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Cellular factors are required to activate bovine papillomavirus-1 early gene transcription and to establish viral plasmid persistence but are not required for cellular transformation

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ABSTRACT

Transcription from the major upstream early gene promoter, P89, of bovine papillomavirus (BPV)-1 is detectable in transfected cells lacking viral gene products yet also responds to viral E2 proteins. In contrast to human papillomaviruses (HPVs), the BPV upstream regulatory region (URR) functions as a transcriptional enhancer in epithelial cells and fibroblasts of bovine, murine or human origin. Mutations of Sp1 and/or two novel transcriptional enhancer factor (TEF)-1 sites within the 5′ URR of the intact BPV-1 genome dramatically reduced P89-initiated mRNA levels, leading to decreased BPV-1 plasmid amplification and inefficient formation of transformed cell foci. However, cell lines transformed with *wt* or mutant BPV-1 genomes contained similar levels of unintegrated BPV-1 DNA, P89 mRNA and E2-dependent transactivation. We conclude that cellular factors necessary for activating viral early gene transcription, establishment of viral plasmid replication and cell immortalization are not required during the maintenance phase of BPV-1 infection.

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Introduction

Papillomaviruses induce a wide range of epithelial neoplastic growths ranging from benign papillomas or warts to invasive cancers in humans and animals. In all known cases, infection with mucosal and cutaneous human papillomavirus (HPV) strains is restricted to epithelial cells of the skin, mucosa or their adnexa. In contrast, some animal papillomaviruses cause fibropapillomas, that is, warts comprising an underlying proliferation of dermal fibroblasts which also harbor persistent viral plasmid genomes. The most extensively studied model, bovine papillomavirus (BPV) type 1, is an example (see Lambert, 1991; Turek et al., 1987 and DiMaio, 1991 for review).

In accord with BPV-1's ability to infect dermal fibroblasts *in vivo*, BPV-1 virions or recombinant BPV-1 DNA can neoplastically transform bovine or rodent fibroblasts in which the viral genome persists as unintegrated, supercoiled circular BPV-1 plasmids (Dvoretzky et al., 1980; Lancaster, 1981; Law et al., 1981; Lowy et al., 1980). Cell transformation absolutely depends on the continued expression of at least some viral genes because fibroblasts that have lost BPV-1 genomes due to interferon treatment revert to the nontransformed phenotype (Turek et al., 1982).

The regulation of BPV-1 early gene expression is still incompletely understood yet it is apparent that it differs from that of mucosal HPVs in several important aspects. Early gene transcripts of mucosal HPVs as well as BPV-1 start at a major promoter immediately upstream of the E6 gene, termed P89 or P2 in BPV-1 (Baker and Howley, 1987; Choe et al., 1989; Stenlund et al., 1985; Szymanski and Stenlund, 1991). In mucosal HPVs, the E6 promoter of HPV types 16 or 31 is one of the promoters active early after viral genome introduction into epithelial cells by transfection (Lace et al., 2008a) and in persistently infected basal keratinocytes; additional promoters are activated only during late stages of infection in differentiating keratinocytes (Meyers et al., 1992; Ozbun and Meyers, 1998). In contrast, BPV-1 early gene transcripts originate at three other promoters, P890 (P3), P2443 (P4) and P3080 (P5) in addition to the P89 promoter early after genome introduction in persistently infected fibroblasts (Szymanski and Stenlund, 1991).

Unlike more complex viral organisms, such as herpesviruses, papillomavirus particles do not include viral gene products, such as the E2 transactivator (E2-TA), that regulate viral gene expression and replication. Therefore, their immediate early transcription is entirely dependent on cellular factors. Mucosal HPVs have complex, cell type-dependent enhancers in their upstream regulatory regions (URRs)

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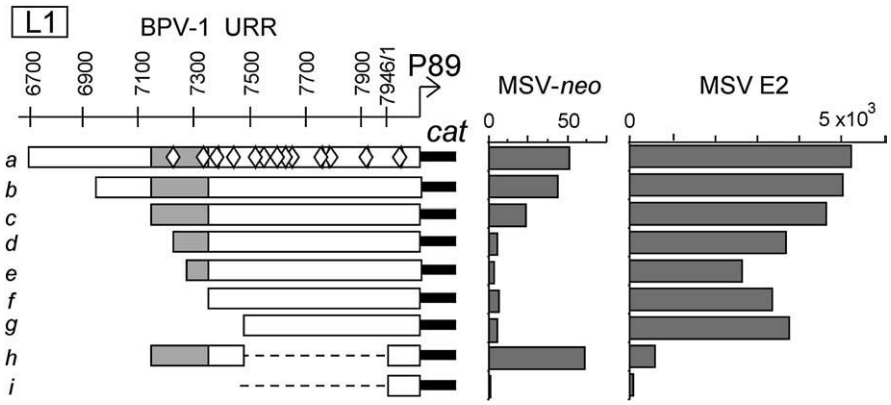

Fig. 1. The BPV-1 P89 promoter is activated by cellular factors in the absence of E2. BPV-1 fragments containing the P89 promoter were linked to the *cat* gene. The relative activity of the constructions was compared to the minimal P89 promoter in pP89-*cat* (nt 1–99; clone *i*) in mouse NIH-3T3 fibroblasts transiently cotransfected with a control plasmid (pMSV-*neo*) or with a clone expressing the full-length BPV-1 E2 protein (pMSV-E2). The baseline (+MSV-*neo*) activity of pP89-*cat* ("i") was ~5-fold lower than that of the enhancer-negative SV40 early promoter in pSVE-*cat* (not shown). Shaded box represents the BPV enhancer while the open diamonds represent the E2 binding sites (illustrated in the first sequence).

that activate early gene transcription (Chin et al., 1988; Cripe et al., 1990; Cripe et al., 1987; Gius et al., 1988; Gloss et al., 1987; Rando et al., 1986; Swift et al., 1987). The HPV enhancers are active in keratinocytes, cervical carcinoma cells and in some epithelial tumor cell lines, but do not function well in fibroblasts (Cripe et al., 1990). Cell type-specific activation of viral transcription thus correlates with the host range of HPV infection restricted to mucosal and cutaneous keratinocytes and related cells (see Bernard and Apt, 1994; Turek and Smith, 1996 for reviews).

