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The specific DNA recognition sequence of the bovine papillomavirus E2 protein is an E2-dependent enhancer

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The upstream regulatory region (URR) of the bovine papillomavirus (BPV) genome contains an enhancer that is activated by a BPV E2 gene product. We have previously found that a bacterially derived E2 fusion protein specifically interacted with several fragments of URR DNA, suggesting that E2 may activate transcription by directly binding to the enhancer. Each of the bound fragments contains at least one copy of a conserved motif ($ACCN_6GGT$). To determine if this motif is required and sufficient for specific E2 binding, we have now constructed a bacterial expression vector that encodes a full-length E2 peptide and developed a refinement of the McKay DNA immunoprecipitation assay that allows the determination, to the nucleotide level, of the minimum sequence required for specific binding. The results show that the E2 recognition sequence is a single copy of this motif and that the variant $ACCGN_4CGGT$ is bound with greater affinity than the minimum $ACCN_6GGT$ motif. An oligonucleotide encoding the motif was able to inhibit E2-dependent *trans*-activation in a transient transfection assay, indicating that the virally encoded E2 also interacts with this sequence in mammalian cells. When present in two or more copies, but not in a single copy, the E2 binding element had intrinsic enhancer activity but only in cells expressing E2. The results indicate that the conserved motif alone is sufficient for E2-mediated enhancement and that the binding of E2 to the motif is probably required for efficient enhancement. Since a single motif did not have a significant enhancer activity, it is likely that bound E2 molecules act cooperatively in activating transcription.

Key words: papillomaviruses/DNA-binding proteins/enhancer/DNA-binding assay/gene expression

Introduction

The upstream regulatory region (URR) that immediately precedes the early genes of the bovine papillomavirus type 1 (BPV) genome contains an enhancer that is activated by a *trans*-acting gene product of the BPV E2 open reading frame (ORF) (Spalholz *et al.*, 1985) (Figure 1). This E2-dependent enhancer has been shown to activate transcription from the BPV p7940 and p89 early promoters (Figure 2) and also from heterologous promoters such as SV40 early and herpes tk (Spalholz *et al.*, 1985; Haugen *et al.*, 1987; Spalholz *et al.*, 1987). Recent reports have demonstrated that deletion mutations in several regions of the URR reduce E2-dependent enhancer activity and that two non-overlapping URR fragments have enhancer activity, indicating that the enhancer is genetically complex (Spalholz *et al.*, 1987; Haugen *et al.*, 1987). E2 *trans*-activation of a URR enhancer appears to be a conserved feature of papillomavirus (PV) gene regulation, since the URRs of other PVs are *trans*-activated by the BPV E2 gene product and the BPV URR is *trans*-activated by the E2s of other PVs (Phelps and Howely, 1987; Hirochiko *et al.*, 1987).

Recent biochemical evidence has suggested that E2 may *trans*-activate by binding to the URR. In two independent studies, bacterial fusion proteins containing BPV E2 encoded peptides were shown to specifically bind multiple sequences within the URR. Using a λ cII-E2 fusion protein, we previously identified four non-overlapping BPV fragments that were specifically bound (Androphy *et al.*, 1987). Three are in the URR and the fourth in the E2 ORF. All of the bound fragments contain at least one copy of a common motif with the consensus sequence: 5'ACC(G)NNNPyCGGT(GC)3' (nucleotides in parentheses are preferred but not invariant, N can be any nucleotide and Py can be C or T). The inverted repeat in this sequence ($ACCN_6GGT$) is found 10 times in the BPV URR (Figure 2) and also numerous times in the URRs of all other papillomaviruses (Dartmann *et al.*, 1986). Using a DNase I protection assay, Moskaluk and Bastia reported that an E2 fusion protein, in which the E2 encoded amino acids were fused upstream of a β-galactosidase peptide through a collagen linker, bound two segments in the URR (Moskaluk and Bastia, 1987); these authors suggested that E2 binding was mediated by the consensus sequence $^{AA}GGCGGNN^{C}$ present in both segments, although the protected regions also contained the inverted repeat noted above.

