The tyrosine kinase inhibitor (tyrphostin) AG 555 selectively interferes with viral transcription in bovine papillomavirus type 1 (BPV-1)-transformed fibroblasts and induces suppression of cyclin-dependent kinase activity and cell cycle arrest. Concomitant with inhibition of viral transcription, c-Jun was strongly up-regulated, which was consistent with the observation that AG 555 treatment also led to an activation of the mitogen-activated protein kinase pathway by enhancing phosphorylation of JNK and p38. Increased JNK and p38 activity resulted in higher phosphorylation of the AP-1 family members c-Jun and activating transcription factor 2. Scanning the BPV-1 genome for potential binding sequences, an intragenic AP-1 site (BAP-1) within the E7 open reading frame was detected. Enhanced dimerization of phosphorylated activating transcription factor 2 together with c-Jun and binding to BAP-1 seem to be responsible for viral dysregulation because both suppression of BPV-1 and induction of c-Jun mRNA could be almost entirely abrogated by simultaneous treatment with SB 203580, an inhibitor of p38 mitogen-activated protein kinase activity. Moreover, dissecting the complex transcriptional pattern of episomal BPV-1 with specific primer sets for reverse transcription-PCR analysis, the repressive effect could be attributed to a selective down-regulation of the mRNA encoding the E2 transactivator function in favor of the E3 repressor, whose mRNA level remained constant during AG 555 treatment. These data indicate that tyrphostin AG 555 disturbs the balance of negative and positive regulatory factors necessary to maintain the homeostasis of a virus-transformed phenotype.

Increased tyrosine phosphorylation is a common feature of many cancers (1, 2). Up-regulation of specific receptors or/and enhanced tyrosine kinase activity concomitantly elevate intra-cellular phosphorylation of many downstream regulatory proteins, which guarantees the maintenance of unscheduled DNA synthesis and cell proliferation (for review, see Ref. 3). In recent years, defined synthetic compounds have been designed which can efficiently block tyrosine kinase activity (tyrphostins) (4) and in turn the proliferative phenotype. In principle, two types of tyrphostin have been developed: one is acting at the substrate binding site of the enzyme, whereas the other functions by competing with the ATP binding domain, which is highly conserved among tyrosine kinases (1). Tyrphostins structurally resemble tyrosine and erstatin moieties and carry hydrophobic residues that enable crossing the cell membrane. The potential application of tyrphostins is not only considered for treatment of malignant disorders but also in other diseases such as atherosclerosis, psoriasis, and septic shock where increased tyrosine kinase activity could be discerned (5, 6). Furthermore, tyrphostins are also useful tools to study regulatory mechanisms where enhanced tyrosine phosphorylation is involved (2).

In the case of papillomavirus-linked diseases, alterations of epidermal growth factor (EGF)/insulin-like growth factor I (IGF-I) signal transduction and in turn increased tyrosine phosphorylation seem to play a pivotal role during multistep progression toward malignancy (7–9). For example, the bovine papillomavirus type 1 (BPV-1) E5 oncoprotein exerts its transforming function through constitutive activation of growth stimulatory pathways via interaction with platelet-derived growth factor and EGF receptors (10, 11). Furthermore, both BPV-1 E5 and the corresponding homolog of the human papillomavirus (HPV) type 16 can cooperate with ectopic EGF receptor expression in cell transformation assays (12, 13). Although the E5 open reading frame (ORF) of HPV-16 is often found to be deleted in cervical carcinoma cells after integration (14), the protein may have a function in premalignant lesions (15), in which the DNA is still episomal (16). Intriguing also is the observation that rodent cells harboring a targeted disruption of the IGF receptor gene (IGF-R1) cannot be transformed by BPV-1 unless a functional IGF-R1 cDNA is provided (8). This supports the notion that enhanced tyrosine kinase activity is apparently a general feature in viral carcinogenesis because elevated expression of EGF/IGF receptors has been reported in a wide proportion of papillomavirus-induced malignancies (17, 18).

A suitable model system, in which enhanced intracellular tyrosine phosphorylation signaling can be studied in the context of viral transcription and transformation, is provided by BPV-1-transformed mouse fibroblasts (ID13 cells) (19). Here, E5 is considered the main oncoprotein (20) because mutations within the ORFs of E6 and E7 (encoding the major HPV-16/18-
transforming proteins) for review, see Ref. 21) have only marginal effects in focus formation assays using rodent cells as recipients (22). However, E6 can also affect tyrosine phosphorylation by interacting with the focal adhesion protein paxillin, known to be involved in coordination of cell spreading and motility (23). Similar to premalignant HPV-positive keratinocytes (24), BPV-1 persists as multicopy episomal nucleoprotein complexes in transformed cells (25, 26). This has the advantage that effects on the multipromoter-driven transcriptional activity (27, 28) can be investigated independently from any position effects (25) often occurring after viral integration into the host genome (29).

