fusion with COS7 cells, since this would imply a competition effect between positive and negative proteins. The time-dependent re-expression of the extinguished marker gene and the accumulation of episomal DNA (Figure 8) rather suggests a direct competition of the amplified DNA with CHX labile repressor molecules at the viral regulatory control region of HPV18.

Although it has been demonstrated that the transforming proteins E6 and E7 of HPV16/18 interact with presumptive tumor suppressor gene products p53 and p105-Rb (Dyson *et al.*, 1989; Werness *et al.*, 1990), this association does not explain the suppression of the tumorigenic phenotype after microcell transfer of chromosome 11 (Saxon *et al.*, 1986; Koi *et al.*, 1989) in cervical carcinoma cells. If there is an involvement of these genes in tumor suppression and negative growth regulation of cervical carcinoma cells, then it seems to be only indirect, since p53 and the p105-Rb retinoblastoma genes are located on different chromosomes (for review, see Sager, 1989). Moreover, the selective absence of HPV18 transcription in non-tumorigenic HeLa × fibroblast hybrids and of HPV16 gene expression in immortalized keratinocytes after inoculation in nude mice (Bosch *et al.*, 1990; Dürst *et al.*, 1991) provides evidence for HPV gene suppression at the level of initiation of transcription rather than by a post-translationally acting mechanism like titration of viral oncogenes by cellular suppressor genes.

Since there is little information concerning negative transcriptional regulation at *cis*-essential DNA target sequences within the viral control region of human papil-

lomaviruses, our experimental system provides an initial attempt to study such a regulatory pathway in greater detail.

Materials and methods

Plasmids

The complete URR of HPV18 (Boshart *et al.*, 1984; Cole and Danos, 1987) was subcloned in the sense orientation as a 1053 bp *Bam*HI fragment at the *Bgl*II site of $pA_{10}CAT_2$ to generate pH18CAT. The vector $pA_{10}CAT_2$ contains the SV40 early promoter and the origin of replication, but lacks the 72 bp repeat enhancer element (Laimins *et al.*, 1984). pBLCAT3 contains the CAT gene without any eukaryotic regulatory signals (Luckow and Schütz, 1987). The plasmid pAG60 harbors the kanamycin resistance gene driven by the HSVtk promoter, and confers G418 resistance to eukaryotic cells (Colbère-Garapin *et al.*, 1981).

Cell lines and the establishment of 'universal fuser' clones

The HPV16 positive cervical carcinoma cell SiHa (Baker *et al.*, 1987; El Awady *et al.*, 1987), the spontaneously immortalized, non-tumorigenic human keratinocyte cell line HaCaT (Boukamp *et al.*, 1988) and the SV40 positive COS7 cells (Gluzman, 1981) were maintained in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum, 1% penicillin, streptomycin, respectively.

To establish 'universal fuser' clones from SiHa cells, four 75 cm^2 tissue culture flasks, each inoculated with 2×10^6 cells were first challenged for growth in the presence of 100 μM 6-thioguanine (6-TG, Sigma) to select for an HPRT deficient phenotype (Caskey and Kruh, 1979). After 4 weeks, the 6-TG resistant clones were pooled, expanded and transfected with the HPV18-driven CAT gene (pH18CAT) together with a dominant selection marker (pAG60) in a 10:1 ratio using the calcium phosphate precipitation technique (Wigler *et al.*, 1978). The transfected, HPRT deficient SiHa cells were subsequently replated at a density of 5×10^5 cells/10 cm dish in medium containing 1 mg/ml G418 (Geneticin, GIBCO) and 100 μM 6-TG. After 4 weeks, 25 clones were picked and individually tested in HAT medium (Sigma) for a stable HPRT deficient phenotype. Those G418 clones which showed no reversion of the HPRT activity (no growth in HAT medium) were finally assayed for constitutive CAT expression. In total 4 CAT^+ 'universal fuser' SiHa clones were obtained harboring a dominant (G418 resistance) and a recessive (inactive HPRT gene) marker. Two of them were used for stable hybrid formation with human keratinocytes (HaCaT) and unmodified SiHa cells.