While these results suggest that a direct interaction between E2 protein and the URR may play an important role in E2-mediated *trans*-activation, the E2 binding sequence remains ambiguous, and the possible dependence of E2 *trans*-activation on this binding has not been determined. In

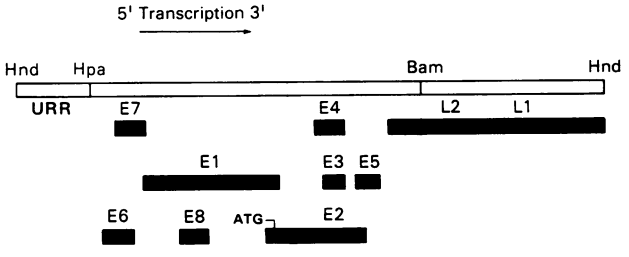

Fig. 1. The 8-kb BPV-1 genome, linearized at its unique *Hin*dIII site. The *Hpa*I site is numbered nucleotide 1. URR is the upstream regulatory region. E1-E8 are the early open reading frames (ORFs), which are expressed in cultured cells. The L1 and L2 ORFs are expressed only in productively infected papillomas. All of the ORFs are on the same DNA strand and transcribed in the direction indicated. ATG indicates the first methionine codon in the E2 ORF.

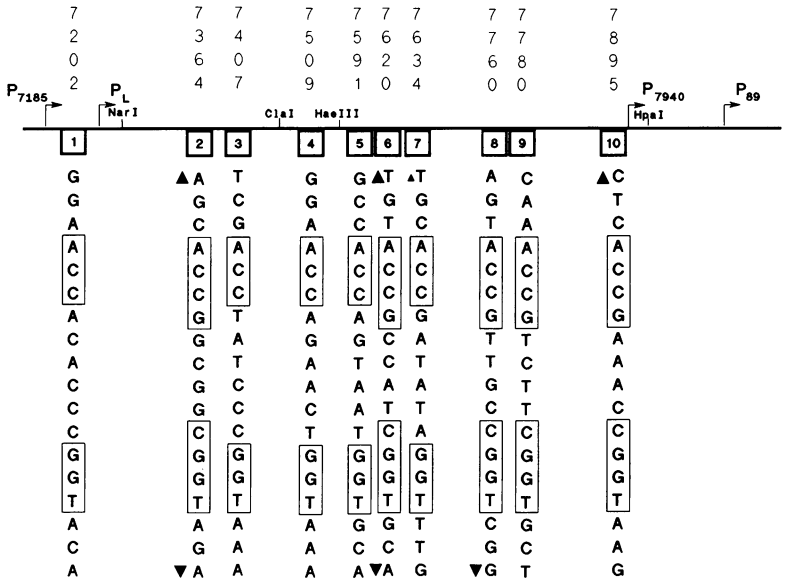

Fig. 2. The potential E2 binding palindromes (E2BP) in the BPV-1 URR are numbered 1–10. The interrupted inverted repeat in each is boxed. The nucleotide number of the A of each palindrome is indicated above the line. The triangles indicate the first nucleotide in the sequence adjacent to the E2BP that was immunoprecipitated in the primer extension assays, and point in the direction that the DNA was synthesized. The smaller triangle adjacent to E2BP 7 denotes weak immunoprecipitation. Sites of transcription initiation are shown immediately above the line (using the designations of Baker and Howley, 1987).

this study we have undertaken the precise mapping of several E2-binding sites and, having determined that the conserved motif is the minimum E2 recognition sequence, we present evidence that this sequence is recognized by the viral E2 in mammalian cells and show that a pair of these binding sites is an E2-dependent enhancer.