In the present study, we show that the tyrphostin AG 555 can selectively suppress BPV-1 transcription through MAP kinase pathway activation and binding of phosphorylated Jun/ATF-2 at a novel intragenic regulatory sequence. We also demonstrate that AG 555 affects the transcription of the major regulatory viral protein E2 by shifting the ratio between E2 transactivator in favor to the repressor function. These data indicate that fine tuning of BPV-1 gene expression in transformed cells is regulated by tyrosine phosphorylation.

EXPERIMENTAL PROCEDURES

Cell Culture and Treatment—ID13 mouse fibroblasts (19) were maintained in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 5% (v/v) fetal calf serum (Invitrogen), 100 units/ml penicillin, and 100 μg/ml streptomycin (Sigma). Cells were seeded at 2.5 × 10⁵ cells/cm² to ensure logarithmic growth. The final concentration of 5 × 10⁴ cells/ml was used for transfection experiments. The expression vector pBR322. c-Jun, encoding the mouse cDNA in a Rous sarcoma virus/H9252 approximately full-length insert of the fibroblast sis gene, was a generous gift from L. Kedes (Medical Center, Palo Alto, CA). For gel retardation assays, cells were washed twice with phosphate-buffered saline and stained with 1 μg/ml propidium iodide (30) (Bio-Rad), and 50 μg of total protein was electrophoresed on 10% SDS-polyacrylamide gels under reducing conditions. After electrophoresis proteins were transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore Corporation), the membranes were incubated for 1 h at room temperature with blocking solution: TBS (20 mM Tris-HCl, pH 7.6, 0.14 M NaCl) containing 0.1% Tween 20 (Sigma) and 5% skim milk powder (Merck). After blocking, the membranes were incubated with the first antibody (diluted in blocking solution) overnight at 4 °C. The membranes were washed three times with TBS containing 0.1% Tween 20 for 30 min and then incubated for 2 h at room temperature with the second antibody (anti-rabbit or anti-mouse IgG conjugated with horseradish peroxidase) diluted 1:5,000 in blocking solution. Finally, the membranes were washed again for 30 min, incubated 2 min with the ECL reagents (Amersham Biosciences), and exposed on Kodak X-Omat films. For stripping, the membranes were incubated with 200 mM NaOH for 5 min and washed with water prior to incubation with an additional antibody. The following polyclonal antibodies were used (all purchased from New England Biolabs): phospho-p38, phospho-JNK, phospho-ERK1/2, phospho-c-Jun, phospho-ATF-2, and ATF-2. c-Jun antibody, and Cdk2 antibody were from Santa Cruz Biotechnology. The BPV E5 monoclonal antibody was a kind gift from R. Schlegel (Georgetown University, Washington, D. C.). Equal protein transfer and loading were routinely controlled by reincubating the film with a specific antibody (anti-His antibody, 926 from (39)).

p38 MAP Kinase Assay—All steps of the nonradioactive kinase assay system were performed exactly according to the manufacturer’s instructions (New England Biolabs). Cells were lysed in buffer containing 20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM-glycerophosphate, 1 mM Na3VO4, and 1 μg/ml leupeptin. The first step includes an immunoprecipitation of p38 MAP kinase from a 100-μg protein extract via immobilized phospho-p38 MAP kinase (Thr180/Tyr182) monoclonal antibody. In vitro kinase assay was performed using purified ATP-2 protein as substrate. ATP-2 phosphorylation was detected by Western blot using phospho-ATF-2 (Thr71) antibody.

Fluorescence-activated Cell Sorter Analysis—For fluorescence analysis, cells were washed twice with phosphate-buffered saline and stained 1 h with 50 μg/ml propidium iodide + 40 μg/ml RNaseA in phosphate-buffered saline in the dark at 4 °C. Analysis was performed using a BD Biosciences flow cytometer (40). Cell cycle distribution and quantification of flow cytometric data were performed according to Dean and Jett (41). For each measurement, 10,000 cells were counted.