Cell fusions

Fusions between the different cell types were done essentially as described (Davidson and Gerald, 1976). Briefly, 1×10^6 'universal fuser' cells were mixed with the same amount of the corresponding fusion partner and seeded in 60 mm dishes. After two washes with phosphate-buffered saline (PBS), lacking Mg^{2+} and Ca^{2+}, the cells were fused with 37°C prewarmed polyethylene glycol (PEG 1500, Boehringer) for exactly 1 min and washed three times with PBS. To reduce the PEG cytotoxicity (Schneiderman *et al.*, 1979) the cells were kept in PBS for 20 min at room temperature and incubated ovrernight in fresh medium. The following day, the cells were replaced at low density and challenged for growth in HAT/G418 medium to select for stable hybrids. The corresponding controls (mixed controls) were not treated with PEG but were processed in the same way. For transient fusions to recover the extinguished marker gene, 2×10^5 CAT^- SiHa × HaCaT hybrids were mixed with 1×10^6 COS7 cells and fused as described above. The PEG treated cells as well as the mixed controls were harvested after 3 and 4 days to monitor for reactivated CAT activity.

Transient transfections and CAT assays

DNA transfections for transient CAT expression assays were performed according to the method of Chen and Okayama (1987) using 1×10^6 recipient cells per 10 cm plate. To correct the transfection efficiencies on SiHa and HaCaT keratinocytes, pBLCAT3 (as a negative control) and pH18CAT plasmids were cotransfected with a luciferase gene, which is under the control of a β-actin promoter. Two days later, the cells were harvested by three cycles of freezing and thawing to monitor the luciferase activity (deWet *et al.*, 1987). The cellular extracts were standardized for CAT assays (Gorman *et al.*, 1982) using equal amounts of luciferase counts. Since the CAT gene is integrated in both the cellular hybrids and the 'universal fuser' SiHa clones, stable CAT expression was tested using equal protein concentrations. The protein content of the cellular extracts was determined as published (Kalb and Bernlohr, 1977).

Cytokeratin analysis

The preparation of the cytoskeletal proteins from the parental SiHa/HaCaT cells and the corresponding hybrids was accomplished by sequential salt extraction exactly as described (Achtstätter *et al.*, 1986). The CKs were analyzed by two-dimensional gel electrophoresis (O'Farrell *et al.*, 1970) with minor modifications (Achtstätter *et al.*, 1986). For examination of the CK expression pattern by immunofluorescence microscopy, the cells were grown on glass coverslips, fixed in methanol (−20°C) and rinsed six times with acetone (−20°C). The procedures for single and double staining were carried out as published (Achtstätter *et al.*, 1985). The following murine monoclonal antibodies were used: Ks18.174, Ks18.18, both directed against CK 18 (Moll *et al.*, 1988); Ks8.1.42, reacting specifically with CK 8 (Franke and Moll, 1988); Ks19.1(A53-B/A2) recognizing CK 19 (Karsten *et al.*, 1985); 6 B10, specific for CK 4 (van Muijen *et al.*, 1986) and Ks13.1 reacting with CK 13 (Moll *et al.*, 1988). Secondary anti-murine goat raised antibodies were obtained commercially as fluorescein isothiocyanate (FITC) or Texas Red conjugates from Dianova (Hamburg, FRG).

DNA and RNA analysis

Genomic DNA was prepared by standard methods (Sambrook *et al.*, 1989), cleaved with the appropriate restriction enzymes and processed for Southern blot analysis as described (Rösl *et al.*, 1989). Total cellular RNA was isolated according to the method of Chomczynski and Sacchi (1987), the corresponding poly(A)$^+$ fraction was enriched by oligo(dT)−cellulose chromatography (Aviv and Leder, 1972) and subjected to Northern blot analysis (Khandijan and Meric, 1986). The GeneScreen plus filters (NEN) were hybridized with specific DNA probes and labeled by random priming (Feinberg and Vogelstein, 1984).

Plasmid rescue and bacterial transformation

Low mol. wt DNA derived from the transient fusion experiments of CAT extinguished SiHa × HaCaT hybrids with COS7 cells prepared by scraping the cells from the plates and resuspending them in 300 μl PBS. Aliquots (100 μl) were removed, denatured by gently mixing with 200 μl of 0.2 M NaOH/1% SDS. After 5 min incubation on ice, 150 μl of 5 M potassium acetate (pH 4.8) was added for neutralization (Ish-Horowicz and Burke, 1981). After additional incubation on ice for 5 min, the precipitated proteins, dodecylsulfate and chromosomal DNA were removed by centrifugation. The supernatant was phenol/chloroform extracted, precipitated with ethanol and the recovered DNA was used for transfection on competent HB101 bacteria (Dagert and Ehrlich, 1979). Transformed bacteria were isolated on ampicillin plates and assayed for CAT expression exactly as described by Gorman *et al.* (1982). Parallel colonies were expanded and processed to isolate the rescued pH18CAT plasmids.

