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Differential regulation of papilloma virus early gene expression in transformed fibroblasts and carcinoma cell lines

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Treatment of bovine papilloma virus (BPV) 1-transformed mouse fibroblasts with cycloheximide led to a 10-fold increase in the amount of viral transcripts, after as little as 1 h of protein synthesis inhibition. Northern blots revealed no qualitative changes in the RNA pattern. Nuclear run-on experiments showed about a 7-fold increase in specific transcriptional activity after cycloheximide treatment. The half-life of BPV1 mRNA was twice as long as in untreated controls. These results indicate that both RNA synthesis and degradation of viral RNA are controlled by labile proteins. Cycloheximide stimulation turned out to be independent of the BPV1 E2 gene activity which enhances viral transcription. Cycloheximide treatment had no effect on the amount of human papilloma virus (HPV) 18 transcripts in cervical carcinoma derived HeLa and C4-1 cells. Transcription of HPV16 in the cervical carcinoma line SiHa was likewise unaffected. The differential regulation of transcription in transformed fibroblasts and cancer-derived cells, and the significance for malignant conversion are discussed.

Key words: bovine papilloma virus type 1/cervical cancer/gene expression/human papilloma viruses/transformation

Introduction

Papilloma viruses represent a heterogeneous group of infectious agents which induce a variety of tumors in skin and mucosa (Pfister, 1984). Individual types have been found to be associated with defined clinical and histological pictures. The proliferations are usually benign and regress spontaneously. Some lesions, however, progress to malignant tumors. This is well established for cottontail rabbit papilloma virus-induced acanthomas of domestic rabbits, which convert into carcinomas in 75% of the cases (Syverton, 1952). In man there exists a continuous spectrum of papilloma virus manifestations from papilloma virus-induced mild dysplasias of the cervix uteri to intra-epithelial neoplasia to invasive cervical cancer (Reid *et al.*, 1984). Human papilloma virus (HPV) 16 or 18 persist in ~60% of the malignant tumors (Dürst *et al.*, 1983; Boshart *et al.*, 1984). This points to a possible role of these viruses in malignant conversion.

Basic genetic experiments concerning the molecular mechanisms of oncogenic transformation were carried out with bovine papilloma virus (BPV)1 which induces fibropapillomas in cattle and fibromas in a number of alien species (Pfister, 1984). Transformation of fibroblasts could be readily reproduced *in vitro* (Dvoretzky *et al.*, 1980). Two independent genes, called E6 and E5, which map at the 5′ and 3′ ends of the early transcriptional unit, respectively, are involved in transformation (Schiller *et al.*, 1984, 1986). Only a few copies of virus-specific mRNA in BPV1-induced fibromas and in transformed cells can be demonstrated (Freese *et al.*, 1982; Heilman *et al.*, 1982; Jaureguiberry *et al.*, 1983). The most abundant species is transcribed from the genes E2 and E5 (Stenlund *et al.*, 1985). There is no transcription of genes coding for structural proteins.

Gene expression seems to play a crucial role in transformation. A clinically non-apparent persistence of multiple copies of papilloma virus DNA was observed in multimammate rats as long as there was no virus-specific mRNA detectable (Amtmann *et al.*, 1984). When tumors developed viral transcripts could be detected.

Most human cell lines established from cervical cancers harbor HPV16 or HPV18 DNA, which is usually expressed (Schwarz *et al.*, 1985; Pater and Pater, 1985; Yee *et al.*, 1985). In contrast to BPV1 DNA, which persists extrachromosomally (Law *et al.*, 1981), HPV DNA in carcinoma cell lines appears integrated in the cellular genome (Schwarz *et al.*, 1985). Integration consistently uncouples the 3′-located genes from the viral promotor. The proximal part of the early region covering the genes E6 and E7 is actively transcribed, however, giving rise to fusion transcripts containing both viral and cellular sequences (Schwarz *et al.*, 1985; F.O.Wettstein, personal communication).

The regulation of viral gene expression is only poorly understood so far. Quite unexpected in view of the low levels of viral RNA, the BPV1 genome revealed sequences which stimulate transcription in *cis* and *trans*. Using the chloramphenicol acetyltransferase test system, an enhancer element was mapped upstream of the early region, which can be stimulated in *trans* by the E2 gene product (Spalholz *et al.*, 1985). These stimulating elements are likely to be counteracted by negative control elements, which could act either as repressors of transcription or by accelerating the degradation of viral transcripts. To test for such proteins, we investigated papilloma virus transcripts after inhibition of protein synthesis, assuming that control elements may be subject to rapid turnover. In particular we compared BPV1-transformed fibroblasts and HPV-containing cell lines, which were established from human malignancies.