In BPV-1, weak E2-independent enhancer activity was localized to a short segment in the distal part of the URR (Stenlund and Botchan, 1990; Stenlund et al., 1987; Vande Pol and Howley, 1990) and observed to activate the E2-TA-coding P2443 promoter (Spalholz et al., 1991; Szymanski and Stenlund, 1991). Deletion of the enhancer abrogated cellular transformation as well as BPV-1 DNA replication in fibroblasts but these activities could be restored by E2-TA (Vande Pol and Howley, 1992). This observation had suggested that the distal URR enhancer activates E2-TA expression from P2443, which in turn triggers the transcription of other viral early genes from P89 and other BPV promoters.

Nevertheless, other early promoters including P89 also are active in the absence of E2-TA. The BPV-1 URR was found to substitute for the promoter of thymidine kinase (*tk*) gene in *cis*, presumably by initiating *tk* transcription from P89 (Campo et al., 1983). Furthermore, P89 as well as other BPV early gene promoters are active in fibroblasts transfected with a BPV-1 E2 mutant (Szymanski and Stenlund, 1991). Taken together, these observations indicate that, similar to HPV major early promoters, transcription at the P89 promoter of BPV-1 is also activated by cellular factors. In contrast to HPVs, BPV-1 is transcriptionally active in fibroblasts in addition to keratinocytes and thus exhibits an extended host cell type range. We have defined the enhancer activity of the BPV-1 URR in greater detail, focusing on the contribution of cellular transcription factors to the broader BPV-1 cell type specificity and on its role in neoplastic transformation and viral persistence.

Results

Regulatory cis elements of the BPV-1 URR

While defining E2-dependent *trans*-activation (Haugen et al., 1987; Haugen et al., 1988), we observed that a BPV-1 P89 promoter fragment, extending ~1500 nt upstream of the transcription start site, had detectable activity in the absence of the E2 transactivator in transfected mouse C127I and NIH-3T3 fibroblasts. To map the *cis* elements of the P89 promoter responsible for its E2-independent activity, we constructed a set of plasmids containing parts of the 5′ *cis* sequences of P89 linked to the *cat* reporter gene (Fig. 1). The resulting pP89-*cat* constructions were cotransfected into uninfected NIH-3T3 cultures together with either a control plasmid (column "+pMSV-*neo*") or a clone expressing the BPV-1 E2 transactivator (column "+pMSV-E2").

P89 promoter activity in the absence of E2 required *cis* sequences between nt 7143 and the transcription start site (Fig. 1, clones *a*–*c*). Deletions in sequences between nt 7143 and nt 7351 (Fig. 1, clones *d*–*g*, *i*) greatly reduced cell-dependent P89 activity. Clone *i* represents the minimal P89 promoter containing its 5′ CCAAT and TATAA elements [BPV-1 nt 1–99 (Linz and Baker, 1988)]. The upstream fragment between nt 7142 and 7477 was sufficient to activate the P89 promoter by cellular factors (Fig. 1, clone *h*). This fragment encompasses the previously described enhancer element within the P7185 promoter (Stenlund and Botchan, 1990; Stenlund et al., 1987; Vande Pol and Howley, 1990). In contrast, all P89 clones containing multiple E2-binding $ACC(N)_6GGT$ motifs were highly stimulated by E2

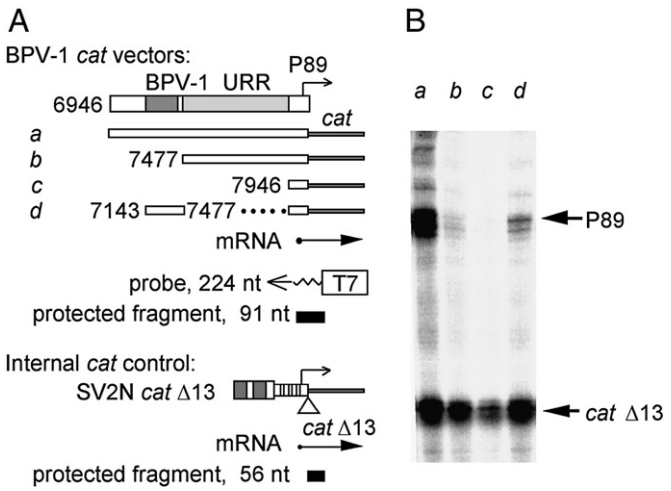

Fig. 2. BPV-1 P89 transcription is activated by cellular *trans*-acting factors interacting with the URR. (A) Diagram of BPV-1 P89 promoter constructs, antisense RNA probes and predicted hybridized, protected RNA fragments. (B) RNA was extracted from NIH-3T3 cells 24 h after transfection with 18 μg of the indicated P89 reporter clones, 8 μg RSV-E2 or RSV-*neo*, and 1 μg pSV2N-*cat*(Δ13), hybridized to uniformly ^{32}P-labeled antisense probes extending to nt 7902, digested with RNase, and the resulting double-stranded RNA fragments were resolved by gel electrophoresis adjacent to molecular weight markers (not shown). The protected bands of 91 nt for P89 and 56 nt for the internal *cat* control transcripts are shown by the arrows.

