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Trans-activation of an upstream early gene promoter of bovine papilloma virus-1 by a product of the viral E2 gene

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The ~1000 nucleotide long upstream regulatory region (URR) of bovine papilloma virus-1 (BPV-1) contains a *cis* element which responds to *trans*-activation by a diffusible factor encoded in the viral E2 open reading frame (ORF). A series of URR DNA fragments have been linked to two heterologous genes, bacterial chloramphenicol acetyl transferase (*cat*) or herpes simplex virus-1 thymidine kinase (*tk*), and tested in transient transfection assays for transcription initiating at the authentic upstream early viral promoter, P89. Transcriptional activity of the P89 promoter was greatly elevated in the presence of the E2 *trans*-activator gene product. The E2-responsive *cis* element (*E2R*) of P89 has been mapped to sequences −277 to −131 nucleotides upstream from the transcription start site (BPV nucleotide 89). The *E2R* element functioned as a strong transcriptional enhancer in *cis* with the SV40 early or the *tk* promoter in the presence, but not in the absence, of the E2 gene product. However, several heterologous promoters which lack sequences related to the *E2R* element were also *trans*-activated in transient co-transfections by a function encoded in the E2 ORF of BPV-1, albeit to a much lesser extent. In addition to activation of early viral gene transcription, the E2 regulatory gene(s) may therefore have the potential to alter cellular gene expression.

Key words: cat gene/cell transformation/HSV-1 *tk* gene/transcriptional enhancer/transient transfection

Introduction

Bovine papilloma virus (BPV) type 1 is a DNA tumor virus which causes benign epithelial skin papillomas (warts) with a dermal fibroblastic component. BPV-1 virions or molecularly cloned BPV-1 DNA neoplastically transform rodent fibroblasts in culture (Dvoretzky *et al.*, 1980; Lowy *et al.*, 1980). This property of BPV-1 may be related to its ability to stimulate dermal fibroblast proliferation *in vivo*. As papilloma viruses do not undergo productive replication in tissue culture, traditional genetic analysis of viral mutants has not been feasible. Transformation of mouse fibroblasts with cloned BPV-1 DNA has therefore served as a model for the elucidation of papilloma viral functions. BPV-1 genomes replicate as unintegrated plasmids in the nuclei of the transformed mouse cells (Lancaster, 1981; Law *et al.*, 1981). Maintenance of transformation and of viral plasmid replication has been shown to depend on the continuous expression of viral functions (Turek *et al.*, 1982).

In vitro dissection and mutagenesis of cloned BPV-1 DNA has permitted the assignment of viral genes involved in cell transformation and plasmid replication to specific potential protein-coding sequences of BPV-1 (open reading frames, ORFs, deduced from viral DNA sequence; Chen *et al.*, 1982; Danos *et al.*, 1982; Ahola *et al.*, 1983). These genes map to a BPV-1 fragment encompassing 69% of the viral genome (the early, 'E', gene region; Lowy *et al.*, 1980). Correct temporal regulation of the early viral genes is required in the establishment and maintenance of cell transformation and plasmid replication, but the regulatory mechanisms involved are poorly understood.

The early genes of BPV-1 are preceded by an 'upstream regulatory region' (URR) of ~1000 nucleotides (nt), which is thought to encompass promoter sequences for early mRNA transcription. Several mature BPV-1 mRNAs initiate immediately 5′ to the coding ORFs. A major 5′ cap mRNA site has been mapped to BPV nt 89 by S_1 nuclease protection and primer extension studies in BPV-1-transformed cells by Stenlund *et al.* (1985), and by sequence analysis of cDNA clones by Yang *et al.* (1985a). The URR segment upstream from this promoter is important for the biological activity of BPV-1. Deletions in sequences 5′ to the promoter inactivate cell transformation. Furthermore, transforming activity of the deletion mutants can be partially restored by insertion in *cis* of strong heterologous enhancer–promoter fragments (Nakabayashi *et al.*, 1983; Sarver *et al.*, 1984; Schiller *et al.*, 1984). Spalholz *et al.* (1985) have found that a large (~1000 nt) fragment of the URR could substitute for a *cis* enhancer of gene expression in the presence of a diffusible gene product of the E2 ORF of BPV-1. These studies, however, have not identified the authentic viral promoter(s) that respond(s) to *trans*-activation by the E2 gene product, nor have they localized the conditional enhancer-like *cis* element. Furthermore, the question whether E2 *trans*-activation influences the levels of mRNA transcription, or operates via another, post-transcriptional mechanism, has not been addressed so far.

In this study, we have identified sequences that are required in *cis* for the functional activity of the upstream P89 promoter, and show that the level of mRNA initiated at the P89 promoter is greatly elevated in cells actively expressing a *trans*-activator gene product of the E2 ORF domain. Deletional mutagenesis has been used to map the E2-responsive *cis* sequence which is required for *trans*-activation (the *E2R* element) between nt −227 and −131 upstream from the P89 promoter. The *E2R* element acts as a strong transcriptional enhancer in the presence of the *trans*-activator gene product, and contains papilloma virus-specific palindromic motifs, $ACC(N)_6GGT$, which may serve as a specific target core for E2 action. The interaction between the *cis E2R* element and the diffusible factor encoded in the E2 ORF of BPV-1 constitutes a well-defined model to study transcriptional gene regulation. However, a gene product of the E2 ORF region also *trans*-activates (albeit to a lesser degree) cotransfected heterologous genes lacking this target element, and may therefore have the potential to alter cellular gene expression.

Results

The upstream early viral promoter, P89, is differentially activated in cells expressing BPV-1 early genes

A promoter immediately upstream of the BPV-1 early gene domain is utilized to initiate transcription of several early BPV mRNAs with a major 5′ cap site at nt 89 (the P89 promoter; Stenlund *et al.*, 1985; Yang *et al.*, 1985a). To map sequences required for the P89 promoter activity, and to assess promoter regulation in response to diffusible factors, restriction fragments of BPV-1 DNA in the P89 promoter region were inserted into the promoter-less plasmid pU-*cat* (Figure 1; Scholer *et al.*, 1986), and the resulting molecular clones were transfected into mouse cells expressing BPV-1 early functions (ID-14), or into uninfected control mouse cell lines that are routinely used to detect BPV transformation (C127I and NIH-3T3).