Results

Turn-over of papilloma virus transcripts after inhibition of protein synthesis

We examined BPV1-specific transcripts after inhibition of protein synthesis to see if proteins are involved in the down-regulation of papilloma virus gene expression. BPV1-transformed C127 cells (C127 B81) and DBA mouse embryo fibroblasts (MEF B84) were incubated with cycloheximide (25 μg/ml) for 1 h–12 h. Total cellular RNA was isolated from treated and untreated cells and analysed by the Northern blot technique using radiolabelled BPV1–DNA as a probe. The amount of viral transcript was strikingly increased after only 1 h inhibition of protein synthesis (Figure 1). No qualitative changes could be observed, however. Long exposure of lanes with RNA from controls revealed all minor transcripts which were detectable after accumulation caused by cycloheximide. No hybridization signals were observed after incubation with a radiolabelled probe specific

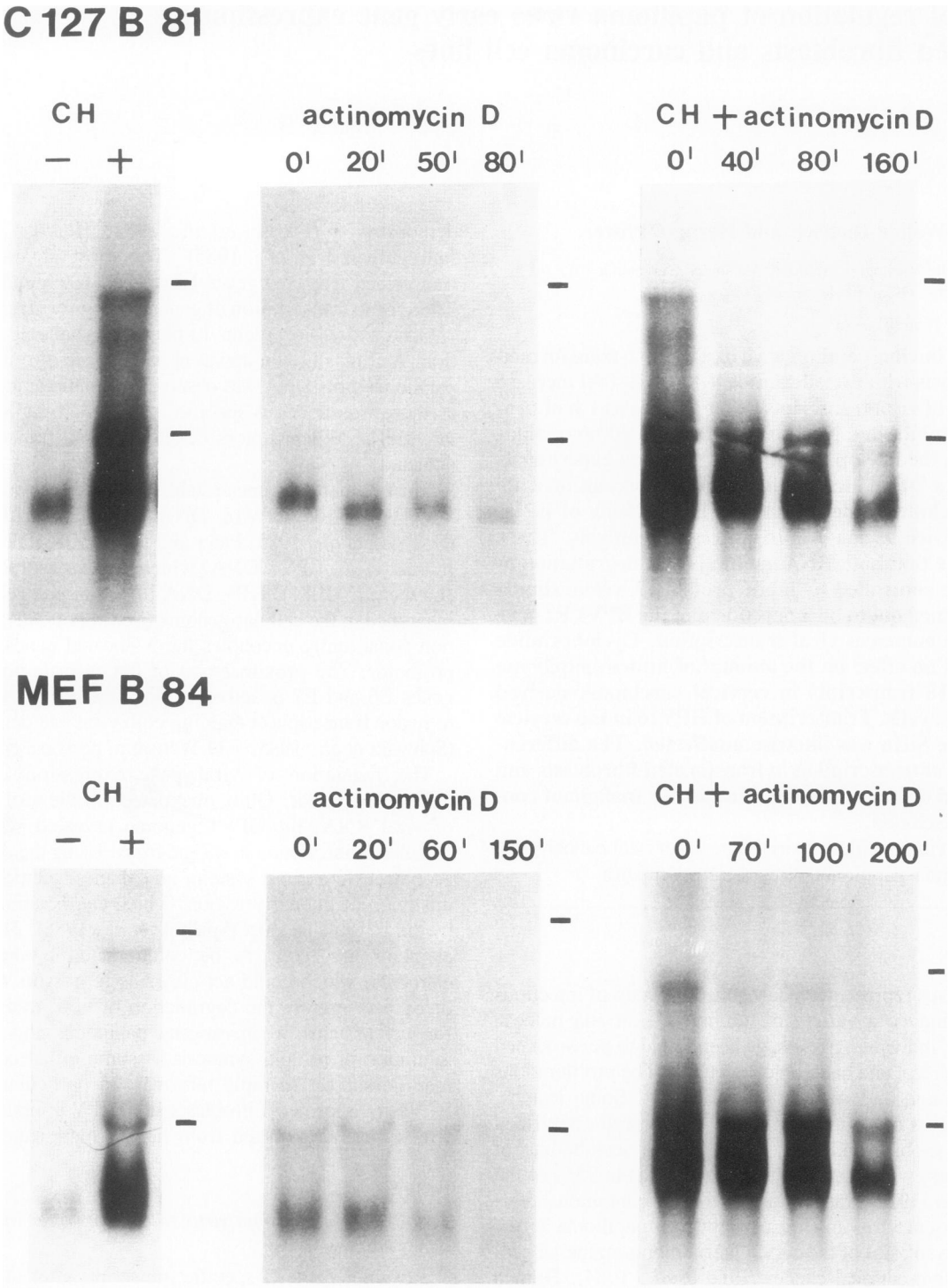

Fig. 1. Effect of protein synthesis inhibition on the amount and the half-life of viral transcripts in BPV1-transformed C127 cells (C127 B81) and DBA mouse embryo fibroblasts (MEF B84). Total RNA (30 μg) was electrophoresed under denaturing conditions, transferred to nitrocellulose and hybridized with nick-translated BPV1-specific DNA. Cells were grown in the absence (−) or presence (+) of cycloheximide (CH, 25 μg/ml for 1 h). Half-life was determined by chasing in the presence of actinomycin D for the time intervals indicated. For these experiments cycloheximide was added 1 h before actinomycin D. The bars indicate the positions of 18S (1.85 kb) and 28S (4.8 kb) ribosomal RNA.

for BPV1 genes encoding structural proteins. This confirmed that there was no switch from early to late gene expression.

The cycloheximide effect was quantitated by scanning the autoradiographic signals in the densitometer and by measuring the radioactivity of the corresponding regions on nitrocellulose filters. Finally, we determined the dilution of RNA from cycloheximide-treated cells which led to the same autoradiographic signal as RNA from untreated controls. The different approaches indicated a 10-fold increase of viral transcripts after inhibition of protein synthesis. Southern blot analysis of DNA from treated and untreated cells revealed no change in the level of viral DNA during 12 h under cycloheximide (data not shown).