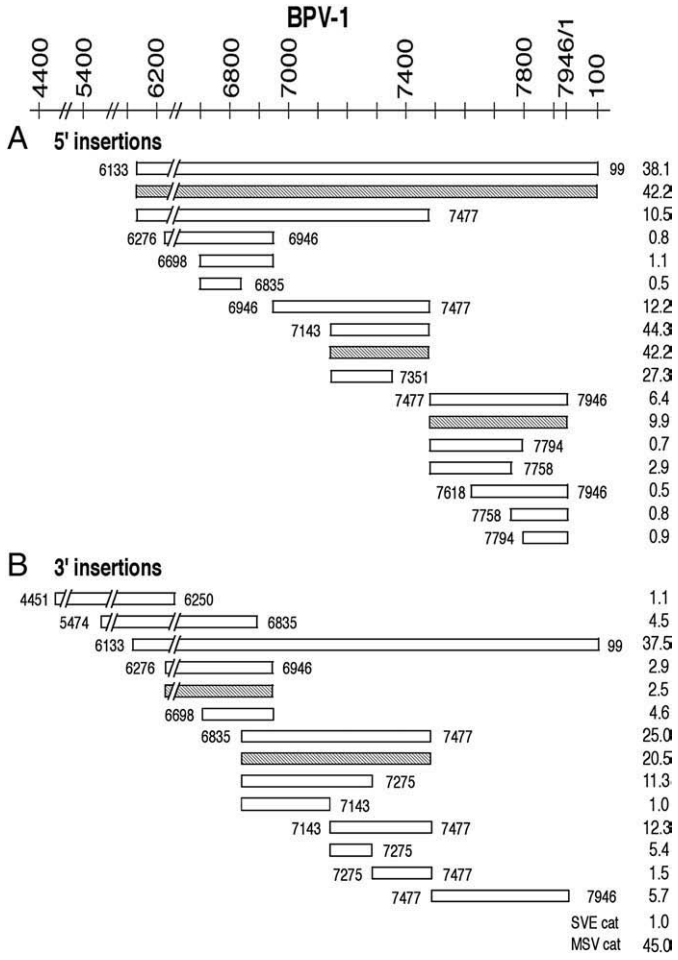

Fig. 3. BPV-1 URR sequences 5′ to P89 contain multiple active *cis* elements. BPV-1 URR fragments were linked either upstream (panel A) or downstream (panel B) from the enhancer-negative SV40 early promoter-driven *cat* gene in the pSVE-*cat* reporter clone. The shaded boxes indicate BPV fragments in the inverted orientation within the SV40 construct. Relative activities are expressed as fold above the parental pSVE-*cat* plasmid in duplicate transiently transfected cultures of NIH-3T3 cells in two to four independent assays and is listed numerically next to the schematic for each plasmid construct.

(Fig. 1, clones *a*–*g*). Clone *h* which encompasses the cell-dependent enhancer but lacks most of the E2 motifs retained only ~10% of the E2 response. The cell-dependent *cis* sequences were therefore distinct from the E2 response elements.

To evaluate steady-state BPV-1 mRNA levels in transiently transfected NIH-3T3 cells, a subset of the clones in Fig. 1 was assayed by RNAse protection (Haugen et al., 1988). P89-initiated mRNA reached ~11-fold higher levels with the full-length upstream promoter fragment between nt 6946 and 99 than with a 5′ deletion to nt 7477 (Fig. 2, compare lane a to lane b). Furthermore, the upstream fragment (nt 7143–7477) linked to the minimal P89 promoter (+1 to +99) increased correctly initiated transcript levels ~10 fold (Fig. 2, lane d versus lane c). The BPV-1 URR *cis* sequences thus enhanced transcription at the authentic P89 promoter. The sequences between nts 7477 and 7946, however, contributed to activation because P89 activity was higher in the complete URR construction (Fig. 2, lane a).

To further characterize the regulatory elements, multiple fragments of the BPV-1 URR and late gene region were inserted upstream or downstream of the enhancer-negative SV40 early promoter-driven *cat* gene in pSVE-*cat* (Haugen et al., 1987). The resulting constructs were assayed for CAT activity in transiently transfected NIH-3T3 cells (Fig. 3). The entire URR with a part of the late region (nt 6133–99) increased the activity of the SVE constructions ~40-fold. Sequences between nt 6698 and 6946 increased CAT activity when placed 3′ to the SVE-*cat* unit, but not in the 5′ location. It is possible that these sequences contain RNA processing signals for late gene transcripts similar to those at the 3′ end of the early region (Baker and Noe, 1989) rather than a classical transcriptional enhancer. In contrast, the fragment between nt 7143 and 7351 activated CAT expression 12 to 44-fold in a position and orientation-independent manner. A smaller fragment between nt 7143 and 7351 (Fig. 3) showed ~30% less activity, indicating that sequences between nt 7351 and 7477 contain additional *cis* elements. Sequences between nt 7477 and 7946 also increased pSVE-*cat* activity 6 to 10-fold in an orientation and position-independent manner. Further deletions in this fragment led to reduction of its activity, demonstrating that it also comprises multiple regulatory elements.

Cell type specificity of BPV-1 cis elements

The BPV-1 genome replicates and is transcriptionally active in fibroblasts as well as in keratinocytes in bovine fibropapillomas (reviewed in Lambert et al., 1988; Turek et al., 1987). In contrast, BPV-4 (Morgan et al., 1999; Vance et al., 1999) and HPV transcription is restricted to keratinocytes and cervical carcinoma cells due to enhancers that are preferentially active in these cell types (Cripe et al., 1990; Cripe et al., 1987; Gius et al., 1988; Gloss et al., 1987; Swift et al., 1987; Thierry and Yaniv, 1987). To determine whether BPV-1 P89 transcription reflected the extended viral host range, selected P89

Table 1
Activity of the BPV-1 P89 promoter and its *cis* elements in different cell types.

Reporter construct	BPV-1 sequences	Transfected cells[a]									
		Bovine		Murine		Human					Monkey
		Fibroblast	Epithelial	Fibroblast	Teratocarcinoma	Fibroblast	Keratinocyte	Cervical carcinoma		Hepatoma	Kidney
		B1	BMGE	3T3	F9	GM3498	HaCaT	HeLa	SiHa	HepG2	CV-1
Promoter clones											
Xba-P89 cat	6133–99	10.5	6.7	39.7	1.1	2.6	1.0	60.9	34.5	1.7	2.8
Bgl-P89 cat	6946–99	nd	nd	17.8	2.3	nd	2.0	34.2	29.5	1.4	3.0
Cla-P89 cat	7477–99	2.9	1.5	1.2	3.8	0.7	1.0	3.0	4.6	0.6	0.8
Hc-Cla-P89 cat	7143–7477, 1–99	12.6	3.5	10.9	1.0	8.0	5.5	65.0	21.9	1.7	4.5
Hpa-P89 cat	1–89	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Enhancer clones											
Xba-99 SVE cat	6133–99	37.7	13.4	38.1	14.0	7.6	2.2	21.0	12.0	2.7	9.3
Cla-Hpa SVE cat	7477–7946	1.9	1.7	6.4	4.9	0.9	3.3	2.6	2.1	1.7	1.4
	7477–7758	nd	nd	3.1	5.2	nd	3.0	2.1	4.0	3.2	nd
	7758–7946	nd	nd	0.4	1.3	nd	2.0	0.8	0.6	0.8	nd
Hc-Cla SVE cat	7143–7477	33.9	17.5	44.3	2.2	8.4	2.4	22.8	15.0	1.8	13.6
SVE cat	None	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