The results of this analysis are shown in Table I. At the 3′ end of the P89 fragment, BPV sequences extending to nt 90 (in the plasmid pXba-90-*cat* 'a') were necessary for detectable P89 promoter activity. Fragments extending to nt 7945 [the URR fragment between nt 6959 and 7945 (*Hin*dIII−*Hpa*I)], or even nt 82 [a fragment terminating at the *Nci*I site (nt 79)], did not suffice to substitute for an active promoter. The *Xba*-90 fragment promoted *cat* gene expression only in the 'sense' ('a') but not in the 'anti-sense' ('b') orientation. The different promoter activity was due to differences in the 3′ sequences, as demonstrated by testing 5′ deletions in the *Xba*-90 fragment (see Figure 4 below). These data, together with the RNase protection experiments described below (Figure 5), demonstrate that the major promoter which directs *cat* expression in the pXba-90-*cat* 'a' construction corresponds to the authentic P89 promoter of BPV-1 mapped *in vivo* (Stenlund *et al.*, 1985; Yang *et al.*, 1985a).

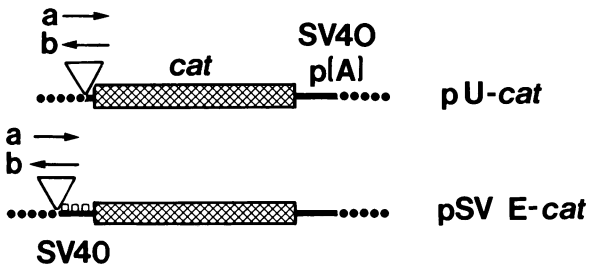

Fig. 1. BPV-1-*cat* target plasmids. Sequences upstream of the viral early coding region were subcloned 5′ to the *cat* gene in the promoter-negative plasmid pU-*cat* to detect promoter activity. To detect enhancer activity, BPV DNA fragments were inserted 5′ to the SV40 early promoter in the enhancer-negative clone pSV E-*cat*. Fragment orientation 'a' corresponds to 'sense', 'b' to 'antisense' in regard to transcription of BPV and *cat* genes.

In contrast to the enhancer−promoter of the Rous sarcoma virus long terminal repeat (RSV LTR) in the control plasmid pRSV-*cat*, the BPV P89 promoter was greater than 5-fold more active in the BPV-transformed ID-14 cells than in uninfected C127I or NIH-3T3 mouse cells (Table I). This was not due to non-specific changes in cellular growth rate or metabolism which accompany neoplastic transformation, since the P89 activity remained low in C127I cell lines transformed with either of two unrelated retroviral oncogenes, v-*fes* or v-*mos* (not shown). We have therefore sought to determine whether a diffusible (*trans*-acting) early viral gene product, presumably expressed in the BPV-transformed ID-14 cells, was responsible for the increase in P89 activity.

Identification of the trans-activator gene region regulating the P89 promoter

A positive, *trans*-acting regulatory gene function has been assigned to the E2 ORF of BPV-1 by Spalholz *et al.* (1985). To determine which BPV early gene(s) encode factors involved in P89 *trans*-activation, we constructed a second set of plasmids, BPV 'expression vectors' (Figure 2). The early gene domain of BPV-1 was subdivided into restriction fragments comprising the upstream transforming region (E6-E7; Schiller *et al.*, 1984), the downstream transforming region (E2-E5; Nakabayashi *et al.*, 1983) and the E1 ORF implicated in replication. High-level transcription of the inserted BPV fragments in transfected cells was directed by the strong promoter−enhancer of the RSV LTR, and transcription termination and mRNA polyadenylation signals were provided by a segment of SV40 DNA (Gorman *et al.*, 1983). pRSV-*neo* (which expresses the bacterial *neo* gene in transfected cells; Gorman *et al.*, 1983) was used as a negative control.

The pRSV-BPV 'expression vectors' were tested for P89 *trans*-activation in transient cotransfection assays in uninfected mouse NIH-3T3 cells, and in monkey CV-1 cells. The latter support transient expression of chimeric plasmids containing the SV40 promoter and mRNA processing sequences, and the use of transient assay eliminates potential clonal variation in BPV gene expression in individual established BPV-transformed cell lines (Spalholz *et al.*, 1985). In preliminary experiments, we had determined that sequences from nt 7476 (*Cla*I) to nt 90 were sufficient to detect regulated promoter activity in this region (see also Figures 4 and 5 below). The BPV P89 promoter clone pCla-90-*cat* 'a' (further referred to as pP89-*cat*) was therefore used as a target to monitor *trans*-activation, and the heterologous SV E promoter, devoid of a functional *cis* enhancer, served as a control (clone pSV E-*cat*; Figure 1).

Table I. Promoter activity of BPV-1 URR fragments[a]

cat vector	BPV fragment[b]	Orientation[c]	CAT activity		
			BPV-1 transformed ID-14	Uninfected C127I	Uninfected NIH-3T3
pSVE-*cat*	None		1.0	1.0	1.0
pRSV-*cat*	None		11.4	8.3	11.8
pHindIII-HpaI-*cat*	6959−7945	a	0.3	0.2	0.1
pHinc-Nci-*cat*	7143−78	a	<0.1	0.1	0.1
pXbaI-90-*cat*	6133−90	a	>20	2.0	4.3
pXbaI-90-*cat*	6133−90	b	0.8	0.5	0.5

[a]CAT enzyme activity in transfected cultures was determined as described in Materials and methods, and is expressed relative to the CAT enzyme of the enhancer-negative clone pSV E-*cat*. Each value is the average of two or more experiments with two or more plasmid DNA preparations.
[b]Numbers give first nucleotide of restriction sites in BPV-1 sequence.
[c]'Sense' (a) or 'antisense' (b) to BPV and *cat* gene transcription.

The results are shown in Table II. As predicted, cotransfection with the pRSV.d69 vector, which contains early BPV coding sequences between nt 1 and 4451, stimulated CAT enzyme activity encoded by the BPV P89 promoter clone, pP89-*cat*, ~5-fold in NIH-3T3 cells. The E2-E5 gene domain vector, pRSV-E2-E5, *trans*-activated the pP89-*cat* clone ~15-fold in NIH-3T3 cells, and greater than 50-fold in CV-1 cells (Table II, and see below). The E1 vector did not consistently modulate target expression in comparison with pRSV-*neo* controls. No significant *trans*-activation of the pP89-*cat* clone was seen with the pRSV-E6,7 clone compared with the pRSV-*neo* control. However, the pSV E-*cat* clone expression was reproducibly elevated 1.5- to 3-fold by cotransfection with the pRSV-E6,7 clone. To our surprise, both the pRSV.d69 and the pRSV-E2-E5 vectors also *trans*-activated the control SV E promoter target clone, pSV E-*cat*, but to a lesser degree (a 3- to 5-fold stimulation was seen in cotransfection with pRSV.d69, and a 5- to 8-fold increase with pRSV-E2-E5). The authentic BPV P89 promoter was therefore strongly *trans*-activated by a gene function encoded in the BPV E2-E5 gene domain (this function is further referred to as the BPV-specific *trans*-activator function, or E2 function A). This *trans*-activator function corresponds to that described by Spalholz *et al.* (1985), as documented below. In addition, the E2-E5 domain was also found to encode a factor which increases the activity of the heterologous SV E promoter in these assays (defined here as 'general' *trans*-activation, function B).