RT-PCR—Total RNA was isolated using the RNaseasy kit from Qiagen according to the instructions of the provider. 30 μg of RNA was treated with 1 unit of DNase for 30 min at 37 °C to remove residual genomic DNA. The RT reaction was performed using the Omniscript RT kit from Qiagen; 2 μg of RNA was used for a 20-μl reaction volume to generate cDNA. A primer set specific for AP-1 was performed with 1 μM of each primer, now containing cDNA, 5 mM of dNTPs, 5 μM of primers, and 0.7 unit of Expand High Fidelity Polymerase (Roche Applied Science) in a 20-μl reaction volume using primers for full-length, amino-terminal, and carboxy-terminal E2 DNA sequences: forward (F) primer, 5'-AGACA-GCAAACTCTTGATGCT-3'; reverse (R) primer, 5'-GAAACTCTTGACACTGATT-3'. The PCR program (MJ Research PTC Cycler) was as follows: step 1, denaturation at 95 °C for 1 min; step 2, denaturation at 95 °C for 30 s; step 3, annealing with touch down 65 °C/Cycle; step 4, elongation at 72 °C for 4 min; step 5, repeat steps 2–4, 19 times; step 6, elongation at 72 °C for 4 min; step 7, annealing at 75 °C for 30 s; step 8, elongation at 72 °C for 4 min; step 9, repeat steps 6–8, 19 times; step 10, elongation at 72 °C for 12 min and cooling at 4 °C. PCR products were separated on 1% agarose gels and detected after ethidium bromide staining under UV.
Suppression of BPV-1 Transcription by AG 555

**RESULTS**

**AG 555 Selectively Down-regulates BPV-1 Transcription**—Because of their structural similarity with tyrosine (Fig. 1, panel A) tyrphostins can block protein-tyrosine kinase activity by binding to the substrate binding site (2). To test the effect of AG 555 on viral transformed cells, BPV-1-transformed mouse fibroblasts (ID13 cells) were treated for different periods of time, and total RNA was examined by Northern blot analysis. As shown in Fig. 1, panel B, cell incubation in the presence of 30 μM AG 555 resulted in a selective down-regulation of the most abundant viral transcripts (27, 43) already 4 h after drug application. To demonstrate that AG 555-mediated BPV-1 suppression was a selective process and not the consequence of a general transcriptional block per se, the RNA filters were rehybridized with endogenous reference genes. Notably, under conditions in which BPV-1 transcription was suppressed, an even costimulatory effect on c-Jun gene expression could be discerned. c-Jun induction was detectable already 2 h after AG 555 addition and remained elevated up to 8–10 h. Subsequent hybridization of the same filters with a housekeeping gene probe (β-actin) demonstrated that suppression was specifically directed against the virus-specific transcription cassette and did not represent the result of a nonspecific transcriptional impairment of the cells by the tyrphostin.

**Down-regulation of BPV-1 Transcription and Cell Cycle Arrest.** Suppression of Cdk2 Activity—Measuring the cell cycle profile by flow cytometric analysis under conditions in which BPV-1 expression was found to be down-regulated, the majority of ID13 cells were growth-arrested with an accumulation of the cells at the late S/early G2 boundary, whereas the G1 phase was diminished (Fig. 2, panel A). For example, considering the fluorescence-activated cell sorter profile 8 h after AG 555 treatment, an increase of the S/G2 phase from 7.7 to 18.5% and from 12 to 25% and a decrease of G1 from 75 to 43%, respectively, could be noted (see Fig. 2, panel A). To analyze whether the inhibition of BPV-1 transcription and cell cycle arrest correlated with cyclin-dependent kinase activity suppression, cyclin-Cdk2 complexes were first immunoprecipitated with a Cdk2-specific antibody and subsequently functionally tested in an in vitro phosphorylation assay using histone H1 as substrate (Fig. 2, panel B, upper part). Compared with the untreated controls, Cdk2 remained active up to 2 h but declined significantly between 6 and 8 h after the addition of AG 555.

To exclude that Cdk2 activity was reduced because of quantitative changes in Cdk2 itself, total cellular extracts were monitored by Western blot analysis. Consecutive incubation of the filter with specific antibodies against Cdk2 and actin as internal loading control confirmed equal Cdk2 expression (Fig. 2, panel C). In addition, consistent with the transcriptional data presented above (Fig. 1, panel B), the level of the E5 oncoprotein was down-regulated in the same time range, whereas the expression of Ha-ras and actin as internal reference was not affected (Fig. 2, panel D). These results suggest that AG 555 was efficiently inducing cell cycle arrest by negatively interfering with BPV-1 transcription and Cdk2 function.