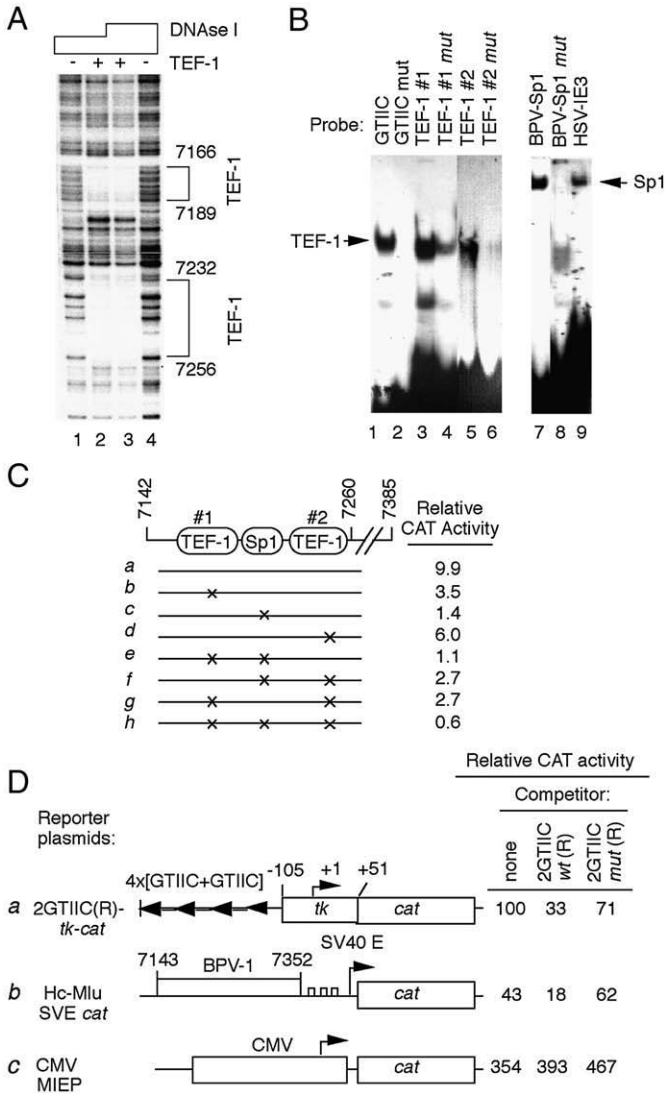

Fig. 4. TEF-1 binding is necessary for BPV-1 enhancer activation *in vivo*. (A) BPV-1 URR contains TEF-1 binding sites. A fragment of BPV-1 URR DNA (nt 7142–7385) was 5′ end-labeled on the lower strand and the binding of vaccinia-expressed TEF-1 protein (lanes 2, 3) or albumin control (lanes 1, 4) was determined by DNAse I footprinting as described (Ishiji et al., 1992). The position of cleavage products was determined by comparison with a sequencing reaction performed on the probe (not shown). (B) URR *cis* mutations reduce or abolish TEF-1 or Sp1 binding. Mobility shift results with double-stranded ^{32}P-labeled *wt* and mutant oligonucleotides (described in Table 2) using purified TEF-1 (lanes 1–6) or Sp1 (lanes 7–9). Specific TEF-1 or Sp1 complexes are indicated by arrows. (C) TEF-1 and Sp1 binding site mutations decrease BPV-1 enhancer activity. CAT activity of pSVE-*cat* reporter clones containing *wt* and mutant BPV-1 sequences (nt 7142–7385) in transiently transfected B1 bovine fibroblasts relative to pSVE-*cat*. Values are from duplicate cultures in two independent assays. (D) Competition with TEF-1 motifs specifically inhibits BPV-1 activation. The reporter plasmids, 9 μg, were cotransfected into HaCaT cells with 18 μg of the competitor plasmid containing either eight copies of the wild-type or mutant TEF-1 enhansons, GTIIC*wt*(R) or GTIIC*mut*(R) as indicated. The column labeled "none" contained 18 μg of pUC18 as competitor. Relative CAT activities are expressed relative to the 2GTIIC(R)-*tk-cat* clone.

promoter and enhancer constructions were tested in fibroblasts, keratinocytes and other epithelial cells of bovine, mouse, human and monkey origin (Table 1).

The upstream enhancer fragment (nt 7143–7477) activated both the P89 promoter and the SV40 early promoter 8 to 65-fold in most cell types tested, including fibroblasts as well as most epithelial cells, except for the human hepatocarcinoma cell line, HepG2, and the undifferentiated mouse teratocarcinoma cell line, F9 (Table 1). In contrast, the promoter-proximal URR fragment (nt 7477–7946) was most active in the mouse NIH-3T3 and F9 cells (6 and 5-fold, respectively) with less activity exhibited in other cell types. The BPV-1 *cis* elements therefore varied in activity among different types of cells but showed an extended host range compared to the keratinocyte-dependent enhancers of human papillomaviruses.

Cooperation of TEF-1 and Sp1 is required for full activity of the upstream enhancer

TEF-1, a factor critical for the function of the HPV-16 E6–E7 promoter, is restricted to keratinocytes and cervical cancer cells (Ishiji et al., 1992). However, TEF-1 also contributes to the activity of the SV40 enhancer (Xiao et al., 1991) in a broad range of cells.

To test for possible binding of TEF-1 to BPV-1 URR *cis* elements, we first performed DNase I protection assays with purified TEF-1 expressed from recombinant vaccinia virus. No strong binding sites were found in the proximal enhancer element (data not shown). However, in the upstream fragment, TEF-1 binding strongly protected sequences between nt 7166–7189 and nt 7232–7256 (Fig. 4A). The protection from DNase I digestion was eliminated by competition with an oligonucleotide containing the SV40 TEF-1 site, GTIIC (Table 2), but not by a mutant oligonucleotide (data not shown). Thus, similar to HPV-16, TEF-1 specifically binds to sites within the BPV-1 enhancer.

To test the functional role of these sites, we synthesized oligonucleotides with mutations that alter the TEF-1 binding motifs (Table 2). In addition, we mutated a region required for enhancer activity in bovine fibroblasts that had been proposed to bind Sp1 (Vande Pol and Howley, 1990). In mobility shift assays, the ^{32}P-labeled double-stranded *wt* oligonucleotide, BPV-1 nt 7196–7227, but not the mutant sequence, formed a complex with purified Sp1 similar in mobility to that with a control oligonucleotide containing an Sp1 binding site of the HSV-1 IE-3 promoter (Forsberg and Westin, 1991), (Fig. 4B, lanes 7–9). TEF-1 site #1 or #2 mutations also reduced binding of purified TEF-1 compared to *wt* motifs (Fig. 4B, lanes 1–6). In competition experiments, the binding affinity for the mutant TEF-1#1 and TEF-1#2 sites was 3 and 5-fold lower, respectively, compared to *wt* (data not shown).