These results suggest that the steady-state level of viral transcripts in BPV1-transformed cells is controlled by (a) rather

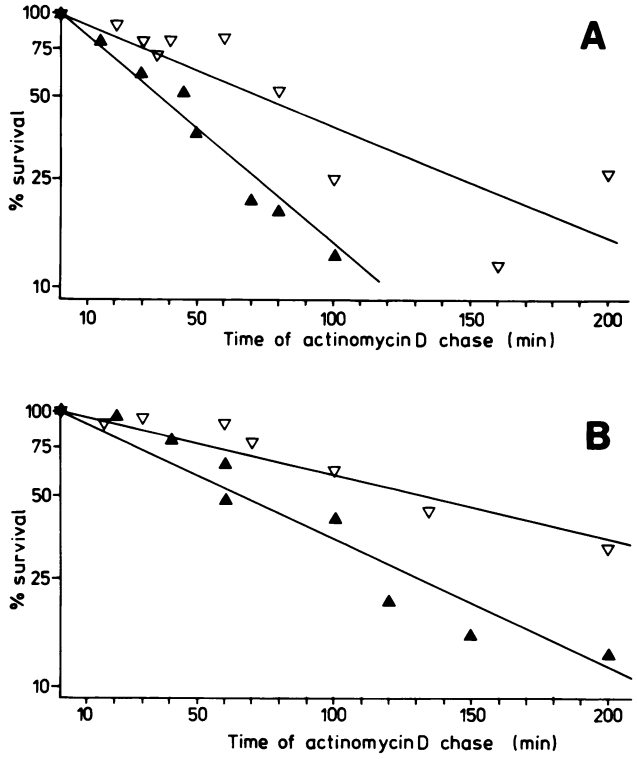

Fig. 2. Half-lives of viral transcripts in C127 B81 **(A)** and MEF B84 cells **(B)** in the absence of (▲) or presence (▽) of cycloheximide. The plot summarizes data from several experiments as shown in Figure 1. Quantitation of mRNAs was performed by densitometry scanning of the autoradiographs, using the zero time of actinomycin D treatment as a reference for 100% survival.

labile protein(s) with a half life of <1 h. The accumulation of BPV1-specific mRNAs after inhibition of protein synthesis could be caused by increased RNA synthesis, or by decreased RNA degradation. Therefore we determined the half-lives of viral transcripts before and after cycloheximide exposure.

Total RNA was extracted before and at various times after treatment with actinomycin D at doses of 6 μg/ml, which blocks all transcriptional activity. Cycloheximide was added 1 h before the actinomycin D chase. Virus-specific mRNA was displayed by Northern blot hybridization (Figure 1) and quantitated by densitometric scanning of the autoradiographs. According to the kinetic analyses the half-life of BPV1 mRNA was 35 min in C127 B81 cells and 65 min in MEF B84 cells. In both cases the half-lives were prolonged ~2-fold in the presence of cycloheximide (Figure 2 A and B).

The amount (A) of any given RNA depends on its synthesis ($A_S = K_S \times t$) and its degradation ($A_D = A_O \times e^{-K_D t}$). The degradation constant K_D is computed from the half-life T ($K_D = (\ln 2)/T$). The quantity of the RNA in the steady state is determined by the proportion of K_S and K_D ($A = K_S/K_D = K_S \times T/\ln 2$). This implies that the 2-fold prolongation of half-life cannot fully explain the cycloheximide effect. One might expect an additional effect on the synthesis of the viral mRNA. We therefore measured the *in vitro* transcriptional activity of nuclei, which were isolated from cycloheximide-treated C127 B81 cells and from untreated controls. Nuclear run-on products were hybridized to an excess of plasmid DNA specific for BPV1 or γ-actin, which was used as a control. As shown in Figure 3 there was a clear effect of cycloheximide on BPV1 transcription. A 7-fold increase was determined by measuring filter-bound

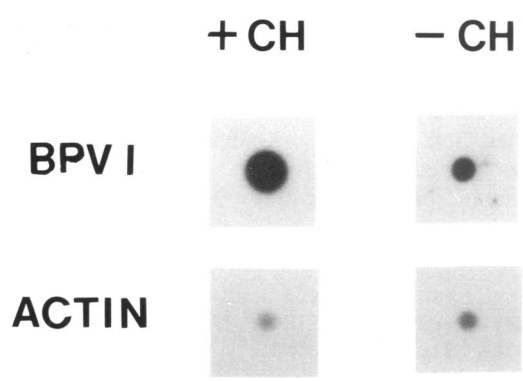

Fig. 3. Effect of cycloheximide on BPV1 and γ-actin gene transcription in isolated nuclei. Nuclei from C127 B81 cells either untreated (−CH) or incubated *in vivo* for 2 h in the presence of 25 μg/ml cycloheximide (+CH) were incubated with [^{32}P]UTP for elongation of nascent RNA chains. RNA was isolated and hybridized to nitrocellulose filters carrying dot spots (10 μg/dot) of denatured DNAs from pdBPV1 (Sarver *et al.*, 1982) and γ-actin (pHFγA-1; Gunning *et al.*, 1983).