**MAP Kinase Pathway Activation by AG 555**—Because c-Jun expression was induced upon AG 555 treatment, we reasoned that tyrphostin treatment may act as a cellular stress signal (for review, see Ref. 44) that activates the MAP kinase pathway and in turn downstream target proteins. Mammalian MAP kinases consist of three groups: ERK, JNK, and the p38 MAP kinases (45). To prove whether AG 555 was affecting MAP kinase activity, Western blot analyses using phosphorylation-specific antibodies raised against ERK, JNK, and p38 were performed. Fig. 3, panel A, shows that only the stress kinases JNK and p38 were phosphorylated, reaching a maximum between 4 and 6 h after the addition of AG 555. In contrast, ERK1 and ERK2 were unaltered under the same experimental conditions. Phosphorylation could be not attributed to an enhanced protein synthesis because control incubations of the filters with nonphosphorylation-specific antibodies revealed that the net amount of the corresponding proteins was the same.

p38 MAP kinases are normally activated by dual phosphorylation on Thr and Tyr within a Thr-Gly-Tyr motif (46). It was therefore unexpected that a tyrphostin, which can potentially interfere with tyrosine phosphorylation (2), can activate p38. Because the p38 antibody used cannot distinguish between these two phosphorylation sites, it was mandatory to test whether phosphorylation was in fact accompanied with an increased enzymatic activity. p38 was therefore first immu-
The Viral Transcriptional Transactivator E2 is Down-regulated, whereas the Level of Short E2 Encoding the Repressor Form Remained Constant during AG 555 Treatment—Al-

Having verified the authenticity of the novel AP-1 binding site within the BPV-1 genome (Fig. 4, panel A), it was of interest to monitor quantitative and qualitative changes after AG 555 treatment. Notably, EMSAs completely failed to detect any increased AP-1 binding affinity using 32P-labeled BAP-1 oligonucleotides as probe (Fig. 4, panel B). Application of a c-Jun antibody in the binding reaction (supershift) demonstrated that c-Jun was only slightly elevated within the AP-1 complex, whereas the amount of phosphorylated ATF-2, which normally binds to the ATF/cAMP-responsive binding protein consensus sequence 5’-TG/A/G/C/TGA/T-3’ (47), became significantly enhanced (Fig. 4, panel C). Elevated association and dimerization of ATF-2 with c-Jun seem to be specific because other AP-1 family members such as c-Fos or Fra-1 were not increased (data not shown).

In the next set of experiments we asked whether MAP kinase and in particular p38 activation was a prerequisite for the negative regulatory effect on viral gene expression. It is known that c-Jun transcription becomes enhanced through an autoregulatory mechanism, which is mediated via binding of c-Jun/ATF-2 heterodimers to its own promoter sequence (34). If there was a divergent regulation on BPV-1 and c-Jun expression, one should expect that in the same way as the transcriptional block on BPV-1 is relieved, the c-Jun steady-state level should be reduced. To answer this question, ID13 cells were treated with AG 555 together with SB 203580, an inhibitor of p38 MAP kinase activity (48). Northern blot analysis revealed that this was indeed the case (Fig. 5). Simultaneous incubation with AG 555 and SB 203580 led to significant re-expression of BPV-1, whereas the c-Jun-specific signal was diminished. These results strongly suggest that p38 activation and phosphorylation of its downstream target ATF-2 play a crucial role in tyrphostin-mediated BPV-1 repression.
though the functional role of BAP-1 is not yet clear, we reasoned that c-Jun/ATF-2 binding could affect BPV-1 expression by perturbing viral transcription initiated at different promoters located both in the upstream regulatory region and within the early coding region (for schematic overview, see Fig. 4, panel D). A well characterized mechanism that inherently interferes negatively with transcription is mediated by the viral E2 protein itself (49, 50). Depending on which promoter is used, two forms of E2 can be synthesized from differently spliced mRNA species: a transactivator, starting from position P2443 (Fig. 4, panel D), consists of the full-length protein harboring an amino-terminal activator domain as well as a carboxy-terminal DNA and dimerization region (51). Alternatively, there also exist two truncated forms of E2, initiating from position P890 and P3080 (Fig. 4, panel D) which lack the trans-activation domain and can therefore act as transcriptional repressors (52). To determine whether treatment of AG 555 had an effect on the ratio between the different transcripts encoding either the repressor (E2-TR) or activator form of E2 (E2-TA), RT-PCR analyses were performed (Fig. 6). Using selected primers diagnostic for the repressor (the smaller carboxyl-terminal part, referred as Fc-R, see Fig. 6, lower panel), a 303-bp PCR product was accumulated whose amount was not quantitatively changed. In contrast, trapping transcripts encoding the complete transactivator (referred as F-Rn, indicative for the amino terminus and therefore encoding the entire E2), the 220-bp PCR product was drastically diminished upon AG 555 treatment. The usage of the cellular glyceraldehyde-3-phosphate dehydrogenase gene as internal control confirmed the selectivity of this process. Because the transactivator function is required to stimulate transcription within the viral promoters (53), E2-TA depletion in favor to E2-TR apparently accounts for the suppressive effect after tyrphostin application.