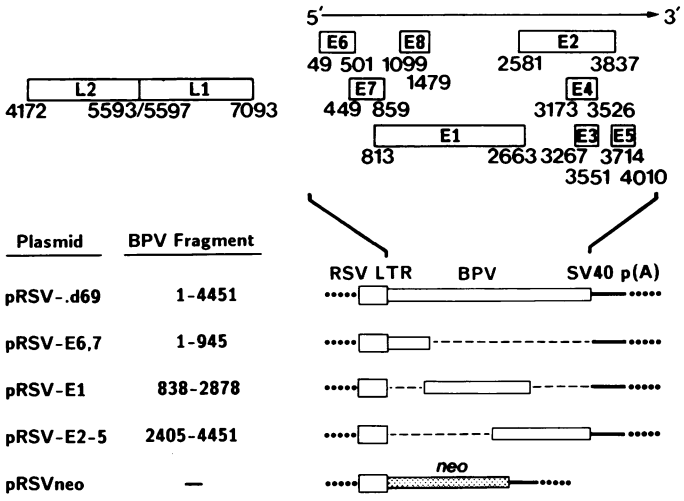

Fig. 2. pRSV-BPV fragment expression vectors. The entire early 69% fragment of BPV-1 or smaller fragments as indicated were subcloned downstream of RSV LTR, and upstream of SV40 splice and polyadenylation signals to assure their optimal expression in transfected mammalian cells.

Table II. *trans*-activation of *cat* gene expression by cotransfection with BPV early gene vectors[a]

DNA	NIH-3T3		CV-1	
	pP89-*cat*	pSV E-*cat*	pP89-*cat*	pSV E-*cat*
pRSV.69	5.5	2.7	ND	4.5
pRSV-E6-E7	0.5	1.5	0.5	2.5
pRSV-E1	0.3	0.8	0.5	0.9
pRSV-E2-E5	18.1	5.4	57.3	8.0
pRSV-*neo*	1.0	1.0	1.0	1.0

[a]Cotransfection experiments were performed with 5 μg of *cat* target vector DNA and 2.5 μg of the respective pRSV-BPV vector (Figure 2). Values represent the average change in CAT expression compared with pRSV-*neo*-cotransfected controls in two to three experiments. Baseline CAT values for the pP89-*cat* (Cla-90) vector in comparison with pSVE-*cat* were ~2- to 4-fold lower in NIH-3T3 cells, and ~9- to 15-fold lower in CV-1 cells.

Trans-activation of P89 requires active expression of the viral E2-E5 genes

Although the pRSV-BPV expression vectors were designed to synthesize BPV gene products in transfected cells, BPV DNA sequences in the molecular clones might provide 'repressor trap' elements that would competitively bind putative repressor-like cellular factors. To investigate this possibility, we constructed

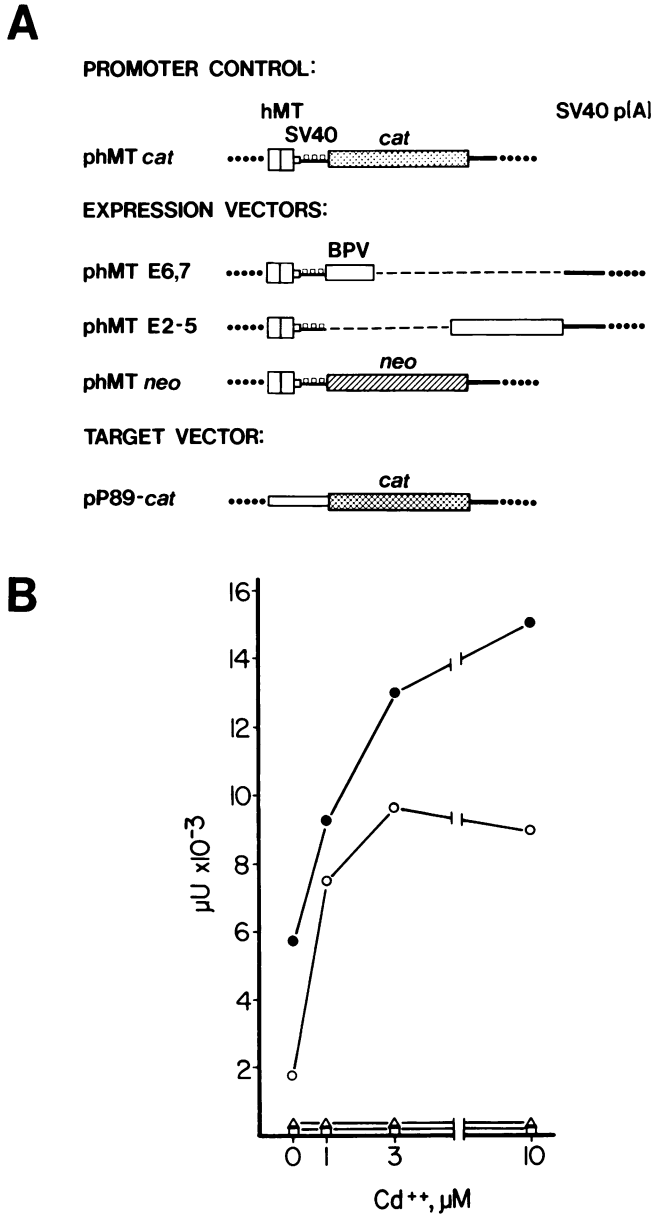

Fig. 3. Cadmium induction of P89-*cat* *trans*-activation by an E2-E5 gene under metallothionein (hMT) promoter control. **(A)** Molecular clones: phMT-*cat* used as a control of hMT promoter induction. phMT-E2-E5 and phMT-E6,7 contain BPV gene domains under control of the hMT promoter; the BPV fragments correspond to pRSV BPV vectors in Figure 2. pP89-*cat* served as target for *trans*-activation. **(B)** Average CAT enzyme activity as a function of Cd induction. Duplicate CV-1 cultures were transfected with 3 μg *cat* DNA together with 1.5 μg phMT expression vector, and treated with increasing Cd^{2+} concentrations. pP89-*cat* with phMT-*neo* vector (□); pP89-*cat* with phMT-E2-E5 (●); pP89-*cat* with phMT-E6,7 (△); phMT-*cat* alone (inducible promoter control) (○).