Results

Expression of a full-length E2 protein in Escherichia coli

The E2 bacterial fusion proteins previously used to identify the DNA binding activity of E2 differed significantly from the predicted full-length viral E2, in one case lacking the amino-terminal third of the protein (Androphy *et al.*, 1987) and in the other case including a large β-galactosidase peptide at the carboxy-terminus (Moskaluk and Bastia, 1987). Although it was likely that these molecules possessed DNA binding activities similar to those of an authentic E2 product, the possibility of artifacts needed to be considered. Prior to beginning a precise mapping of the E2 binding site, we therefore decided to synthesize an E2 protein in bacteria that more closely resembled the predicted full-length viral protein, since this molecule would be even more likely to have the same DNA-binding activity as the authentic viral protein and would also be more likely to function in future studies of E2-dependent enhancement. A full-length E2 expression vector was constructed by cloning the *Sph*I to *Bam*HI fragment of BPV (nt 2617–4450) into the *Bam*HI site of the expression vector pOTV. pOTV contains the inducible λ P_L promoter and the λ cII ribosome binding site upstream of the cII initiation codon which overlaps the unique *Bam*HI site (Devare *et al.*, 1984). The cII ATG was joined in frame to the E2 coding sequences, using an adapter to supply the missing E2 amino acids. The adapter had one

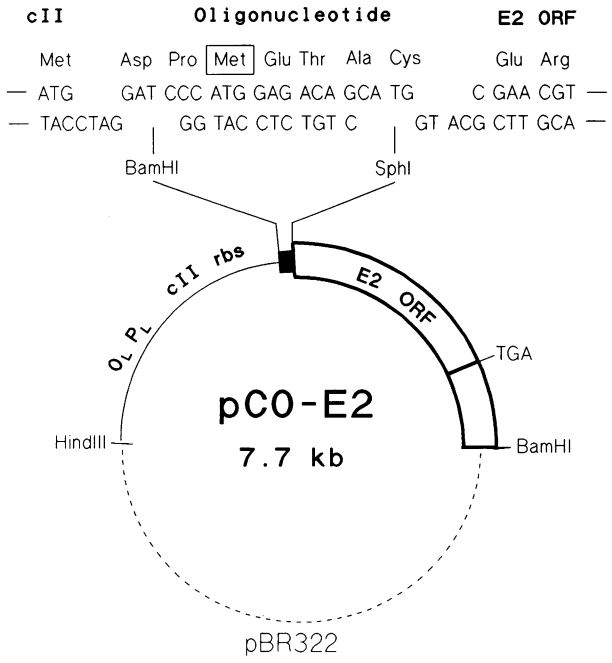

Fig. 3. Full-length E2 bacterial expression vector. Solid line, phage λ sequences that include the late operator and promoter (O_L and P_L) and cII ribosome binding site (cII rbs); black rectangle, synthetic oligonucelotide (sequence shown above) used to link the cII initiation codon to the BPV sequences; open box, BPV-1 sequences (nt 2617-4450); TGA, stop codon of the E2 ORF; interrupted line, pBR322 sequences. In the sequence of the oligonucleotide, the first methionine in the E2 ORF is boxed.

*Bam*HI and one *Sph*I cohesive end to join the cII and E2 sequences, so that the resulting ORF would direct the synthesis of a fusion protein consisting of the entire E2 coding