DISCUSSION

Tyrphostins are useful tools for negatively interfering with enhanced tyrosine phosphorylation in many malignant disorders (2). Some tyrphostins were shown to possess strong antiproliferative activity without having identified the exact target. Using BPV-1-transformed C127 mouse fibroblasts (ID13 cells) as the model system, we studied the effect of the tyrphostin AG 555 in the context of virus-induced carcinogenesis. Treatment of ID13 cells with 30 μM AG 555 for different periods of time resulted in a selective down-regulation of viral transcription (Fig. 1, panel B). However, the mRNA decline followed a different kinetic compared with actinomycin D, known to block RNA polymerase transcription nonspecifically by intercalating into the DNA (data not shown). The slight initial increase and the subsequent drop of the viral transcripts either argued for delayed regulatory pathway acting at the level of initiation of transcription or for a post-transcriptional control mechanism. Although the steady-state level of BPV-1 mRNA can be enhanced by cycloheximide (54), short term blockage of protein synthesis for 1 h was not abrogating the suppressive effect on viral transcription after a 7-h AG 555 treatment (data not shown). This favored the assumption that AG 555 apparently induced an alternative mode of transcriptional control rather than through post-transcriptional labilization of the BPV-1-specific RNAs (see below).
As further demonstrated, down-regulation of viral transcription temporarily correlated with growth arrest and cyclin-dependent kinase suppression (Fig. 2, panels A and B). However, it should be stressed that this coincidence may not necessarily be the consequence of the reduction of the major viral oncoprotein E5 (Fig. 2, panel D) because the same situation can be found when the nontransformed, but still immortalized, C127 mouse fibroblasts were monitored (data not shown). On the other hand, it seems that whenever there is enhanced tyrosine phosphorylation to threshold levels, tyrphostins have growth-inhibitory properties (55). Consistent with this notion is that AG 555 is capable of blocking cell proliferation of HPV-16-immortalized human keratinocytes (56, 57) and psoriatic keratinocytes, where persistent autocrine/paracrine stimulation of the EGF receptors by transforming growth factor-β (TGF-β) can be discerned (58).

The expression of immediate-early genes such as c-Jun, found to be activated upon AG 555 addition (Fig. 1, panel B), is tightly controlled by the MAP kinase pathway (for review, see Ref. 44). We therefore anticipated that tyrphostin treatment may act as stress signal, similar to the situation described recently in human platelets treated using genistein as a tyrosine kinase inhibitor (59). As depicted in Fig. 3, panel A, AG 555 selectively induced JNK and p38 phosphorylation, whereas ERK1/2 MAP kinase was not affected. Enhanced c-Jun expression can be explained by phosphorylation of pre-existing c-Jun and ATF-2 proteins (Fig. 3, panels B and C), which in turn stimulate transcription through binding of c-Jun/ATF-2 dimers to related cis-regulatory sequences within the c-Jun promoter (for review, see Ref. 60).

EMSA revealed that c-Jun became dimerized with increased levels of phosphorylated ATF-2, which bind to a novel identified intragenic AP-1 sequence motif (BAP-1) within the E7 ORF (Fig. 4, panels A and C). Notably, even though c-Jun mRNA was increased, the net amount of DNA binding at the BAP-1 sequence was not affected (Fig. 4, panel B). In contrast to “high risk” HPV-16s, where the transcription factor AP-1 and its composition are apparently part of an intracellular surveil-