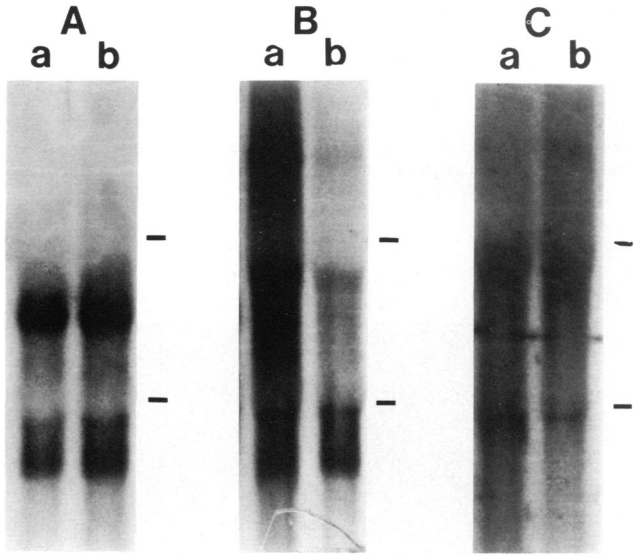

Fig. 4. Northern blot analysis of viral transcripts in cell lines derived from cervical carcinomas. HeLa **(A)**, C4-1 **(B)** and SiHa cells **(C)** were maintained in the absence (a) or in the presence of cycloheximide for 4 h (b). The filters were incubated with ^{32}P-labelled HPV18 **(A,B)** or HPV16-specific DNA **(C)**. The bars indicate the positions of 18S (1.85 kb) and 28S (4.8 kb) ribosomal RNA.

radioactivity in a liquid scintillation counter.

Cell lines which are persistently infected with human papilloma viruses, were also treated with cycloheximide to see if other papilloma viruses are subject to comparable control mechanisms. Both HeLa and C4-1 cells harbor HPV18 DNA whereas SiHa cells contain HPV16 DNA. The viral DNAs are actively transcribed (Schwarz *et al.*, 1985; Yee *et al.*, 1985). The amount of viral transcripts did not change after inhibition of protein synthesis by cycloheximide for up to 7 h (Figure 4).

Effect of the E2 gene on viral transcription

The human papilloma virus DNAs are integrated into the cellular genome in HeLa, C4-1 and SiHa cells. Open reading frame E2 is consistently affected by extensive deletions (Schwarz *et al.*, 1985; Pater and Pater, 1985). Because E2 was recently shown to play an active role in regulation of transcription, as mentioned above, we designed experiments to see if the E2 function is

necessary for the cycloheximide effect. A BPV mutant was constructed by insertion of *Xba*I linkers into the open reading frame E2 (Figure 5). The linker carries a TAG stop codon thus terminating translation of E2 within the amino-terminal moiety. Transfected into C127 cells, the mutant DNA showed only 20% focus-forming activity compared to wild-type BPV1 DNA. The foci did not morphologically differ from those induced by wild-type (Figure 6A) and the transformed cells formed colonies in soft agar. The viral genome was integrated in tandem orientation at multiple sites as deduced from cleavage analysis with several restriction enzymes (Figure 6B). In spite of comparable DNA copy numbers the amount of viral RNA was at least 10-fold reduced when compared to wild-type transformed cells (Figure 6C). Treatment with cycloheximide (25 μg/ml) for 2 h led to a 10-fold increase of viral mRNAs roughly restoring wild-type conditions. This result indicates that stimulation by cycloheximide is independent of E2 activity.

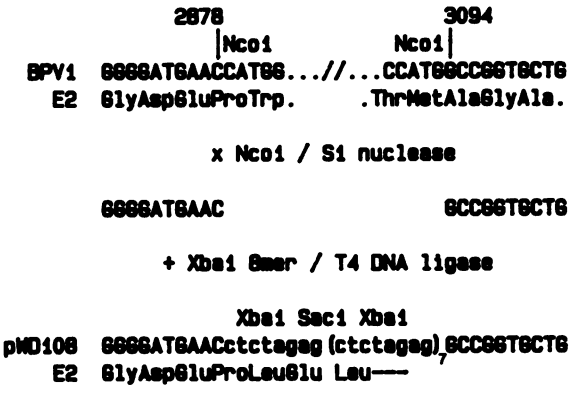

Fig. 5. Construction of the BPV1 mutant pWD 108. Cloned BPV1 DNA (pdBPV1) was cleaved with *Nco*I and treated with S1 nuclease. *Xba*I linker octamers were inserted. The predicted E2 protein of 420 amino acids is terminated at amino acid 103 by the TAG codon within the linker insertion.

Discussion

After inhibition of protein synthesis in BPV1-transformed mouse fibroblasts the level of viral transcripts increased by about one order of magnitude. As early as 1 h after cycloheximide treatment the maximal effect was observed. This suggests a short half-life of at least some protein involved in the regulation of viral gene expression. Inhibition of protein synthesis induced only quantitative changes. There was no preferential induction of individual mRNAs, nor did transcripts of the late region appear, i.e. there was no evidence that the productive replication cycle was brought about by cycloheximide treatment.