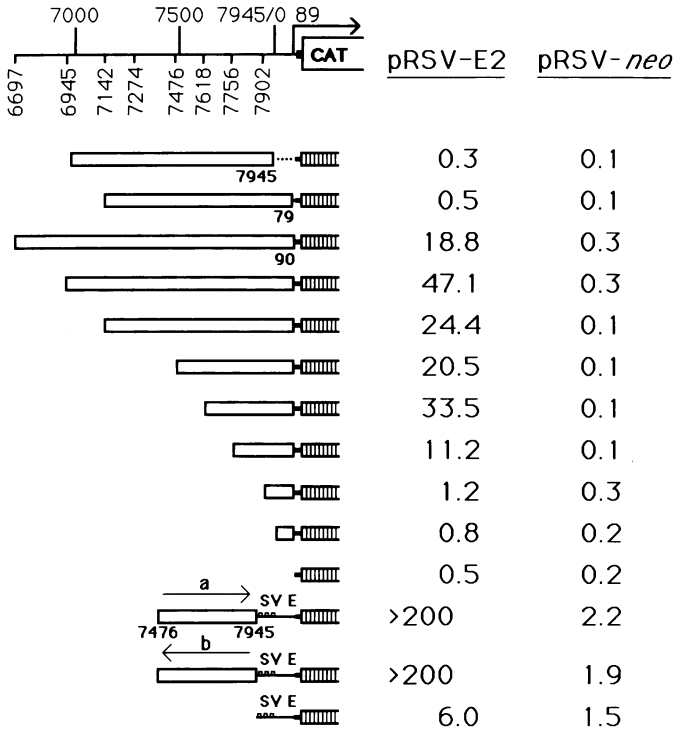

Fig. 4. Deletion mapping of 3′ and 5′ boundaries of the BPV-1 P89 promoter and the *E2R* target element. Different BPV URR fragments between nt 6133 and 90, encompassing both putative promoters upstream of the BPV early genes (TATAA boxes at 7109 and 58) were tested for promoter activity in the promoter-less plasmid pU-*cat* upon cotransfection into CV-1 cells with pRSV-E2, expressing the E2 function of BPV-1 (described in text), or with pRSV-*neo*. The *E2R* region upstream of P89 was examined for enhancer activity in pSV E-*cat* in the presence and absence of E2. CAT enzyme activity is given in milliunits/culture × h.

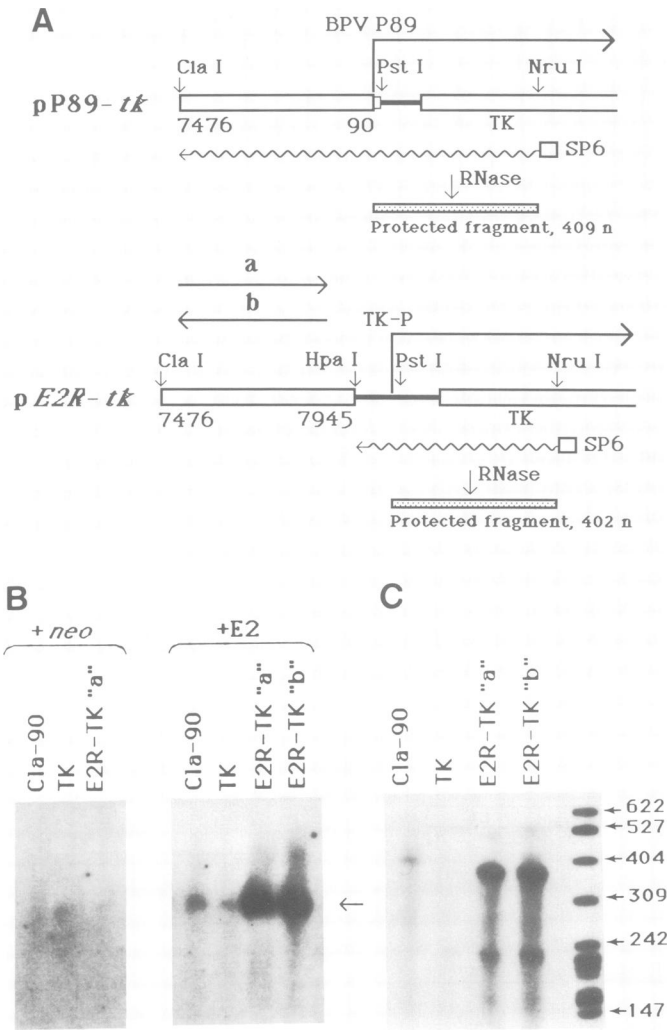

Fig. 5. Analysis of BPV P89 and *E2R tk* mRNA in CV-1 cells cotransfected with *tk* target vectors, and pRSV-E2-E5 or pRSV-*neo* (control). **(A)** pP89-*tk* and p*E2R-tk* target vectors, and SP6 clones used to synthesize complementary riboprobes. Predicted sizes of RNase-protected fragments are indicated by bars. **(B)** Northern blot analysis of *tk* mRNA levels 60 h after transfection. **(C)** Mapping of 5′ ends of *tk* mRNA by RNase protection.

cadmium-inducible E2-E5 and E6-E7 plasmids (Figure 3A). BPV gene transcription in these constructions was modulated by a duplicated heavy metal-inducible enhancer of the human metallothioneine gene-II_A (hMT-II_A; Haslinger and Karin, 1985), attached in the 'sense' orientation to the SV E promoter (Scholer *et al.*, 1986). These inducible vectors were cotransfected with the BPV P89 promoter clone pP89-*cat* (Figure 3B) into CV-1 cells, and CAT enzyme levels were determined after treatment of the recipient cultures with increasing $CdCl_2$ concentrations.

The phMT-*cat* clone (Figure 3A; Scholer *et al.*, 1986), in which the chimeric hMT-SV E promoter directs CAT enzyme synthesis in *cis*, served as a control of promoter response to Cd^{2+} induction. The hMT-SV E promoter (open circles in Figure 3B) showed a moderately high activity in the absence of induction. CAT enzyme levels were stimulated up to ~6-fold in response to increasing Cd^{2+} concentrations under these conditions. In contrast, expression of the BPV P89 promoter clone, pP89-*cat*, was not induced by Cd^{2+} treatment in *trans* in cotransfection with the phMT-*neo* clone which served as a negative control (squares in Figure 3B).