To assess the role of Sp1 and TEF-1 binding in transcriptional activation, the *cis* mutations were first introduced singly or in combination into the reporter clone 7143–7385 SVE-*cat* (depicted in Fig. 3A), and the resulting constructs were transfected into B1 bovine fibroblasts (Fig. 4C). The mutation in the upstream TEF-1 site #1 reduced activity 3-fold while the mutations in the downstream TEF-1 site #2 further lowered reporter activity, albeit slightly (less than 2-fold). The Sp1 site mutation reduced activity 6-fold. These data show that mutations that alter the binding of TEF-1 and Sp1 decrease the activity of the upstream enhancer element. The combination of mutations had an additive effect, suggesting that activation requires cooperation between TEF-1 and Sp1.

To confirm that TEF-1 interaction *in vivo* with its cognate motifs is necessary for activation, we tested the activity of the 7143–7385 SVE-*cat* reporter clone in transfections with competitor plasmids carrying multiple wild-type or mutant GTIIC enhansons of the SV40 enhancer (Ishiji et al., 1992; Xiao et al., 1991). The prototype TEF-1-dependent enhancer clone, 2GTIIC(R)-*tk-cat* (Fig. 4D, clone *a*), with four copies of two closely spaced GTIIC enhansons, was active in HaCaT cells. Cotransfection with excess wild-type GTIIC sequences reduced its activity 3-fold, and the reporter plasmid containing the BPV-1 nt 7143–7385 enhancer element (Fig. 4D, clone *b*) was reduced 2.5 fold. In contrast, cotransfection with the mutant GTIIC enhansons that do not bind TEF-1 led to no reduction in activity, indicating that the *wt*, but not the mutant GTIIC competitor specifically titrated active TEF-1 in the cells. In control experiments, the TEF-1-independent major immediate early enhancer/promoter of human cytomegalovirus was not influenced by excess quantities of competing TEF-1 sites (Fig. 4D, clone *c*). Taken together, our mutagenesis and competition results

Table 2
Oligonucleotides used in this study.

Site		Source or reference	Sequence[a]
TEF-1 #1	*wt*	BPV-1 nt 7165–7195	CACTTAATAGCAATGTGCTGTGTCAGTTGTT
	Mutant		CACTTAATAGCAcgtTGCTGTGTCAGTTGTT
Sp1	*wt*	BPV-1 nt 7193–7125	GTTTATTGGAACCACACCCGGTACACATCCTGT
	Mutant		GTTTATTcGAACCctctCCGGTACACATCCTGT
TEF-1 #2	*wt*	BPV-1 nt 7231–7253	ATTTGCAGTGCGTGCATTGAATT
	Mutant		ATTTGCAGTGCGTGacgTGAATT
GTIIC	*wt*	SV40 (Ishiji et al., 1992)	CAGCTGTGGAATGTGTGTCAGTT
Sp1		HSV-1 IE-3 gene (Forsberg and Westin, 1991)	ctagaaTATTCCGGCCCCGCCCATtctgtc[b]

[a] Lower case letters designate mutations or added cloning sites as indicated.
[b] Top strand of a double-stranded oligonucleotide with Xba I and Xho I compatible ends.

show that binding of TEF-1 is required for the upstream enhancer of BPV-1 *in vivo*.

TEF-1 and Sp1 binding to the 5' sites is necessary for efficient early gene transcription and initial BPV plasmid amplification

To test the role of enhancer activity on viral early gene transcription, TEF-1 and Sp1 mutations were introduced into the plasmid BPV-1/pML. This clone contains the entire BPV-1 genome and can stably replicate in and transform mouse cells (Fig. 5A). Total RNA was harvested 18 h after transfection of 3T3 cells with these plasmids. The mutations in both of the TEF-1 sites or the Sp1 site reduced P89-initiated transcripts by two and threefold, respectively, while a construct with all three mutations (TEF-1/Sp1 mut) had 5-fold lower activity (Fig. 5B). Thus, factor binding to these sites was necessary for P89 activity in the context of the complete viral genome.

The Sp1 and TEF-1 mutations also affected the initial amplification of the BPV-1 plasmids. After transfection of mouse NIH 3T3 cells, BPV-1 replication was assessed by the accumulation of BPV-1 DNA which lost bacterial methylation and thus became resistant to Dpn I digestion (Fig. 5C). Although replication of BPV-1 sequences was observed in all cases, mutations in the TEF-1 or Sp1 motifs or their combination resulted in decreased BPV-1 DNA accumulation compared to the *wt* after 108 h (2, 4 and 7-fold lower than *wt* DNA, respectively).

Full enhancer activity is necessary for efficient cell transformation and establishment of replication but it does not control viral gene expression or copy numbers of BPV-1 plasmid genomes in established transformants

Since the expression of viral early genes is a prerequisite of efficient transformation of C127 cells by BPV-1 we tested the *wt* and mutant BPV-1 constructions in transformation assays. As shown in Fig. 6A, compared to *wt*, the numbers of transformed cell foci observed with the TEF-1 and Sp1 mutants were reduced 3 and 8-fold, respectively, while the combined mutant was 12-fold lower, in agreement with reduced expression of viral early genes in the mutants.

Because some replication-defective BPV-1 mutants have been shown to integrate in transformed cells, we further analyzed *wt* and mutant BPV-1 transformants to determine the physical state of viral