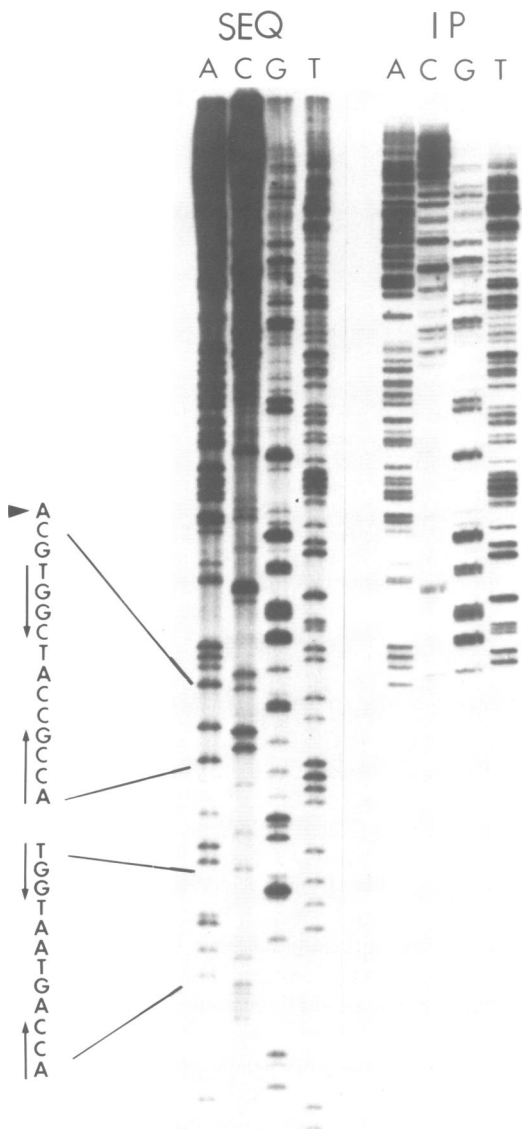

Fig. 4. Primer extension immunoprecipitation assay. The **left hand panel** shows the dideoxy sequencing reactions using an M13mp18 single-stranded vector with BPV nt 7587–7945 (*Hae*III–*Hpa*I) inserted into the *Sma*I site of the polylinker as a template and the −20 M13 17-mer (New England Biolabs) as a primer. The sequence of E2BP 6 (top) and 5 (bottom) are indicated. The **right hand panel** shows the fragments from each of the sequencing reactions that were immunoprecipitated by the bacterially synthesized full-length E2 protein. The arrowhead next to the sequence on the left indicates the 3′ terminal nucleotide of the first fragment that is specifically immunoprecipitated.

sequence (from the first methionine) plus three additional amino acids (encoding the *Bam*HI site) at the amino terminus (Figure 3). The resulting plasmid, designated pCO-E2, was introduced into *E.coli* strain N6405, which carries a temperature-sensitive cI repressor. After thermal induction and partial purification by differential solubility (Androphy *et al.*, 1987), the expected 45-kd protein was specifically recognized by the previously characterized E2 antisera in immunoprecipitation and western blotting assays (data not shown), indicating that this protein was E2 encoded.

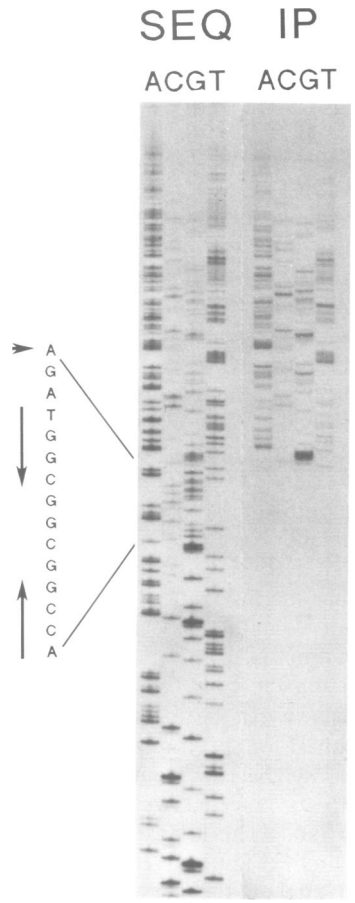

Fig. 5. Primer extension immunoprecipitation assay of an M13mp18 clone with BPV nt 7274–7476 (*Nar*I to *Cla*I) inserted into the *Acc*I site of the polylinker. The sequence of E2BP 2 is shown on the left. Designations are the same as in Figure 4.