The increase of viral transcripts is mainly due to a greater transcriptional activity. In addition the rather short half-life of BPV1 transcripts seems to be slightly prolonged. This indicates that labile proteins are involved in the down regulation of viral

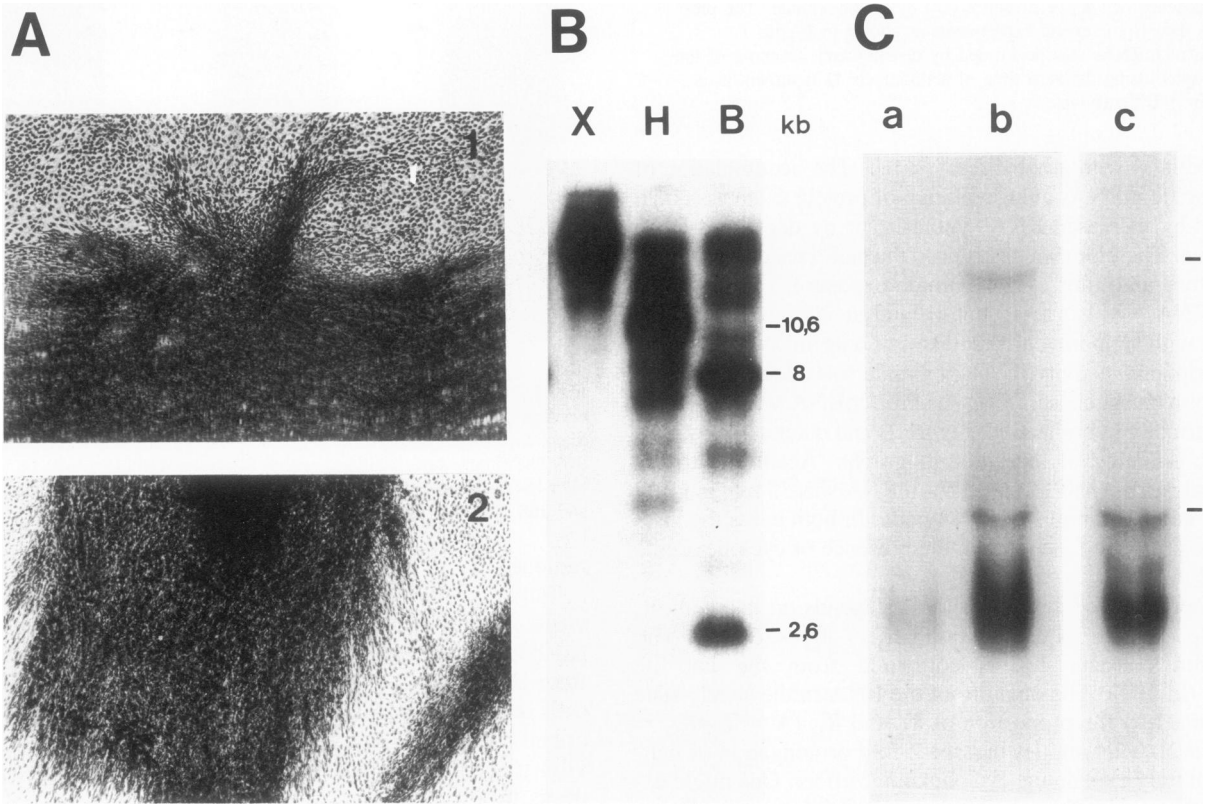

Fig. 6. A: Morphology of foci of transformed C127 cells induced by pdBPV1 DNA (1) or pWD 108 DNA (2). **B:** State of the viral DNA in pWD 108 transformed C127 cells. Total cellular DNA (10 μg) of C127p108/2 was cleaved with the restriction endonucleases *Xho*I (X), *Hin*dIII (H) or *Bam*HI (B), separated by electrophoresis, transferred to nitrocellulose and hybridized with ^{32}P-labelled pdBPV1 DNA. Viral DNA was only detected in the high molecular weight range after digestion with *Xho*I which does not cleave pWD 108 DNA. *Hin*dIII cuts once and *Bam*HI twice in pWD 108. Numerous off-size bands indicate integration into the cellular genome at multiple sites. Tandem integration is suggested by prominent bands at the positions expected for cleaved plasmid DNA. **C:** Amount of BPV1-specific transcripts in C127p108/2 cells in the absence (a) or presence (b) of cycloheximide compared with transcrips in C127 B81 cells in the absence of cycloheximide (c). For experimental details see legend to Figure 1. The bars indicate the positions of 18S (1.85 kb) and 28S (4.8 kb) ribosomal RNA.

gene expression and in the rapid degradation of BPV1 mRNAs.

Transcription of HPV16 and HPV18 was not affected by cycloheximide treatment of cervical carcinoma-derived cell lines. Even after a 7 h drug exposure there was no increase in the amount of viral transcripts. It is noteworthy that the 160 min half-life of HPV18 mRNAs in untreated HeLa cells (data not shown) is considerably longer than the half-life of BPV1 mRNAs even after inhibition of protein synthesis. The increased RNA stability may be explained by the replacement of the viral 3′ terminus with cellular sequences and/or by the absence of a specific protein involved in degradation. The HPV transcripts, which cover the open reading frame E6, are clearly more abundant than homologous transcripts of BPV1 (Schwarz *et al.*, 1985; Stenlund *et al.*, 1985). Due to the integration of the HPV18 genome the early transcription unit is disrupted and there are no mRNAs comparable with the more prominent BPV1 transcripts. The open reading frame E6 is also actively transcribed in C4-1 cells and in the SiHa line, harboring HPV16 DNA (Schwarz *et al.*, 1985; F.O.Wettstein, personal communication). These data suggest that the human virus-cell systems lack the down-regulation mechanisms which were observed with BPV1-transformed fibroblasts.