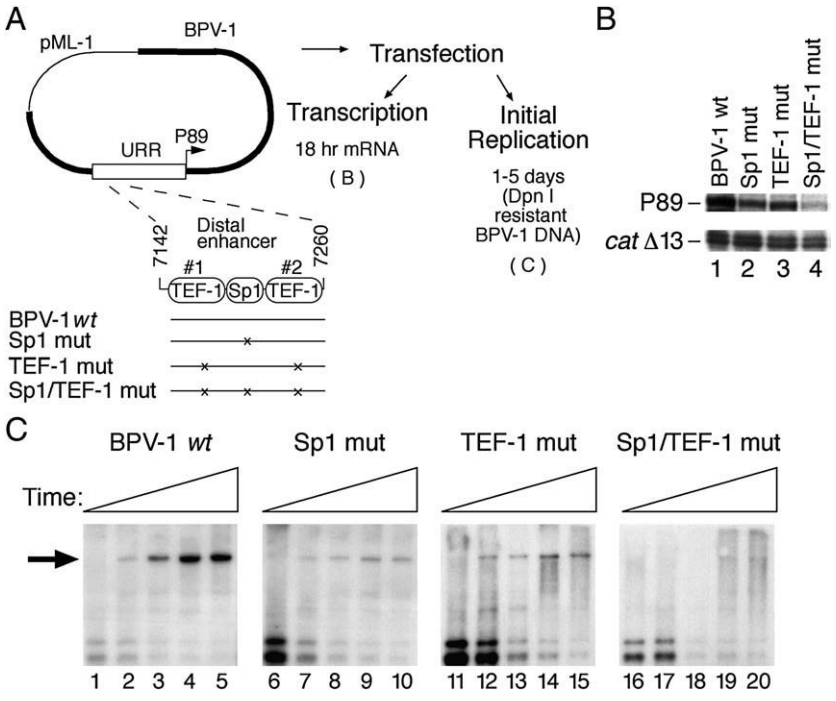

Fig. 5. Activation of viral early gene transcription by cellular factors is limiting for BPV-1 plasmid amplification. (A) Experimental design for the evaluation of initial events after transfection with *wt* and mutant BPV-1 genomes. (B) Mutations in TEF-1 and/or Sp1 sites in the entire BPV-1 genome reduce P89 mRNA levels. Cellular RNA was extracted from NIH-3T3 cells 24 h after transfection with *wt* BPV-1/pML or mutants together with pSV2-*cat*(Δ13) control, hybridized to ^{32}P-labeled antisense RNA probes against BPV-1 P89 (nt 310 to nt 1) and *cat* (nt 69 to 1), digested by RNase and analyzed by denaturing gel electrophoresis. Protected bands of 221 nt for P89 and 56 nt for the internal Δ13-*cat* control transcripts are shown by the arrows. (C) TEF-1 and/or Sp1 site mutations reduce initial accumulation of BPV-1 plasmid DNA. After transfection of NIH-3T3 cells with *wt* or mutant BPV-1 plasmids, DNA was harvested from the cultures at 18, 36, 60, 84 and 108 h, digested with Dpn I and HinD III, and analyzed by agarose gel electrophoresis followed by Southern blot hybridization with a BPV-1 probe. The arrow indicates the migration position of replicated, Dpn I-resistant BPV-1 DNA. The lower bands correspond to the Dpn-I digestion products derived from input plasmid DNA. Lanes 1–5 were exposed for 24 h and lanes 6–20 for 48 h to visualize replicated DNA.

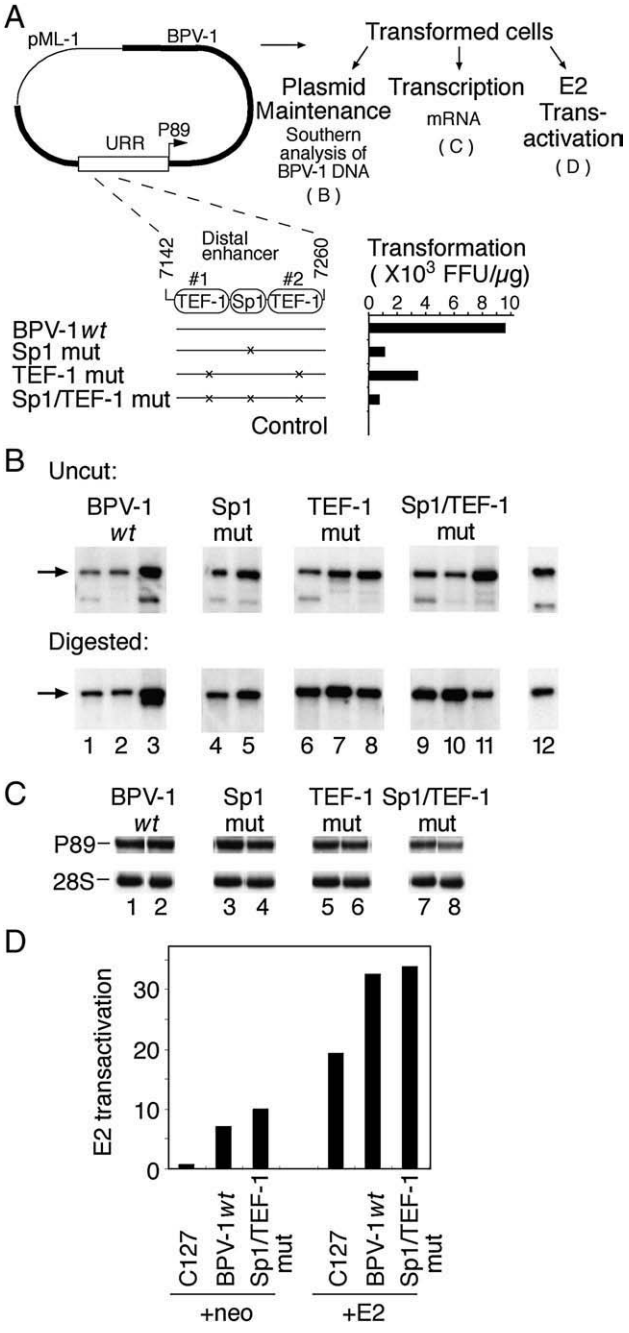

Fig. 6. Cellular factors are not required for viral gene expression in persistent infection. Cellular transformation by mutant BPV-1 genomes is reduced. C127 cells were transfected with *wt* and mutant BPV-1/pML. The average focus-forming units/1 μg BPV-1 clone DNA in duplicate C127I cultures in two experiments are shown. (B) Established transformed cell clones harbor comparable levels of *wt* or mutant extrachromosomal BPV-1 DNA. Uncut and DpnI/HindIII-digested DNAs from C127I cultures transformed independently by *wt* or mutant BPV-1 were analyzed by Southern blot hybridization. Uncut cellular DNAs were run next to 100 pg *wt* BPV-1/pML input DNA ("control"). The same DNAs were also tested after digestion with Dpn I and HinD III (except in "control" where Dpn I was omitted). BPV-1 mutations in the transformants were confirmed by PCR and sequencing. (C) Transformed cell clones with *wt* and mutant BPV-1 express comparable levels of P89 transcripts. RNA was extracted from duplicate independent cultures of C127 cells transformed by *wt* or mutant BPV-1/pML, and analyzed by RNase protection using ^{32}P-labeled antisense RNA probes against BPV-1 P89 (as illustrated in Figs. 2 and 5) or 28S ribosomal RNA. The protected bands of 221 nt for P89 and 115 nt for the 28 S ribosomal RNA control transcripts are shown. (D) *wt* and mutant BPV-1 transformants express comparable levels of active E2 transactivator. C127 cells or transformed cell clones were cotransfected with 1 μg of a baseline reporter plasmid *tk-cat*, or the E2 responsive reporter pE2×2 *tk-cat* and with pCG E2 or pCG *neo* as indicated. The CAT activity was determined as in Fig. 3. The E2 transactivation is expressed as the ratio of the observed activities of the E2 responsive reporter plasmid to non-responsive *tk cat* plasmid. A ratio of 1 indicates no E2 activity.