Expression of the BPV P89 promoter in the pP89-*cat* clone was strongly *trans*-activated in Cd^{2+}-treated cultures cotransfected with the phMT-E2-E5 inducible expression vector) up to ~100-fold over the phMT-*neo* control; closed circles in Figure 3B). Furthermore, induction of *trans*-activation followed the same $CdCl_2$ dose response as that of the hMT-SV E promoter in the plasmid phMT-CAT. These data indicate that induced transcription of BPV gene(s) in the phMT-E2-E5 plasmid increases the *trans*-activating effect, and that the *trans* effect seen in cotransfections is not due to passive binding of potential cellular repressors to BPV DNA sequences in the E2-E5 region. The pP89-*cat* expression was unaffected by E6-E7 function(s) in these experiments (open triangles in Figure 3B).

Localization of the E2-responsive promoter element

To identify specific E2-responsive (*E2R*) promoters of the P89 promoter, we tested the effect of 3′ and 5′ deletions in the BPV sequences of pXba-90-*cat* on promoter activity. Clones with a modified P89 region were cotransfected into CV-1 cells either with the pRSV-*neo*, or with the BPV-1 E2-expressing vector, pRSV-E2 (pRSV-E2-E5-*Bst*X-d, which has a mutated E5 ORF see below). At the 3′ end, BPV sequences to nt 90 were required for promoter activity, as had also been true in ID-14 cells (Table I). A series of 5′ deletions in the active clone pXba-90-*cat* was then assayed in cotransfection with pRSV-E2 (Figure 4). Deletions of upstream sequences down to nt 7618 (*Rsa*I) did not impair the essential E2 response of the P89 promoter. The deletion between nt 7618 and 7756 (*Rsa*I) reduced P89 promoter

response to *trans*-activation ~3-fold. Residual fragments encompassing nt 7902 (*Hpa*II) −90 or nt 7945 (*Hpa*I) −90 lacked significant promoter response to E2 cotransfection in these experiments. Activity of all P89 fragments remained low in the absence of E2.

The specific response of P89 to the E2 *trans*-activator gene product was therefore mediated by a *cis* sequence (the *E2R* element), located in the URR immediately upstream of the putative CAAT box of P89 (Chen *et al.*, 1982; Danos *et al.*, 1983). When a fragment encompassing the *E2R* element (7476−7945; *Cla*I−*Hpa*I) was linked to the SV E promoter in the plasmid pSV E-CAT in either orientation (Figure 4), it functioned as an enhancer-like *cis* element dependent on an E2 gene function. These results confirm and extend the work of Spalholz *et al.* (1985) who have previously detected an E2-dependent enhancer within the URR. We have mapped the essential *E2R* element sequences to nt −277 to −131 upstream of the expected cap site at nt 89.

Trans-activation of the E2R element leads to enhanced mRNA levels

Since the *E2R* element was found to be located outside of the predicted transcriptional unit of pP89-*cat*, and since ligation of the *E2R* element in *cis* in either orientation conferred high susceptibility to E2 *trans*-activation upon a heterologous promoter, *trans*-activation of the P89 promoter would be expected to occur at the level of mRNA transcription. To examine directly the effect of E2 *trans*-activation on mRNA levels we have constructed target plasmids based on the HSV-1 thymidine kinase (*tk*) gene (Figure 5). HSV-1 *tk* mRNA transcripts are more stable in transiently transfected cells than chimeric *cat* mRNAs (M.Karin, unpublished), and this facilitates their quantitation and mapping. In the plasmid pP89-*tk*, the *Cla*-90 (7476−90) BPV fragment was linked to the *tk* gene at nt +14 (*Pst*I). In this construction, predicted transcription initiation depends on the BPV P89 promoter. In the two p*E2R-tk* clones 'a' and 'b', the *E2R* element-containing BPV-1 fragment between nt 7476 (*Cla*I) and 7945 (*Hpa*I) was linked upstream of the intrinsic *tk* promoter at nt −109 in the clone p*tk*(−109) (Karin and Richards, 1982). Transcriptional activity of both these clones depends on the activity of the *E2R* element as a *cis* enhancer.

These constructions, as well as the enhancer-negative control clone p*tk*(−109) (Karin and Richards, 1982), were cotransfected into CV-1 cells with pRSV-E2-E5 (Figure 5B, panel labeled +E2), or pRSV-*neo* as a control (Figure 5B, panel +*neo*). HSV-1-specific, poly(A)-selected *tk* RNA 60 h after transfection was detected by Northern blot analysis (Figure 5B), and quantitated by densitometric scanning. pP89-*tk* mRNA (lanes labelled Cla-90 in Figure 5B) of the predicted size (~1.4 kb) was detectable only in the presence of the E2 gene product(s). *tk* mRNA was detectable in cells cotransfected with the target clone p*tk*(−109), containing the intrinsic *tk* promoter, and the pRSV-*neo* control, as a faint band in Figure 5B (lane labelled TK in panel +*neo*), and was readily detectable on longer exposure. Linkage in *cis* of the *E2R*-containing fragment in either orientation increased *tk* mRNA levels less than 1.5-fold in the absence of E2 (lane E2R-TK a in panel +*neo*, and results not shown). Cotransfection of either p*E2R-tk* clone ('a' or 'b') with the E2 expression vector (shown in panel +E2) resulted in a 34- and 39-fold increase in steady-state mRNA levels, respectively. The enhancer-negative control clone p*tk*(−109), when cotransfected with pRSV-E2-E5, exhibited a 3- to 4-fold higher *tk* mRNA level in three independent experiments (Figure 5B, lane TK in panel +E2, and not shown). Since the increase in steady-state *tk* mRNA correlated closely with the changes in *cat* enzyme activity in response to E2 *trans*-activation, we conclude that both *trans*-activation functions of the E2 gene domain, the BPV-specific *trans*-activation mediated in *cis* by the *E2R* element (function A), as well as the 'general' *trans*-activation of heterologous promoters, independent of the specific *E2R cis* element (function B) act primarily at the level of mRNA transcription or stabilization.