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**Fig. 4. Detection of an AP-1 binding element in the BPV-1 genome.** Panel A, the left part demonstrates binding selectivity at the BAP-1 sequence. Competition can be achieved by addition of 100-fold molar amounts of unlabeled BAP-1, AP-1, and TRE oligonucleotides, but not with oligonucleotides harboring a SP1 recognition site. The right part shows that BAP-1 can also compete binding to labeled AP-1 consensus oligonucleotides. Panel B, EMMA of BAP-1 binding after AG 555 incubation for different periods of time. Panel C, supershift EMMA after the addition of AG 555 using antibodies against c-Jun and phosphorylated ATF-2 (P-ATF-2). Panel D, linear map of the BPV-1 genome. The localization of the different promoters (P7685, late promoter; P7685, P1; P7949, P2; P890, P3; P2443, P4; P3080, P5)) relative to the ORFs (E, early; L, late) are indicated. URR, upstream regulatory region.
lance mechanism regulating both E6/E7 transcription and tumorigenicity (38, 61, 62), a similar role for AP-1 and its corresponding binding site has not yet been described for BPV-1. Although it is currently not clear how this binding site affects the usage of BPV-1 upstream and downstream localized promoters in the whole genomic framework (see the schematic overview in Fig. 4, panel D), divergent transcriptional regulation of c-Jun and BPV-1 gene expression became drastically abrogated by blocking p38 activity with SB 203580 (63) (Fig. 5). The explanation of why no complete reversion to initial steady-state levels could be accomplished is probably due to the redundancy of the MAP kinase network as both JNK and p38 can phosphorylate c-Jun and ATF-2 (44). It is reasonable to assume that phospho-ATF-2 may negatively influence BPV-1 expression. Although ATF-2 is generally considered as a positive regulator (34), the involvement of ATF-2 in gene repression is not without a precedent. Notably, as shown for hepatitis B virus, ATF-2 can down-regulate hepatitis B virus X promoter activity by competing for the AP-1 binding site through enhanced Jun/ATF-2 heterodimer formation (64). How this is exactly mediated in the case of BPV-1 remains to be elucidated.

Considering the small size, the existence of at least five as yet characterized promoters and the circularity of DNA (a circular permuted viral template), BPV-1 can be regarded as an episomal enhanceosome, where the occupancy of the novel intragenic cis-regulatory element at position 505–525 by phosphorylated Jun/ATF-2 can also affect other promoters, in particular promoters controlling the transcription of the E2 protein (see Fig. 4, panel D). E2 seems to be the master regulator protein controlling not only the viral copy number (65) but also the transcriptional fine tuning by utilizing different promoters (28). The E2 protein can be synthesized from differently spliced mRNA species found in both transformed cells and differentiating cells. When started from the promoter at position P2443 (Fig. 4, panel D), a full-length transactivator protein is generated, harboring an amino-terminal activator domain as well as a carboxyl-terminal DNA and dimerization region (51). On the other hand, there also exist two shortened forms of E2, initiating from positions P890 and P3080, respectively (Fig. 4, panel D). The E8/E2 as well as the E2-TR gene products lack the transactivation domain and can therefore act as transcriptional repressors (52). In the context of the entire genome, however,
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Dysfunction of the E8/E2 protein has little effect on viral gene expression, whereas disruption of the E2 repressor E2-TR resulted in an increase of the transformation potential (50).

Dontase I footprinting analyses have revealed that there are 17 different E2 binding sites within the BPV-1 genome, which differ in their affinities and equilibrium constants (66). In addition to the E2 binding sites within the upstream regulatory region, there are also recognition positions for E2 immediately upstream of the P2443 and the P3080 promoter (67). The former, which is controlled by an E2 autoregulatory loop (68), is responsible for the synthesis of the most abundant mRNA encoding the transforming protein E5 (27). AG 555 is apparently triggering a cascade of distinct regulatory steps, which resulted in a disturbance of the ratio between E2-specific RNAs encoding either the transactivator (E2-TA) or the transrepressor function (E2-TR) of E2 (Fig. 6). Because the P2–P5 promoters (Fig. 4, panel D) are all stimulated by the E2 transactivator function (28), the selective reduction of E2-TA in favor of E2-TR probably accounts for the ultimate suppression of viral transcription. These findings indicate that tyrosine kinase signaling obviously plays an important role in the maintenance of the BPV-1 transforming activity.

Are these results transferable to human papillomaviruses? With the exception of the HPV-16-positive W12 cells (69) or the HPV-31-containing CIN612 cells (70), not many human keratinocyte lines harbor stable episomes. In most cervical carcinoma cells, HPV is found to be integrated where the balanced repression in favor of E2-TR (Fig. 6C) is destroyed (for review, see Ref. 21). However, with respect to the distribution of additional regulatory elements upstream from and within the E2 ORF (71, 72), it would be interesting to test AG 555 and the role of E2 on cell lines carrying HPV in an episomal state.

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