Determination of the minimum E2 recognition sequence

To determine to the nucleotide the sequence requirement for E2 recognition, we developed a primer extension DNA immunoprecipitation assay. In this assay, we first cloned into an M13 vector various URR fragments that contained one or more of the conserved motifs. These constructions were then used as templates in standard dideoxy sequencing reactions to generate a series of progressively longer double-stranded DNAs with single-stranded tails. The reactions were then subjected to S1 nuclease digestion to remove the unpaired single stranded template, since we had noted previously that single-stranded DNA interacts nonspecifically with E2 (Androphy *et al.*, 1987), and so might mask specific binding to the double-stranded fragments containing the E2 recognition site. This digestion resulted in a series of double-stranded fragments that differed in length by single nucleotide increments at the 3′ end of the newly synthesized strand. When subjected to the McKay DNA immunoprecipitation assay (McKay, 1981) using the full-length E2 synthesized in bacteria and the previously characterized E2 antiserum (Androphy *et al.*, 1987), we reasoned that fragments that were terminated prior to the synthesis of a complete E2 recognition sequence would not be immunoprecipitated in the assay, while all fragments that were terminated after the synthesis of a complete binding site would be recovered. By comparing the immunopreciptated fragments with the in-

put synthesis reactions, the precise sequence of the smallest precipitated fragment, and hence the specific sequence required for E2 recognition, could be determined.

The sequencing reactions in which the BPV sequences from nt 7587 to nt 7945 (*Hae*III to *Hpa*I, Figure 2) were synthesized and the E2 immunoprecipitations of these reactions are shown in Figure 4. The above predictions were fulfilled in that precipitation was noted for all fragments containing a specific sequence; all fragments that extend past the A at nt 7634 were specifically bound by bacterial E2 protein, while those that terminated prior to this nucleotide were not. This A is three nucleotides downstream of the last nucleotide of the inverted repeat ($ACCGN_4CGGT$) of the conserved motif 6 and immediately adjacent to the preferred GC at the 3′ end of the consensus sequence. As shown in Figure 2, the immunoprecipitated fragments also contain conserved motif 5 (nt 7589−7605), but fragments containing this motif without the downstream motif, were not specifically immunoprecipitated. This motif has a 3-bp inverted repeat ($ACCN_6GGT$) rather than the 4-bp repeat of the downstream motif (Figure 2), suggesting that the minimum conserved motif is not as efficiently bound by the BPV E2, at least not in the presence of fragments containing the more complete consensus sequence.

We do not believe that the above results indicate that two motifs are required for specific binding, since when motif 2 (nt 7364) was synthesized from the *Nar*I site (nt 7274), specific binding was detected two to three nucleotides after the motif even though no related element is apparent upstream of this motif (Figure 5). In the synthesis of motif 2 from the *Cla*I site on the opposite strand, motif 3 (nt 7407−7418) was synthesized prior to the motif at 7364, but no specific binding was seen at motif 3, which also has a 3-bp inverted repeat instead of the 4-bp repeat of the consensus binding site and also lacks the 3′ GC (data not shown). Fragments containing only motif 7, which has the 3-bp repeat, were weakly bound but those that also contained motif 6, a 4-bp repeat element just upstream of 7, were bound to a greater extent (not shown). Similar analyses were conducted using motifs 8 and 10, which contain 4-bp repeats, and in each case specific E2 binding began after synthesis of three (±1) nucleotides past the last nucleotide in the inverted repeat of the conserved motif (results summarized in Figure 2). These results indicate that a single motif with the consensus sequence ($ACCGN_4CGGT$), when flanked by a few apparently random nucleotides, consitutes an E2 binding site and that the shorter inverted repeat ($ACCN_6GGT$) is less efficiently recognized by E2. In addition, these results make it extremely unlikely that the other proposed consensus sequence ($^{AA}_{TT}GGCGGNN^{C}_{G}$) is an important determinant of E2 specificity, since fragments that did not contain this sequence, but that did contain motifs 7, 8 or 10, were specifically immunoprecipitated as noted above.