DNA. We found that BPV-1 genomes with mutations in the TEF-1 and/or Sp1 sites persisted in all independently transformed cultures as extrachromosomal, supercoiled circular plasmids (Fig. 6B and data not shown). Furthermore, they were indistinguishable not only in structural DNA forms but also in quantity (i.e. plasmid copy number per cell) in the *wt* BPV-1 transformed cells (Fig. 6B, "uncut"). Analysis of the DNA linearized with HinDIII (Fig. 6B, "digested") showed, within the limits of analysis by gel electrophoresis, no deleted or rearranged forms with either *wt* or mutant viral genomes. Sequence analysis of the enhancer URR region amplified by PCR from the transformed cultures confirmed that all replicating BPV-1 plasmids retained the *cis* mutations of the input mutant constructs.

Furthermore, in contrast to the decreased levels of steady-state P89 mRNA encoded by the BPV-1 mutants 18 h post-transfection, multiple independent cultures transformed by *wt* BPV-1 or by the TEF-1, Sp1 or TEF-1/Sp1 mutants expressed comparable P89-initiated mRNA levels (Fig. 6C and additional data not shown). Thus, the effect of mutations in the TEF-1 or Sp1 binding sites was to preferentially interfere with viral transcription and DNA replication during the establishment phase of infection, but not in established transformed cells.

In agreement with this conclusion, we found that *wt* and mutant BPV-1 transformants expressed comparable levels of functional E2 gene products (Fig. 6D). The E2 responsive promoter plasmid, E2×2 *tk-cat*, was activated 7 to 10-fold upon transfection in *wt* BPV-1 or TEF-1/Sp1 mutant-harboring cell lines but not in uninfected C127 cells. Cotransfection with a plasmid expressing the BPV-1 E2 transactivator, pCG-E2, resulted not only in reporter activation in uninfected C127 controls but also in further stimulation of reporter activity in the *wt* and mutant BPV-1-expressing cell lines. This observation demonstrated that both *wt* and mutant BPV-1-transformed cells expressed comparable levels of active E2-TA. Taken together, these results indicate that full transcriptional activation by cellular factors interacting with the URR is not limiting for the maintenance of viral gene expression or plasmid persistence in established transformants.

Discussion

In this study, we examined the transcriptional regulation of BPV-1 early genes by cellular transcription factors. The BPV URR contains regulatory elements that interact with viral and cellular factors as do the URRs of HPV strains. In contrast to the restricted, epitheliotropic host range of the HPV enhancers, activities of the BPV-1 URR elements reflect the ability of the virus to persist in fibroblasts. Full transcriptional activation by cellular factors, TEF-1 and Sp1, was found to be limiting during the establishment phase for the initial accumulation of BPV-1 plasmid genomes and cell transformation but not required for the maintenance of viral transcription or genome copy number in BPV-1-transformed fibroblasts.

Structure of BPV-1 URR

The URR of BPV-1 contains *cis* active sequences required for virus gene expression, cell transformation and viral replication. Although the URRs of both BPV and HPV have been known to contain multiple conserved E2 binding motifs (Sanchez et al., 2008) that are required for viral gene expression and replication (Haugen et al., 1987; Spalholz et al., 1987) and (Lace et al., 2008b; Stubenrauch et al., 1998), they also respond to regulation by cellular transcription factors. We identified multiple fragments spanning the BPV-1 late gene region and URR that can independently influence transcription in *cis*. The URR fragment between BPV-1 nt 7143 and 7477 was a strong enhancer in fibroblasts as well as in most other cell types while the nt 7477–7946 segment was preferentially active in some but not all cells of epithelial origin. We have shown that cellular factors Sp1 and TEF-1 are both necessary

for the activity of this fragment. Interestingly, TEF-1 (Jacquemin et al., 1996) was previously identified as a factor critical for the function of the HPV-16 E6–E7 promoter in keratinocytes and cervical cancer cells (Ishiji et al., 1992). TEF-1 also contributes to the function of a broadly-active SV40 enhancer (Xiao et al., 1991). It is possible that TEF-1, together with or independent of related factors (e.g. TEF-3, TEF-4 and AP-2), mediates the activation of enhancers in different cells. In addition to the three Sp1 motifs defined previously (Sandler et al., 1996), URR sequences proximal to the P89 promoter also appear to interact with multiple cellular factors as shown by deletion mutagenesis (this study). For example, a binding site for transcription factor AP-2 has been demonstrated within this proximal segment, by DNase I footprint analysis (Imagawa et al., 1987). However, only imperfect matches were identified for additional putative AP-2 consensus motifs.

It is likely that overall BPV enhancer activity reflects cooperative interactions of TEF-1 and Sp1 with other, as yet undefined factors. We predict that these factors differ from those that restrict the transcription of HPV E6–E7 promoters to keratinocytes and cervical carcinoma cells. The identification of such factors will require further mutagenesis and *in vitro* DNA binding assays to define these functional differences.

Role of cellular transcription factors in the establishment of papillomaviral infection

Since the binding of papillomaviral particles to cells appears to lack tissue specificity (Müller et al., 1995; Roden et al., 1994; Volpers et al., 1995), transcription is dependent on cell factors that vary between cell types and may provide a mechanism for the restriction of viral infection to specific tissues. Consistent with this model, the ability of BPV-1 to establish viral gene expression and DNA replication in both epithelial and fibroblastic cells is reflected by the observed activity of its URR segments in a variety of cell types.