Flow cytometry

To confirm the tetraploid state of SiHa × SiHa hybrids, the cells were washed twice in PBS, resuspended in 1 ml of 0.9% NaCl solution and immediately injected in 10 ml of ethanol for fixation. After 1 h, the cells were recovered and stained with the AT specific dye DAPI (4,6-diamidino-2-phenylindol, Serva) as described (Stöhr, 1976). Flow cytometry analysis was carried out with the cytofluorograph 30-L (Ortho Diagnostic Systems), connected to a personal computer (Compac, Deskpro 386/16). Data acquisition, real time display, cell cycle analysis and graphical documentation were realized in the 'C' language. The interface between flow cytometer and personal computer is the DT-2828 board (data translation; Futterman and Stöhr, 1989). The UV lines (351 and 364 nm) of an organ laser (Spectra Physics 2025-055) were used for DAPI excitation. DAPI fluorescence was collected through a low pass filter at 450 nm.

DNA probes for hybridization

The following recombinant plasmids were used in this study: pHPV16 contains the full-length HPV16 DNA cloned at the *Bam*HI site (Dürst *et al.*, 1983); pEJras contains the 6.6 kb *Bam*HI fragment of the human Ha-rasI proto-oncogene (Parada *et al.*, 1982); pHFβ-A1, harboring an approximately full-length cDNA *Bam*HI insert of the human fibroblast β-actin gene (Gunning *et al.*, 1983). pRNA represents a genomic *Hin*dIII fragment of the ribosomal transcription unit of *Xenopus laevis* (Rungger *et al.*, 1979). The CAT gene was isolated from pBLCAT (Luckow and Schütz, 1987) after *Eco*RI digestion. The corresponding inserts were excised from their cloning vector, gel purified (Tautz and Renz, 1983) and used as hybridization probes.

Acknowledgements

We thank P.Boukamp for providing the HaCaT cells, L.Turek for the pH18CAT construct, and M.Rentrop and H.Lange for the luciferase reporter

plasmid. We also thank B.Klevenz for excellent technical assistance, and L.Gissmann, J.Kleinschmidt and J.Schlehofer for critical reading of the manuscript. We are grateful to K.Zawatzky and K.Freund for secretarial help, and R.Webler for the photographic work. This work was supported by the Deutsche Forschungsgemeinschaft (Rö. 784/1-1).