The in vitro E2 binding motif blocks E2 trans-activation in vivo

Having determined that the bacterially expressed E2 protein recognized the consensus motif *in vitro*, we then sought evidence of an interaction between the viral E2 and this motif in mammalian cells by testing the ability of the motif to competitively inhibit E2-dependent enhancement by the URR. Three synthetic oligonucleotide copies of the consensus motif, or a derivative with a single nucleotide change in the inverted repeat, were cloned into the polylinker of the bacterial pUC12 plasmid. These plasmids were cotransfected with the E2-dependent CAT expression vector p407, which contains the URR upstream of the SV40 early promoter and the CAT gene (see below), and pRSVE2E5 (Haugen *et al.*, 1987), an LTR activated E2 gene. CAT activity was measured in a standard CAT assay after transient expression of the plasmids (Figure 6). When compared to the parental pUC12 control plasmid, the plasmid containing the consensus motif significantly inhibited CAT expression from the E2-dependent vector (compare lanes 4 with 6 and 7 with 9). The plasmid containing three copies of the altered motif, which was not efficiently immunoprecipitated by the bacterial E2 *in vitro* (data not shown), did not inhibit E2 *trans*-activation (lanes 5 and 8). The failure of the non-binding derivative to block *trans*-activation rules out the possibility that the inhibitory activity seen with the plasmid containing the consensus motif results from the disruption of the pUC polylinker or from the several nucleotides that flank the motif in the synthetic oligonucleotides. These results strongly suggest that the viral E2 protein in the transfected cells specifically binds the motifs on the pUC plasmid and that, when bound to these plasmids, the E2 molecules are prevented from efficiently *trans*-activating transcription from the E2-dependent promoter in the p407 CAT expression vector.

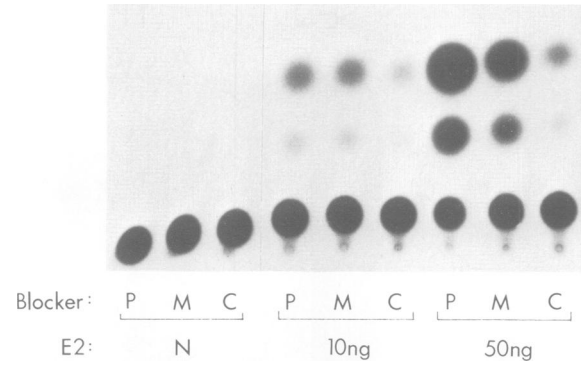

Fig. 6. Inhibition of E2 *trans*-activated p407-1 expression by the E2BP. An autoradiograph after thin layer chromatography of the unacetylated chloramphenicol (lower spots) and two monoacetylated forms (two upper spots) is shown. P, cotransfection with pUC12 DNA; M, cotransfection with pUC12 containing three copies of the mutated E2BP; C, cotransfection with pUC12 containing three copies of the consensus E2BP. N, 10 ng, and 50 ng indicate cotransfection with no, 10 ng, or 50 ng of pRSVE2E5.

Enhancer activity of the E2 binding element

To determine if the element that was recognized by E2 *in vitro* and *in vivo* had intrinsic enhancer activity, an oligonucleotide with the consensus sequence was cloned into the enhancerless $pA_{10}CAT$ vector, which contains the SV40 TATA element and 21-bp repeats but produces little CAT RNA because it lacks the SV40 enhancer (the 72-bp repeats) (Figure 7a). This vector has been previously used to show that the entire BPV URR functions as an E2-dependent enhancer when cloned either 100 bp upstream of the start of transcription (the *Bgl*II site; clone p407-1) or 1700 bp downstream of the transcription start site (the *Bam*HI site; clone p731-1). We therefore cloned oligonucleotides that