The transcript sizes (~1.4 kb) in all cases implied the utilization of the predicted promoters (Figure 5A). To map the transcription initiation sites more precisely, we performed RNase protection analysis of the 5′ ends of the *tk* mRNAs. Uniformly labelled, single-stranded RNA probes, complementary to the mRNA coding strand, were synthesized *in vitro* with SP6 polymerase as shown diagrammatically in Figure 5A, and annealed to poly(A)-selected RNA from CV-1 cells 60 h after cotransfection with pRSV-E2-E5. After RNase digestion, protected RNA fragments were resolved on urea−acrylamide gels (Figure 5C). RNA from cells cotransfected with the pP89-*tk* plasmid yielded a protected RNA fragment of ~400 nt, in good agreement with the size predicted for a 5′ cap site at nt 89 of BPV-1 (409 bp; Figure 5A). As fragments of other sizes were not readily detectable, we conclude that the major promoter which responds to E2 *trans*-activation in the BPV fragment between nt 7476 and 90 most likely represents the authentic viral early promoter previously mapped to nt 89 (Stenlund *et al.*, 1985; Yang *et al.*, 1985a).

Analysis of RNAs from cells cotransfected with either the p*E2R-tk* 'a' and 'b' resolved a slightly shorter RNA fragment, in agreement with the predicted smaller size of the protected region (402 nt; Figure 5A). A minor protected fragment of ~220 nt was also observed in both these lanes; it is unclear whether it represented mRNA moieties starting at a cryptic promoter within the *tk* coding sequence (Karin and Richards, 1982). Longer exposure of the fluorogram revealed a fragment of identical size in RNA from cells cotransfected with the p*tk*(−109) clone and pRSV-E2-E5 (faintly visible in Figure 5C). Therefore, *cis* linkage of the *E2R* element strongly activated the authentic *tk* promoter in response to an E2 gene product, and the transcriptional response was not due to activation of promoters within the upstream BPV *E2R* or pUC plasmid sequences. Furthermore, specific *trans*-activation required the presence of the *E2R* element upstream from the transcription start site, strongly favouring promoter activation, rather than post-transcriptional events, as the mechanisms of E2 gene product action.

Specific and general trans-activation functions are inactivated by a mutation in the E2 ORF

Activation of the BPV P89 promoter (function A; Table II, and see also Figures 4 and 5), specific *trans*-activation of the *E2R* element (Figures 4 and 5) and general *trans*-activation of heterologous genes (function B; Table II and Figures 4 and 5) could represent related effects of the same BPV function or, alternatively, could be encoded in different viral genes in the E2-E5 region. We have examined the effect of molecular mutations in the pRSV-E2-E5 expression vector to test these possibilities, and to confirm or exclude the identity of the *trans*-activator(s) as the product(s) of the E2 ORF (Spalholz *et al.*, 1985). The mutation in pRSV-E2-E5-*Nco** inserts termination codons into all three possible peptide reading frames at the *Nco*I site (nt 2878). This site is located in the N-terminal region of the E2 ORF which does not overlap with other ORFs (E3 or E4). Deletion of 4 nt

Table III. Transformation and *trans*-activation by BPV expression vectors and their mutants

BPV plasmid[a]	*trans*-activation[b]				C127I transformation[c]
	pP89-*cat*	p*E2R*-SV E-*cat*-'b'	pRSV-*cat*	pMSV-*cat*	
BPV/pMLd	ND	ND	ND	ND	393 ± 194
pRSV-d69	ND	12.8	6.8	ND	26.0 ± 11.2
pRSV-E2-E5	97.3	>120.0	10.7	8.3	27.6 ± 11.6
pRSV-E2-E5 *Nco**[d]	1.7	1.8	1.6	2.3	48.0 ± 16.8
pRSV-E2-E5 *Bst*Xd[d]	75.2	>120.0	11.3	18.2	<0.25
pRSV-*neo*	1.0	1.0	1.0	1.0	<0.25

[a]BPV plasmids and mutants are described in the text.
[b]The average increase in CAT enzyme activity in duplicate CV-1 cultures cotransfected with 3 μg *cat* vector and 1.5 μg pRSV-BPV vector, compared with pRSV-*neo* cotransfected controls, in two experiments.
[c]Average (± SD) focus-forming units/1 μg BPV clone DNA in duplicate C127I cultures in two experiments.
[d]Results represent average obtained with two independently constructed mutant clones.

at the *Bst*XI site (nt 3881) in the pRSV-E2-E5-*Bst*X-d clone results in a frameshift mutation in the E5 ORF, and inactivates the E5 transforming gene (Groff and Lancaster, 1986; Schiller *et al.*, 1986; DiMaio *et al.*, 1986). These mutants were compared with wild-type pRSV-E2-E5 for *trans*-activation and cell transformation. In addition to the *cat* target clones tested previously, we have also assayed for potential *trans*-activation of two highly active *cat* vectors driven by the strong enhancer–promoter regions of a chicken and a murine retroviral LTRs (pRSV-*cat* and pMSV-*cat*).

The results are shown in Table III. Specific *trans*-activation of the pP89-*cat* target vector (function A) as well as the activation of the *E2R* enhancer-SV E promoter clone p*E2R*-SV E-*cat* 'b' was reduced from very high to background levels by the E2 termination mutation (Table III). The pRSV-*cat* and pMSV-*cat* clones, containing LTR enhancer–promoter regions of unrelated retroviruses, were *trans*-activated by the E2-E5 clone to a lesser degree, but the E2 mutant also lacked the capability to *trans*-activate these heterologous promoter clones. In contrast, the E2 mutation did not influence the capacity of the pRSV-E2-E5 vector to transform C127I cells. Cell transformation was abolished by the E5 mutation which did not influence either specific or general *trans*-activation. These data confirm the assignment of the downstream transforming gene to the E5 ORF (Schiller *et al.*, 1986; Groff and Lancaster, 1986; DiMaio *et al.*, 1986; Yang *et al.*, 1985b). Therefore, both the specific and general *trans*-activation functions were found to require translation of an intact peptide product of at least the N-terminal portion of the E2 ORF, but not that of the E5 ORF.

Discussion

The data presented here show that the P89 early gene promoter of BPV-1 is strongly *trans*-activated by the regulatory gene product encoded in the viral E2 ORF. The authenticity of the promoter has been verified by genetic dissection as well as by direct mapping of the 5′ end of the chimeric mRNA to the predicted correct transcription initiation site by RNase protection experiments. The E2-mediated *trans*-activation of the P89 promoter results in greatly increased levels of correctly initiated mRNA (Figure 5). Although we have examined only steady-state mRNA, we strongly favor the hypothesis that P89 activation is the result of increased transcriptional rates rather than mRNA stabilization. This is because the E2-mediated, specific increase in mRNA levels depends on the presence of a short *cis*-linked element, the *E2R* sequence, which is located outside of the transcriptional unit. The *E2R* element acts as a strong transcriptional enhancer in either polar orientations both with the authentic BPV P89 early promoter, as well as with two heterologous promoters, the SV40 early promoter and the HSV *tk* promoter. The specific interaction between the *cis* element, *E2R*, and the diffusible E2 gene product therefore provides a well-defined model for genetic and biochemical studies on mammalian gene activation.