This result may reflect differential control mechanisms of individual virus and/or cell types. Studies of BPV1 were carried out with transformed fibroblasts, whereas HPV16 and HPV18 were examined in cells of epithelial origin. The control of papilloma virus gene expression does not reflect basic differences in the regulation of viral transcription by the cells in question. Early transcripts of SV40 in C127 B81 cells were not affected by cycloheximide treatment whereas adenovirus Ela mRNAs were augmented ~5-fold by cycloheximide treatment of HeLa cells (data not shown), which is in line with previously published data (Eggerding and Heschel, 1978). A fundamentally different control of papilloma virus gene expression in fibroblasts and epidermal cells may also be doubted in view of recent data on transcription of HPV6 in condylomata accuminata (Lehn *et al.*, 1984). The pattern of viral mRNAs from these purely epithelial tumors was qualitatively and quantitatively similar to that from BPV1-transformed mouse fibroblasts or BPV1-induced hamster tumors (Stenlund *et al.*, 1985; Freese *et al.*, 1982).

A lack of E2-specific transcripts seems to be a common characteristic of carcinoma-derived cell lines harboring HPV16 or HPV18 DNA, but our analysis of a BPV1 E2 mutant rendered it unlikely that the down-regulation depends on an active E2 gene. As expected from previous *in vitro* assays (Spalholz *et al.*, 1985), mutant-transformed cells revealed an extremely low level of viral transcripts because E2 was no longer able to activate the early viral transcription unit. Inhibition of protein synthesis, however, led to an increase of viral transcripts by the same order of magnitude as observed in wild-type transformed cells.

BPV1-transformed fibroblasts and the HPV-containing cell lines differ last but not least in their tumorigenicity. The fibroblasts must be still regarded as benign, whereas HeLa, C4-1 and SiHa cells are derived from malignant tumors. The changed physiology of the cancer cell could account for the inability to control viral gene expression. On the other hand it is attractive to speculate that the loss of down-regulation of viral transcription plays a role in malignant conversion. It was recently shown that enhanced expression of open reading frame E6 of BPV1 under control of a retroviral large terminal repeat leads to an increased transforming activity when tested with C127 cells (Schiller *et al.*, 1984). The amino acid sequence of HPV16 open reading frame E6 reveals significant homologies with E6 of BPV1 (Seedorf *et al.*, 1985).

One could imagine that increased expression of such a gene pushes the cell towards the malignant phenotype. It will be interesting to see if the lack of down-regulation of HPV transcripts is a consistent feature of all the cervical cancer derived systems, and if it is due to a change in the viral or cellular genome.

Materials and methods

Cell lines and cell culture

MEF B84 cells were obtained by infection of primary mouse embryo fibroblasts (MEF) with BPV1, which had been prepared from a bovine wart. MEF cells had been established from 18-day-old embryos of a DBA-mouse according to a standard protocol (Mayr *et al.*, 1974). Infection of C127 cells with BPV1 led to the cell line C127 B81. C127 cells are derived from a mammary tumor of a RIII mouse (Lowy *et al.*, 1978). C127p108/2 cells are derived from one focus of transformed cells obtained by transfection of C127 cells with pWD108 DNA. HeLa cells are derived from a cervical adenocarcinoma (Gey *et al.*, 1952). C4-1 (Auersperg, 1964) and SiHa cells (Friedl *et al.*, 1979) were both established from squamous cell carcinomas of the cervix.

All cell lines were maintained in Eagle's minimum essential medium (Gibco) supplemented with penicillin (100 i.u./ml), streptomycin (100 μg/ml) and 5% fetal calf serum (10% for C4-1 and SiHa cells). Cycloheximide (obtained from Boehringer, Mannheim) and actinomycin D (purchased from Sigma, Munich) were added at concentrations of 25 μg/ml and 6 μg/ml, respectively.

Construction of pWD108–DNA

Cloned BPV1 DNA (pdBPV; Sarver *et al.*, 1982) was digested to completion with *Nco*I and the protruding 5′ ends were removed by S1 nuclease treatment. The blunt-ended molecules were religated by phosphorylated *Xba*I linker octamers (CTCTAGAG, purchased from N.E. Biolabs). Transformed *Escherichia coli* HB101 colonies were screened for plasmids with at least two inserted linkers by cleaving DNA from quick lysates (Birmboim and Doly, 1979) with *Sac*I (a *Xba*I linker tandem creates a *Sac*I cleavage site). One clone was partially sequenced by the Maxam–Gilbert technique (Maxam and Gilbert, 1980) in order to confirm the integrity of the junctions at the linker insertion site. This clone contains eight linkers and was designated pWD 108. The TAG codon of the second linker terminates the E2 open reading frame.

Transfection and soft agar selection

C127 cells were transfected with pWD 108–DNA by the calcium phosphate precipitation method described by Graham and van der Eb (1973) with the modifications introduced by Stow and Wilkie (1976). Transformed cells were selected in soft agar, according to the protocol of MacPhesson and Montagnier (1964).