References

Achtstätter,T., Moll,R., Moore,B. and Franke,W.W. (1985) *Exp. Cell Res.*, **173**, 17–35.
Achtstätter,T., Hatzfeld,M., Quinlan,R.A., Parmelee,D.C. and Franke,W.W. (1986) *Methods Enzymol.*, **134**, 355–371.
Aviv,H. and Leder,P. (1972) *Proc. Natl. Acad. Sci. USA*, **64**, 1408–1412.
Avvedimento,A.V., Musti,A., Fusco,A., Bonapace,M.J. and Dilauro,R. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 1744–1748.
Baker,C.C., Phelps,W.C., Lindgren,V., Braun,M.J., Gonda,M.A. and Howley,P.M. (1987) *J. Virol.*, **61**, 962–971.
Banks-Schlegel,S.P. and Howley,P.M. (1983) *J. Cell Biol.*, **96**, 330–337.
Bernard,B.A., Bailly,C., Lenoir,M.C., Darmon,M., Thierry,F. and Yaniv,M. (1989) *J. Virol.*, **63**, 4317–4324.
Bosch,F.X., Schwarz,E., Boukamp,P., Fusenig,N.E., Bartsch,D. and zur Hausen,H. (1990) *J. Virol.*, **64**, 4743–4754.
Boshart,M., Gissmann,L., Ikenberg,H., Kleinheinz,A., Scheurlen,W. and zur Hausen,H. (1984) *EMBO J.*, **3**, 1151–1157.
Boukamp,P., Petrussevska,R.T., Breitkreutz,D., Hornung,J., Markham,A. and Fusenig,N.E. (1988) *J. Cell Biol.*, **106**, 761–771.
Caskey,C.T. and Kruh,G.D. (1979) *Cell*, **16**, 1–9.
Chandler,L.A., Ghazi,H., Jones,P.A., Boukamp,P. and Fusenig,N.E. (1987) *Cell*, **50**, 711–717.
Chen,C. and Okayama,H. (1987) *Mol. Cell. Biol.*, **7**, 2745–2752.
Chomczynski,P. and Sacchi,N. (1987) *Anal. Biochem.*, **162**, 156–159.
Colbère-Garapin,F., Hordoniceanu,F., Kourilsky,P. and Garapin,A.C. (1981) *J. Mol. Biol.*, **150**, 1–14.
Cole,S.T. and Danos,O. (1987) *J. Mol. Biol.*, **193**, 599–608.
Conrad,S.E., Liu,C.P. and Botchan,M.R. (1982) *Science*, **218**, 1223–1225.
Dagert,M. and Ehrlich,S.D. (1979) *Gene*, **6**, 23–26.
Davidson,R.L. and Gerald,P.S. (1976) *Somatic Cell Genet.*, **2**, 165–176.
deWet,J.R., Wood,K.V., Deluca,M., Helinski,D.R. and Subramani,S. (1987) *Mol. Cell. Biol.*, **7**, 725–737.
Dürst,M., Gissmann,L., Ikenberg,H. and zur Hausen,H. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 3812–3815.
Dürst,M., Petrussevska,R.T., Boukamp,P., Fusenig,N.E. and Gissmann,L. (1987) *Oncogene*, **1**, 251–256.
Dürst,M., Bosch,F.X., Glitz,D., Schneider,A. and zur Hausen,H. (1991) *J. Virol.*, **65**, 796–804.
Dyson,N., Howley,P.M., Münger,K. and Harlow,E. (1989) *Science*, **243**, 934–937.
El Awady,M.K., Kaplan,J.B., O'Brien,S.J. and Burk,R.D. (1987) *Virology*, **159**, 389–398.
Feinberg,A. and Vogelstein,B. (1984) *Anal. Biochem.*, **137**, 266–267.
Franke,W.W. and Moll,R. (1988) *Differentiation*, **36**, 145–163.
Franke,W.W., Weber,K., Osborn,M., Schmid,E. and Freudenstein,C. (1978) *Exp. Cell Res.*, **116**, 429–445.
Franke,W.W., Schiller,D.L., Moll,R., Winter,S., Schmid,E., Engelbrecht,I., Denk,H., Krepler,R. and Platzer,B. (1981) *J. Mol. Biol.*, **153**, 933–945.
Futterman,G. and Stöhr,M. (1989) *PC Applications in Flow Cytometry: Hardware and Software Architecture*. Flow-Cytometric Symposium, Heidelberg.
Gluzman,Y. (1981) *Cell*, **23**, 175–182.
Gorman,C.M., Moffat,L.F. and Howard,B.H. (1982) *Mol. Cell. Biol.*, **2**, 1044–1051.
Gorman,C.M., Rigby,P.W.J. and Lane,D.P. (1985) *Cell*, **42**, 519–526.
Gunning,P., Ponte,P., Okayama,H., Engel,J., Blau,H. and Kedes,L. (1983) *Mol. Cell. Biol.*, **3**, 787–795.
Harris,H. (1990) *J. Cell Sci.*, **97**, 5–10.
Holliday,R. (1987) *Science*, **238**, 163–170.
Ish-Horowicz,D. and Burke,J.F. (1981) *Nucleic Acids Res.*, **9**, 2989–2998.
Jenkins,F.J., Howett,M.K. and Rapp,F. (1983) *J. Virol.*, **45**, 478–481.
Jones,P.A. (1985) *Cell*, **40**, 485–486.
Junker,S., Pedersen,S., Schreiber,E. and Matthias,P. (1990) *Cell*, **61**, 467–474.
Kalb,V.F. and Bernlohr,R.W. (1977) *Anal. Biochem.*, **82**, 362–371.
Karsten,U., Papsdorf,G., Roloff,G., Stolley,P., Abel,H., Walthers,J. and Weise,H. (1985) *Eur. J. Cancer Clin. Oncol.*, **21**, 733–740.
Kashanchi,F. and Wood,C. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 2157–2161.
Khandijian,E.W. and Meric,C. (1986) *Anal. Biochem.*, **159**, 227–232.