Androphy, Lowy and Schiller (personal communication) have recently determined that a purified E2 peptide prepared by molecular manipulations in bacteria specifically binds *in vitro* to several restriction fragments of cloned BPV DNA. Two E2 binding sites with the strongest affinity are located within the *E2R* element we have mapped in *in vivo* transfections (nt 7758–7902), and another two are found in the 5′ adjacent URR segment which further potentiates E2 response (nt 7618–7758; Figure 4). The core sequence for E2 peptide binding corresponds to the palindrome, $ACC(N)_6GGT$, which is also present in the URR of other papilloma viruses (Dartmann *et al.*, 1986). The URR of HPV-16 (T.Cripe, T.Haugen and L.Turek, unpublished) as well as the HPV-8 URR (R.Seeberger, T.Haugen, L.Turek and H.Pfister, preliminary data) also specifically respond to the BPV E2 *trans*-activator in enhancer tests. It may well be possible that the specific *trans*-acting E2 function A is mediated by direct binding of the E2 ORF gene product to the *E2R* target element. This would be of great interest since most viral *trans*-acting proteins, such as the E1A proteins of adenoviruses, interact with cellular factors rather than bind directly to specific DNA promoters (Nevins, 1986).

The E2 *trans*-activator gene resembles the adeno E1A gene, however, in that its product is also capable of non-specific *trans*-activation of several heterologous genes in transient cotransfections (this adeno E1A-like function is designated 'general' *trans*-activation, or E2 function B, in this paper). Unrelated promoters which appear to respond to E2 function B *trans*-activation so far included the SV40 early promoter with or without the 72 nt enhancer repeat (Tables II and III, and data not shown), the long terminal repeat enhancer–promoter regions of RSV and MSV (Table III), and the authentic HSV-1 *tk* promoter (Figure 5B). It is unclear whether function A and function B reside in the same peptide molecule, or are carried out by proteins which result from differential mRNA splicing of the E2 region. In the latter case, general E2 *trans*-activation (function B) might act via another mechanism, such as mRNA stabilization, since it increases the expression of all the chimeric promoter clones we have tested so far.

One attractive model is that an E2 *trans*-activator protein would

have at least two separate domains for *E2R* sequence binding, and for transcriptional activation. E2 function B would then represent the general capability of this E2 gene product to stimulate promoter utilization. Specific binding of the peptide to the *E2R* element would effectively increase the concentration of the activator domain at the selected promoter, and mediate function A. In at least two other specific *trans*-activators of eukaryotic mRNA transcription, the glucocorticoid receptor (Giguere *et al.*, 1986) and the GCN4 protein of yeast (Hope and Struhl, 1986), DNA binding and regulatory domains on the same protein molecule have been identified by site-specific mutagenesis. Since the *Nco** mutant, located in the N-terminal portion of the putative E2 peptide, inactivates both specific and general E2 *trans*-activation, it will be of interest to see if E2 functions A and B can be separated by mutations localized further towards the carboxyl terminus of the E2 ORF. These possibilities need to be resolved in complementation tests with different E2 mutants, and by direct characterization of the physical and binding properties of the wild-type and mutant proteins.

As the transcriptional map of the early region of BPV-1 is still incomplete, we can only speculate about which viral genes are influenced by E2 action. Viral mRNAs with a transcription start site at nt 89 are spliced to encode an intact E6 peptide, and a potential spliced E6/7 gene product. The E6 protein represents one of the two transforming gene products of BPV-1 (Schiller *et al.*, 1984; Yang *et al.*, 1985a; Androphy *et al.*, 1985), and both the E6 as well as the E6/7 gene products are required for positive modulation of plasmid copy numbers in stable plasmid maintenance (the *cop* function; Lusky and Botchan, 1985; Berg *et al.*, 1986a,b). This could explain why mutations in the E2 ORF impair transformation efficiency, and abolish unintegrated plasmid replication (Sarver *et al.*, 1984; Lusky and Botchan, 1985; DiMaio, 1986; Groff and Lancaster, 1986). In addition, P89-initiated mRNAs with coding potential for E6/4 and E6/1 proteins of unknown function have been found in BPV-transformed mouse cells (Yang *et al.*, 1985a; Stenlund *et al.*, 1985). Whether the P89 promoter directs the synthesis of less abundant mRNAs representing other genes remains to be determined. Other viral gene functions are likely to directly or indirectly modulate P89 transcription since the level of P89 initiated mRNAs are low in established BPV transformants (Stenlund *et al.*, 1985).

It is also possible that the E2 gene product regulates other viral promoters. First, the *E2R* element can act as an enhancer at a distance from the promoter (Spalholz *et al.*, 1985). Second, the E2 *trans*-activator gene can modulate mRNA levels of unrelated genes via its function B. Third, Androphy, Lowy and Schiller (personal communication) have identified two additional E2 binding domains outside of the region upstream of P89; these may mediate specific regulation of additional viral promoters. Finally, since the E2 *trans*-activator gene(s) have the capability to influence heterologous gene expression, they could potentially contribute to cell transformation by activating cellular genes. The E2 ORF region itself does not transform established mouse cell lines. The molecular constructions described here will enable us to determine whether the E2 *trans*-activator gene product(s) play(s) a role in cell immortalization or transformation in primary recipient cells.

Materials and methods

Target plasmids containing BPV-1 promoter and enhancer fragments

The promoter-less plasmid pU-*cat* contains the bacterial chloramphenicol acetyl transferase gene, *cat* (E.C. 2.3.1.28), and 3′ sequences from SV40, in pUC8 (Scholer *et al.*, 1986). BPV-1 fragments tested for promoter activity were excised at restriction sites shown in Table I, and inserted at a *Hin*dIII site 5′ to *cat* (Figure 1); coordinates (*n*) correspond to the first nucleotide of the enzyme recognition site in BPV-1. Molecular cloning followed standard protocols (Maniatis *et al.*, 1982). The 3′ end at nt 90 was obtained from a linker insertion mutant, pXH731 (a gift of J.Schiller), which has an 8-mer *Xho*I linker (CCTCGAGG) between nt 90 and nt 99 (Schiller *et al.*, 1984); the *Xho*I site was used for cloning. The *Cla*I (7476) to 90 (*Xho*I in pXH731) fragment-containing *cat* clone is referred to as pP89-*cat* in Tables II and III, and in Figure 3.