Preparation of radio-labelled probes

BPV1-specific DNA pdBPV1 (Sarver *et al.*, 1982), cloned HPV16 DNA (Dürst *et al.*, 1983) and cloned HPV18 DNA (Boshart *et al.*, 1984) were labelled by nick translation (Rigby *et al.*, 1977) to a specific activity of 10^8 c.p.m./μg. Plasmid pWD 114 was used to prepare a probe specific for the late region of the BPV1 genome. This plasmid is a derivative of pdBPV1 with a destroyed *Tth*111 I site at position 3235. Digestion of pWD 114 with *Tth*111 I led to linearization of the plasmid by cleavage of the remaining recognition site at position 5075. Starting at the 3′ ends ~2000 nucleotides were labelled in both directions with T4 DNA-polymerase (Boehringer, Mannheim) as described by O'Farrel (1981). The specific activity was ~10^7 c.p.m./μg. Labelled DNA was digested with *Hin*dIII and the 1.91-kb *Tth*111 I/*Hin*dIII fragment, which covers the open reading frame L1 and part of L2, was prepared by gel electrophoresis.

Northern blot technique

Cells from confluent monolayers were collected by trypsinization. Total RNA was prepared by the guanidium thiocyanate method (Chirgwin *et al.*, 1979). RNA samples (30 μg) were denatured in 50% formamide, 2.2 M formaldehyde in MOPS buffer (20 mM MOPS pH 7, 5 mM Na-acetate, 1 mM EDTA). After heating for 10 min at 65°C, samples were electrophoresed on 1% agarose gels containing 2.2 M formaldehyde in MOPS buffer. Transfer of RNA to nitrocellulose filters was performed in 20 × SSC (3 M sodium chloride/0.3 M sodium citrate). Filter-bound RNA was hybridized with ^{32}P-labelled probes (5 × 10^4 c.p.m./cm^2) for 48 h in the presence of 50% formamide, 5 × SSC, 50 mM Pipes pH 6.5, 5 × Denhardt solution and 0.1 mg/ml yeast RNA. Autoradiography was on Kodak X-AR5 film at −70°C, using intensifying screens. Viral mRNAs were quantitated by scanning the autoradiographs in a DD2-densitometer joined to a BD5-micrograph (Kipp and Zonen, Delf).

Southern blot analysis

Total cellular DNA was prepared according to the protocol of Ebeling *et al.* (1983) with slight modifications. Cells from confluent monolayers were harvested by

trypsinization and lysed with 500 μl (per 10 cells) of 2% sodium lauroyl sarcosinate, 50 mM Tris-HCl (pH 7.5), 10 mM EDTA. Treatment with 100 μg/ml RNase (Sigma Chemical Co., Munich) for 1 h at 37°C was followed by incubation with 500 μg/ml proteinase K for the same time at the same temperature. The DNA was extracted twice with phenol–chloroform (1:1) by shearing in a 0.9-mm tubule. After ethanol precipitation the DNA was redissolved in distilled water and stored at −20°C.

DNA samples of 10 μg were digested with several restriction enzymes (purchased from Boehringer Mannheim and Bethesda Research Laboratories Neu-Isenburg) and electrophoresed on 1% agarose gels. Blotting analyses were performed according to the protocol of Southern (1975). Hybridization to labelled BPV1-specific DNA was carried out as described previously (Fuchs *et al.*, 1985).

In vitro transcription in isolated nuclei

Nuclear run-on transcription assays were basically performed as described by Greenberg and Ziff (1984). Cells from four confluent 150-cm² tissue culture flasks were harvested by trypsinization. After centrifugation at 500 *g* for 5 min the cell pellet (4×10^7 cells) was resuspended in 5 ml NP-40 lysis buffer [10 mM Tris–HCl pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% (v/v) NP-40, 0.1 mM PMSF], incubated for 5 min on ice and centrifuged at 500 *g* for 5 min. The nuclear pellet was washed once with 5 ml NP-40 lysis buffer and centrifuged again. The nuclei were resuspended in 50 mM Tris–HCl pH 8.3, 40% (v/v) glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM PMSF at a concentration of 10^7 nuclei per 100 μl and frozen in liquid N_2. For the run-on transcription assay, 400 μl of the nuclear suspension were thawed and mixed with 50 μl of 10 × run-on buffer (10 × consists of 50 mM Tris–HCl pH 8.0, 25 mM $MgCl_2$, 1.5 M KCl and 2.5 mM triphosphates of A, G and C) and 50 μl of α-^{32}P-labeled uridine triphosphate (= 500 μCi, 3000 Ci/mol). After incubation for 30 min at 30°C the nuclei were immediately lysed by repipetting with a 10-ml pipette after the addition of 6 ml guanidium thiocyanate buffer (5 M guanidium thiocyanate, 50 mM Li-citrate, 0.1% Li-laurylsulfate, 0.1 M β-mercaptoethanol) and RNA was purified as described by Chirgwin *et al.* (1979). The run-on products were hybridized to plasmid DNA immobilized on nitrocellulose according to Marzluff and Huang (1984) with slight modifications: the prehybridization and hybridization buffer consisted of 50% deionized formamide, 5 × SSC, 1 × Denhardt, 0.1% SDS, 50 mM Pipes pH 7.0, and 250 μg/ml *E. coli* RNA. After hybridization, the filters were washed twice for 15 min in 0.1% SDS, 2 × SSC at room temperature and then washed for 30 min in 0.1% SDS, 0.1 × SSC at 52°C. Filters were then exposed to Kodak XOMAT film.