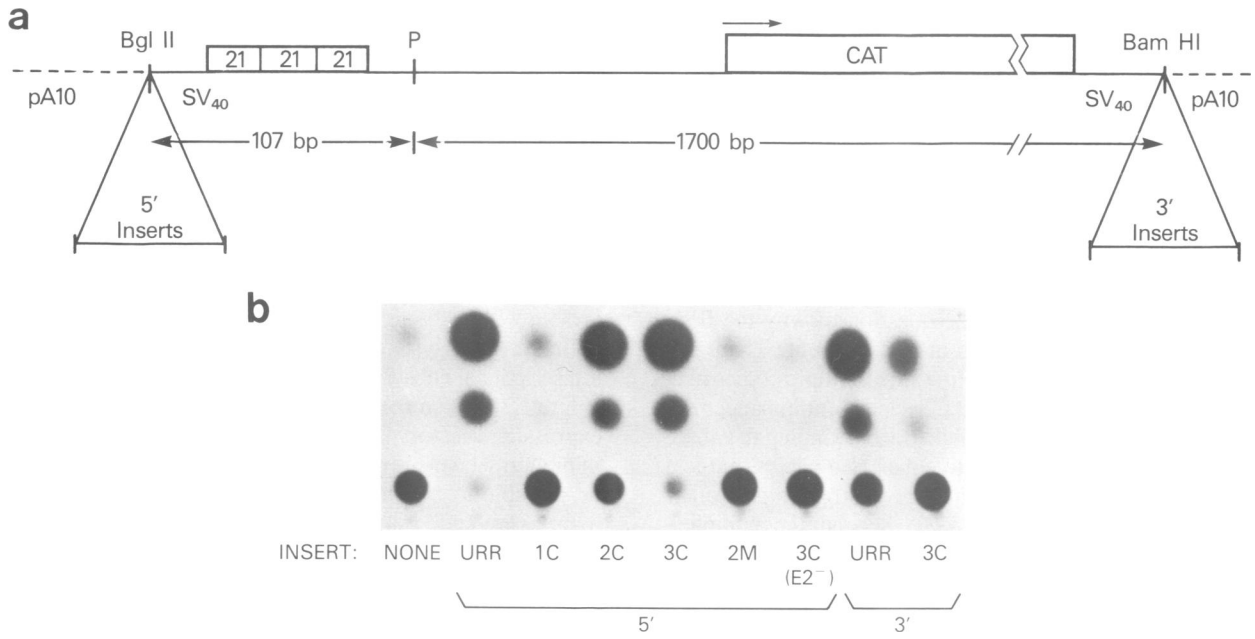

Fig. 7. Enhancer activity of the E2BP. PA10CAT is depicted at **a**. The distances between the sites of E2BP insertion (*Bgl*II and *Bam*HI) and the SV40 early promoter (P) are shown. Boxed 21s, SV40 21 bp repeat elements; CAT, chloramphenicol acetyl-transferase gene. At **b**, an autoradiograph from a representative CAT assay is shown. To visualize the weakly active lysates, the assays of the more active constructions were allowed to go beyond the linear range. Insert designations: 1C, 2C and 3C indicate the insertion of one, two or three copies of the consensus E2BP into pA10CAT respectively; 2M indicates the insertion of two copies of the mutated E2BP. Insertions 5′ of the promoter are bracketed on the left and those inserted 3′ are bracked on the right. (E2-) indicates that a derivative of the E2 expression vector with an inactive E2 gene was co-transfected in place of the wild-type E2 expression vector used in the other assays.