The plasmid pSV E-CAT contains the SV40 early promoter (SV E), but not a functional enhancer [SV40 sequences from nt 128 (*Sph*I) to nt 5171 (*Hin*dIII)], inserted into pU-*cat*. A *Cla*I linker site upstream of SV E (at nt 128) was used to insert putative BPV enhancer fragments (Figures 1 and 4). All HSV-1 thymidine kinase (*tk*) clones were derived from the clone p*tk*(−109) (Karin and Richards, 1982) as described in Figure 5A and in the text. Plasmids used in transfection experiments were purified twice on CsCl gradients. Concentration, configuration and absence of contaminating RNA were verified on ethidium bromide-stained agarose gels.

Vectors expressing BPV-1 gene domains

The pRSV-*neo* plasmid contains the neomycin resistance gene of transposon Tn*5* (E.C. 2.7.1) under transcriptional control of the RSV LTR promoter, and 3′ signals from SV40 (Gorman *et al.*, 1983). Mammalian cells transfected with pRSV-*neo* are resistant to the aminoglycoside antibiotic G418 (Geneticin, GIBCO). This plasmid served as a selective marker in transformation experiments. pRSV-BPV expression vectors were made by replacing the *neo* gene cassette (*Hin*dIII to *Bam*HI) with fragments of the early region of BPV-1 DNA (Figure 2). Although the E1 ORF starts at nt 813, the first methionine triplet, ATG, is located at position 849; the E1 clone therefore contains the potential E1 peptide-coding sequence in its entirety. Inducible BPV vectors are described in the test and in Figure 3A. In pRSV-E2-E5-BstX-d, a frameshift mutation (a 4 nt deletion) in the E5 ORF was made at the *Bst*XI site (nt 3881) by digesting overhanging 3′ ends with T4 DNA polymerase, followed by ligation. In pRSV-E2-E5-Nco*, termination codons were introduced into all three reading frames in the N-terminal part of the E2 ORF at the *Nco*I site (nt 2878) by insertion of an *Xba*I 8-mer linker, followed by *Xba*I digestion, fill-in of ends, and insertion of a *Hpa*I 6-mer linker, resulting in the final sequence CTC*TAGGTTAACCTAG*AG.

Cells and transfections

Mouse C127I fibroblasts (passage 3), NIH-3T3 cells, CV-1 monkey cells and BPV-transformed ID-14 cells (C127I fibroblasts transformed with BPV-1 virion DNA; Law *et al.*, 1981; Turek *et al.*, 1982) were transfected by the calcium phosphate coprecipitation procedure as described (Sherr *et al.*, 1979). For transient *cat* expression assays, cells were plated at $3-4 \times 10^5$ cells per 35 mm well. Each DNA combination was transfected into duplicate wells at 6 μg (adjusted with calf thymus DNA) precipitated in 0.2 ml per well. C127I, ID-14, other C127I-derived lines and CV-1 cells were treated with 24% DMSO in 1× Hepes-buffered saline 4−5 h after transfection; NIH-3T3 cells were treated with 17% glycerol. All murine cells were incubated with 0.06 mM chloroquine between DNA addition and treatment. CAT enzyme was assayed in freeze-thaw cell lysates 60 h later, using [^{14}C] chloramphenicol (Cm; 40−60 mCi/mmol; NEN) as substrate, and 1 mM acetyl coenzyme A (PL Inc) as acetyl group donor (Gorman *et al.*, 1983). Acetylated Cm fraction was separated by thin layer chromatography and scintillation counted.

Relative concentration of cell lysates was determined by absorbance at 260 nm of a 1:100 dilution in 10% SDS. Standard curves were obtained by serial 2-fold dilutions of bacterial CAT (PL Inc.) in uninfected cell lysate; their slope was used to convert relative acetylation values to CAT enzyme activity in microunits (μU). Enzyme activity was linear from 20 to ~5000 μU per sample per hour (50% conversion under our conditions). Values above this range would underestimate *cat* expression in transfected cells, and such samples were assayed at dilution where required.

For transformation, 1.5×10^5 C127I cells were seeded per 35 mm well, and transfected 24 h later with coprecipitated BPV vector DNA, and 0.4 μg pRSV-*neo* plasmid. The cultures were replated in 100 mm dishes in media containing 375 μg/ml active G418 48 h later, re-fed twice a week with G418 media for 12−16 days, and then with non-selective media. Transformed cell foci were counted 16−28 days post-transfection under a microscope. Transformation-negative cultures were maintained for up to 10 weeks. Although final focus numbers were not significantly influenced, foci of transformation appeared earlier and reached larger size under initial G418 selection.

RNA analysis

Cellular RNA was prepared by the procedure of Chirgwin *et al.* (1979), poly(A)-selected once, and analyzed by Northern blotting (Thomas, 1980) at 2 μg/lane, using SP6 riboprobes complementary to *tk* mRNA. Quantitative RNase mapping (Zinn *et al.*, 1983) was performed using uniformly labelled SP6 riboprobes (Melton *et al.*, 1984) from linearized DNA templates in Figure 5A. 5 μg of poly(A) RNA

were annealed to labelled riboprobes at 45°C for 8–12 h. The RNase resistant fragments were resolved on 5% acrylamide gels containing 7 M urea. SP6 riboprobe clone construction in the Gem II plasmid (Promega Inc.) and predicted size of protected fragments are given in Figure 5A.

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Work in this paper was presented in part at the meeting 'Cancer Cell 5: Papillomaviruses', held at the Cold Spring Harbor Laboratory, USA, September 3–7, 1986. Additional data to be given in detail elsewhere show that the BPV P89 early promoter *trans*-activation requires at least two single ACCG(N)$_4$CGGT palindromes near the boundaries of the E2R element mapped by deletions (Figure 4). The E2 response of P89 is abolished in deleted promoter fragments containing only one palindromic site, but the *cis* activity of the deleted fragments in response to the E2 (function A) is fully restored by insertion of a second, synthetic consensus ACCG(N)$_4$CGGT site. These data also suggest a possible mechanism for the high specificity of P89 promoter *trans*-activation by the E2 (function A) gene product, involving cooperative binding of the regulatory factor(s) to two or more ACCG(N)$_4$CGGT target sites.