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References

Amtmann,E., Volm,M. and Wayss,K. (1984) *Nature*, **308**, 291–292.
Auersperg,N. (1964) *J. Natl. Cancer Inst.*, **32**, 135–163.
Birnboim,H.C. and Doly,J. (1979) *Nucleic Acids Res.*, **7**, 1513–1518.
Boshart,M., Gissmann,L., Ikenberg,H., Kleinheinz,A., Scheurlen,W. and zur Hausen,H. (1984) *EMBO J.*, **3**, 1151–1157.
Chirgwin,J.M., Przybyla,A.E., MacDonald,R.J. and Rutter,W.J. (1979) *Biochemistry*, **18**, 5294–5299.
Dürst,M., Gissmann,L., Ikenberg,H. and zur Hausen,H. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 3812–3815.
Dvoretzky,I., Schober,R., Chattopadhy,S.K. and Lowy,D.R. (1980) *Virology*, **103**, 369–375.
Ebeling,A., Keil,G.M., Knust,E. and Koszinowski,U.H. (1983) *J. Virol.*, **47**, 421–433.
Eggerding,F. and Heschel,J.R. (1978) *Virology*, **91**, 312–320.
Freese,U.K., Schulte,P. and Pfister,H. (1982) *Virology*, **117**, 257–261.
Friedl,F., Kimura,I. and Osato,T. (1979) *Proc. Soc. Exp. Biol. Med.*, **135**, 543–545.
Fuchs,P., Rüger,R., Pfister,H. and Fleckenstein,B. (1985) *J. Virol.*, **53**, 13–18.
Gey,G.O., Coffman,W.D. and Kubicek,M.T. (1952) *Cancer Res.*, **12**, 264–265.
Graham,F.L. and van der Eb,A.J. (1973) *Virology*, **52**, 456–467.
Greenberg,M.E. and Ziff,E.B. (1984) *Nature*, **311**, 433–438.
Gunning,P., Ponte,Ph., Okayama,H., Engel,J., Blau,H. and Kedes,L. (1983) *Mol. Cell. Biol.*, **3**, 787–795.
Heilmann,C.A., Engel,L., Lowy,D.R. and Howley,P.M. (1982) *Virology*, **119**, 22–34.
Jaureguiberry,G., Favre,M. and Orth,G. (1983) *J. Gen. Virol.*, **64**, 1199–1204.
Law,M.F., Lowy,D.R., Dvoretzky,I. and Howley,P.M. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 2727–2731.
Lehn,H., Ernst,T.N. and Sauer,G. (1984) *J. Gen. Virol.*, **85**, 2003–2010.
Lowy,D.R., Rands,E. and Scolnick,E.M. (1978) *J. Virol.*, **26**, 291–298.
MacPhesson,I. and Montagnier,L. (1964) *Virology*, **23**, 291–294.
Marzluff,W.F. and Huang,R.C.C. (1984) In Hames,B.D. and Higgins,S.J. (eds), *Transcription and Translation — A Practical Approach*. IRL Press Limited, Oxford, pp. 112–115.
Maxam,A. and Gilbert,W. (1980) *Methods Enzymol.*, **65**, 499–560.
Mayr,A., Bachmann,P.A., Bibrack,B. and Wittmann,G. (1974) *Virologische Arbeitsmethoden I.* Gustav Fischer Verlag, Stuttgart, p. 345.
O'Farrel,P. (1981) *Focus* (Bethesda Research Laboratories), **3**, 1–3.
Pater,M.M. and Pater,A. (1985) *Virology*, **145**, 313–318.
Pfister,H. (1984) *Rev. Physiol. Biochem. Pharmacol.*, **99**, 111–181.
Reid,R., Crum,C.P., Herschman,B.R., Fu,Y.S., Braun,L., Shah,K.V., Agronow.S.J. and Stanhope,C.R. (1984) *Cancer*, **53**, 943–953.
Rigby,P.W.J., Dieckmann,M., Rhodes,C. and Berg,P. (1977) *J. Mol. Biol.*, **113**, 273–286.
Sarver,N., Byrne,J.C. and Howley,P.M. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 7147–7151.
Schiller,J.T., Vass,W.C. and Lowy,D.R. (1984) *Proc. natl. Acad. Sci. USA*, **81**, 7880–7884.
Schiller,J.T., Vass,W.C., Vousden,K.H. and Lowy,D.R. (1986) *J. Virol.*, **57**, in press.
Schwarz,E., Freese,U.K., Gissmann,L., Mayer,W., Roggenbuck,B., Stremlau,A. and zur Hausen,H. (1985) *Nature*, **314**, 111–114.
Seedorf,K., Krämmer,G., Dürst,M., Suhai,S. and Röwekamp,W.G. (1985) *Virology*, **145**, 181–185.
Southrn,E.M. (1975) *J. Mol. Biol.*, **98**, 503–517.
Spalholz,B.A., Yang,Y.C. and Howley,P.M. (1985) *Cell*, **42**, 183–191.
Stenlund,A., Zabielski,J., Ahola,A., Moreno-Lopez,J. and Pettersson,U. (1985) *J. Mol. Biol.*, **182**, 541–554.
Stow,N.D. and Wilkie,N.M. (1976) *J. Gen. Virol.*, **33**, 447–458.
Syverton,J.T. (1952) *Ann. N.Y. Acad. Sci.*, **54**, 1126–1140.
Yee,C., Krishnan-Hewlett,I., Baker,C.C., Schlegel,R. and Howley,P.M. (1985) *Am. J. Pathol.*, **119**, 361–366.

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