Role of cellular transcription factors in BPV-1 plasmid maintenance

BPV URR segments also contain multiple binding sites for the viral transcription factor E2. These sites are required for transcription activation as well as plasmid maintenance by E2 gene products. Once BPV replication is established, E2 isoforms will be present within the cell that may regulate transcription from P89 and other viral promoters. Since transformed cultures derived from *wt* and mutant BPV-1 genomes exhibited similar ranges of replicating plasmid BPV-1 DNA and P89 mRNA levels, we conclude that the critical transcription activation functions of TEF-1 and Sp1 at the upstream URR segment are replaced by other cellular and viral factors, including forms of the viral activator protein E2. Similar levels of E2 transactivation activity detected in cell lines transformed with *wt* and mutant BPV-1 genomes support this concept.

However, cellular factors are nonetheless critical in the maintenance of BPV-1 persistence; for example, the E2-TA activates transcription in cooperation with Sp1 and other cellular transcription factors (Steger et al., 1996; Ushikai et al., 1994). In HPV-16 and other mucosal HPVs, the proximal E2 sites mediate transcriptional repression at the E6 promoter by limiting levels of E2-TA and E8^E2. This repression is required for maintenance of papillomaviral replication and limits initial plasmid amplification (Lace et al., 2008a, 2008b). This suggests a mechanism for negative feedback control of early gene synthesis is critical for HPV infection. In BPV-1, E2-TA is thought to activate all early region promoters (Szymanski and Stenlund). Since the plasmid levels and P89 mRNA levels are similar for BPV wt and mutant cell lines (this study), it suggests that a mechanism to limit replication and transcription is also present in BPV-1. Although it is not clear how this modulation may occur in BPV-1, regulation by E2 repressor forms appears to play a role since E2 mutant genomes are unable to replicate in stably transformed cultures (Choe et al., 1989; Lambert et al., 1990; Riese et al., 1990).

Materials and methods

Plasmid constructions

Molecular cloning followed established protocols (Cripe et al., 1987; Haugen et al., 1987; Haugen et al., 1988; Ushikai et al., 1994). The plasmid constructions are illustrated in the respective figures. Restriction enzyme fragments of BPV-1 DNA are numbered by the first nucleotide of the endonuclease recognition site (Haugen et al., 1987; Haugen et al., 1988). BPV-1 promoters are designated by the nucleotide coordinates of their major transcription start site (Ahola et al., 1987; Baker and Howley, 1987; Stenlund et al., 1985); their alternative names, P1 through P5 (Choe et al., 1989; Stenlund and Botchan, 1990; Stenlund et al., 1987), are given in the Introduction.

The precursor plasmid to the chloramphenicol acetyl transferase (*cat*) reporter clones was pSVE-*cat* (Cripe et al., 1987; Haugen et al., 1987; Haugen et al., 1988; Ushikai et al., 1994). Restriction fragments of the BPV-1 URR were inserted into upstream or downstream polylinker restriction sites. The pP89-*cat* deletion mutants were described previously (Haugen et al., 1987) or constructed by deletions or insertions in pP89-*cat*. Binding motif mutations were introduced into reporter clones or BPV-1/pML plasmids via a two-stage polymerase chain reaction (PCR) method with oligonucleotides containing 3 nt substitutions listed in Table 2 (Ishiji et al., 1992; Ushikai et al., 1994). The pMSV-E2, pCG-*neo*, pCG-E2, p*tk-cat*, and pE2×2*tk-cat* clones were as described (Haugen et al., 1987). All molecular constructions were verified by DNA sequencing.

Cells and transfections

The bovine embryo fibroblast strain B1 and the bovine breast duct epithelial cell line BMGE (Schmid et al., 1983) were a kind gift of Drs. M. Blessing and W. Franke, German Cancer Research Center, Heidelberg, Germany. HaCaT, a spontaneously immortalized human keratinocyte cell line that retains its capacity for *in vivo* differentiation (Boukamp et al., 1988), was a kind gift from Drs. P. Boukamp and N. Fusenig, German Cancer Research Center, Heidelberg, Germany. Human fibroblasts (strain GM3498) were from the American Type Culture Collection, Rockville, MD. All cell cultures were grown in Dulbecco's MEM with 7% iron-supplemented newborn calf and 1% fetal calf serum except for NIH-3T3 cells in which the fetal calf serum was omitted. All cells, except the HaCaT line, were transfected in duplicate by calcium phosphate coprecipitation followed by glycerol or DMSO treatment 4 to 6 h later (Cripe et al., 1987). HaCaT cells were transfected by lipofection (BRL/Gibco, Gaithersburg, MD). To assess transfection efficiency, control *cat* plasmids driven by the SV40 promoter-enhancer or the murine sarcoma virus LTR were included in each transfection experiment. Enzymatic CAT assays and RNase protection assays were performed as described (Cripe et al., 1987; Haugen et al., 1988). The 28S rRNA probe was transcribed from the plasmid template pTRI-RNA-28S (Ambion, Austin, TX).

Transformation and replication assays

Transformed foci of C127I cells were scored 2–3 weeks after transfection with *wt* BPV-1/pML or mutant plasmids (Haugen et al., 1987). For all replication assays, DNAs were extracted from transfected NIH 3T3 cells or from transformed C127I cells and analyzed by agarose gel electrophoresis and alkaline Southern blot transfer onto positively-charged Nylon membranes (Zeta-probe, Bio-Rad, Hercules, CA) as described (Ustav and Stenlund, 1991). Probes were generated by random priming using α-^{32}P-labeled dCTP and dATP with BPV-1/pML DNA as a substrate.

DNAse I footprinting

The substrate for footprinting experiments was a DNA fragment of the BPV-1 regulatory region, nt 7142–7385, 5′ end-labeled using T4 polynucleotide kinase. Reaction mixtures containing vaccinia-expressed TEF-1 protein were preincubated at 30 °C for 10 min in a final volume of 50 μl and processed as described (Ishiji et al., 1992).

Mobility shift assays

Oligonucleotides used as probes and competitors are shown in Table 2. Binding was performed in a 25 μl reaction volume as described (Ishiji et al., 1992) with DNA affinity-purified TEF-1 protein from HaCaT nuclear extracts or recombinant Sp1 (Promega) and 15,000 cpm (~4–8 fmol) of ^{32}P-labeled double-stranded oligonucleotide probe in the presence of 30–100 ng of poly(dI-dC) (Pharmacia) at 30 °C for 30 min. Protein–DNA complexes were resolved from the unbound probe on non-denaturing 6% acrylamide gels and visualized by autoradiography.

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