Koi,M., Morita,H., Yamada,H., Satoh,H., Barrett,J.C. and Oshimura,M. (1989) *Mol. Carcinogen.*, **2**, 12–21.
Krontiris,T.G., DiMartino,N.A., Colb,M. and Parkinson,D.R. (1985) *Nature*, **313**, 369–374.
Laimins,L.A., Gruss,P., Pozzatti,R. and Khoury,G. (1984) *J. Virol.*, **49**, 183–189.
Lenardo,M.J., Staudt,L., Robbins,P., Kuang,A., Mulligan,R.C. and Baltimore,D. (1989) *Science*, **243**, 544–546.
Luckow,B. and Schütz,G. (1987) *Nucleic Acids Res.*, **15**, 5490.
Loh,T.P., Sievert,L.L. and Scott,R.W. (1990) *Mol. Cell. Biol.*, **10**, 4045–4057.
McClelland,M. (1981) *Nucleic Acids Res.*, **9**, 5859–5866.
Moll,R., Achtstätter,T., Becht,E., Balcarova-Ständer,J., Ittensohn,M. and Franke,W.W. (1988) *Am. J. Pathol.*, **132**, 123–144.
Nir,U., Walker,M.D. and Rutter,W.J. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 3180–3184.
O'Brien,W., Stenman,G. and Sager,R. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 8659–8663.
O'Farrell,P.Z., Goodman,H.M. and O'Farrell,P.H. (1977) *Cell*, **12**, 1133–1137.
Parada,L.F., Tabin,C.J., Shih,C. and Weinberg,R.A. (1982) *Nature*, **297**, 474–478.
Pereira-Smith,O.M. and Smith,J.R. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 6042–6046.
Pirisi,L., Yasumoto,S., Feller,M., Doninger,J. and Di Paolo,J. (1987) *J. Virol.*, **61**, 1061–1066.
Ponder,B. (1988) *Nature*, **335**, 400–402.
Romanczuk,H., Thierry,F. and Howley,P.M. (1990) *J. Virol.*, **64**, 2849–2859.
Rösl,F., Dürst,M. and zur Hausen,H. (1988) *EMBO J.*, **7**, 1321–1328.
Rösl,F., Westphal,E.M. and zur Hausen,H; (1989) *Mol. Carcinogen.*, **2**, 72–80.
Rungger,D., Ackerman,H. and Crippa,M. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 3957–3961.
Sager,R. (1989) *Science*, **246**, 1406–1412.
Sambrook,J., Fritsch,E. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 2nd edition.
Saxon,P.J., Srivatsan,E.S. and Stanbridge,E.J. (1986) *EMBO J.*, **5**, 3461–3466.
Schneiderman,S., Farber,J.L. and Baserga,R. (1979) *Somatic Cell Genet.*, **5**, 263–269.
Schwarz,E., Schneider-Gädicke,A. and zur Hausen,H. (1987) In Brandsma,J. *et al.* (eds), *Papillomaviruses*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 47–53.
Sleigh,M.J. (1987) *Nucleic Acids Res.*, **15**, 9379–9395.
Smits,H.L., Raadsheer,E., Rood,J., Mehendale,S., Slater,R.M., van der Noordaa,J. and ter Schegget,J. (1988) *J. Virol.*, **62**, 4538–4543.
Smits,P.H.M., Smits,H.L., Jebbink,M.F. and ter Schegget,J. (1990) *Virology*, **176**, 158–165.
Stanbridge,E.J. (1976) *Nature*, **260**, 17–20.
Stanbridge,E.J. (1984) *Cancer Surveys*, **3**, 335–350.
Stöhr,M. (1976) *Pulse Cytophotometry*, **2**, 39–45.
Tada,H., Lashgari,M., Rappaport,J. and Khalili,K. (1989) *J. Virol.*, **63**, 463–466.
Tautz,D. and Renz,M. (1983) *Anal. Biochem.*, **132**, 14–19.
Thierry,F., Heard,J.M., Dartmann,K. and Yaniv,M. (1987) *J. Virol.*, **61**, 134–142.
Tripputi,P., Guerin,S.L. and Moore,D.D. (1988) *Science*, **241**, 1205–1207.
Van Muijen,G., Ruiter,D., Franke,W.W., Achtstätter,T., Haasnoot,W., Ponec,M. and Warnaar,S.O. (1986) *Exp. Cell Res.*, **162**, 97–113.
von Knebel Doebertiz,M., Oltersdorf,T., Schwarz,E. and Gissmann,L. (1988) *Cancer Res.*, **48**, 3780–3786.
Weintraub,H., Cheng,P.F. and Conrad,K. (1986) *Cell*, **46**, 115–122.
Werness,B.A., Levine,A.J. and Howley,P.M. (1990) *Science*, **248**, 76–79.
Wigler,M., Pellicer,A., Silverstein,A. and Axel,R. (1978) *Cell*, **14**, 725–731.
Zaller,D., Yu,H. and Eckhardt,L. (1988) *Mol. Cell. Biol.*, **8**, 1923–1939.
zur Hausen,H. (1977a) *Curr. Top. Microbiol. Immunol.*, **78**, 1–30.
zur Hausen,H. (1977b) *Behring Inst. Meth.*, **61**, 23–30.
zur Hausen,H. (1986) *Lancet*, **ii**, 489–491.
zur Hausen,H. (1989) *Cancer Res.*, **49**, 4677–4681.
zur Hausen,H. and Schneider,A. (1987) In Howley,P. and Salzman,N.P. (eds), *The Papovaviridae*. Plenum Press, New York, pp. 245–263.

Received on January 18, 1991; revised on February 21, 1991