contained the consensus E2 binding element into these sites and assayed the CAT activity of the resulting constructions. As a specificity control, we also tested a derivative that carried two nucleotide changes in the inverted repeat. When co-transfected with an E2 expression vector, the clone that contained a single copy of the consensus motif in the 5′ site did not activate CAT expression above the background level of the parental $pA_{10}CAT$ plasmid (Figure 7b, lanes 1 and 3). In contrast, the clones that contained two or three copies of the motif exhibited relatively high levels of CAT expression, with the three elements producing more than twice as much activity as two elements (Table I). This level of activity was usually less than the activity generated by the URR in the same 5′ site (lane 2). The activation is E2 dependent, since co-transfection with an isogenic expression vector that encoded a mutant E2 gene product that is unable to *trans*-activate the URR (RSVE2E5 Nco*, Haugen *et al.*, 1987) did not lead to enhancement (lane 7). A clone containing two copies of the derivative with the alterations in the inverted repeat, which was not specifically immunoprecipitated by E2 *in vitro* (data not shown), had no detectable enhancer activity (Figure 7b, lane 6). These controls indicate that the *trans*-activation observed was due to the E2 binding elements rather than to an element fortuitously generated by the flanking nucleotides. *Trans*-activation was also detected when three of the consensus elements were cloned 3′ to the CAT gene, thus satisfying the standard genetic criteria for an enhancer. Consistent with previous studies of the intact URR (Spalholz *et al.*, 1985, and Figure 7b, lane 8) and other enhancers, less *trans*-activation was observed when the E2 binding elements were located 3′ of the target promoter.

Table 1. Enhancer activity of E2BP

Clone	Description[a]	E2[b]	Relative activity[c]			
			Exp 1	Exp 2	Exp 3	Avg[d]
pA10CAT	No insert	wt	1	1	1	1
p407-1	URR 5′	wt	139	91	54	95
pC515-11	1 E2BP 5′	wt	0.1	0.8	0.5	0.5
pC515-12	2 E2BP 5′	wt	12	11	16	13
pC515-9	3 E2BP 5′	wt	27	24	66	39
pC521-10	2 mut E2BP 5′	wt	0.7	0.7	0.4	0.6
pC515-9	3 E2BP 5′	mut	0.4	0.6	0.3	0.4
p731-1	URR 3′	wt	14	7	9	10
pC315-21	3 E2BP 3′	wt	2.3	1.9	1.7	2.0

[a]E2BP, oligonucleotide with consensus E2 binding palindrome; mut E2BP, oligonucleotide with mutated E2 binding palindrome.
[b]wt, cotransfected with wild-type E2 gene; mut, cotransfected with *trans*-activation defective E2 gene.
[c]Percent of chloramphenicol converted to acetylated form relative to the percent converted by the pA10CAT control.
[d]Average of activities from experiments 1, 2 and 3.

Discussion

The data presented in this study show that a single copy of the sequence $ACCGN_4CGGT$ constitutes a specific E2 binding site and that two tandem copies of this motif are sufficient by themselves to generate an E2-dependent enhancer. These results, together with the observations that the virally encoded E2 appears to interact with this motif *in vivo* and that alterations in the motif that abolish specific binding also abolish the enhancing activity of the motif, strongly imply that efficient transcriptional enhancement involves direct binding of the E2 protein to the motif.

The requirement for at least two elements suggests a cooperativity of interaction among DNA-bound E2 molecules. Since the minimum binding element contains an inverted repeat, it is possible that an E2 dimer is required for high affinity specific DNA binding and that the functional *trans*-

activator may therefore be a tetramer. Experiments are currently in progress to determine the stoichiometry of E2 binding. The activity of many other enhancers, such as the SV40 and polyoma, also require multiple elements (Veldman *et al.*, 1985; Herr and Clarke, 1986; Zenke *et al.*, 1986). However, multiple enhancer elements are not invariably required, since a single copy of a TPA-responsive element has recently been shown to function as a phorbol ester-inducible enhancer (Angel *et al.*, 1987).

The determination that two of the E2 binding motifs can constitute an enhancer raises the question of why the BPV URR contains so many copies of this element. There are several possible explanations. First, the multiple copies may contribute incrementally to the E2-dependent enhancer activity. Consistent with this possibility is our finding that three copies of the motif are more active than two copies, but less active than the intact URR (which probably contains other non-E2-dependent enhancers as well). Second, the multiplicity of elements may be involved in the differential activation of the URR promoters. In other enhancers, the introduction of a second promoter between an enhancer and a promoter can reduce the activity of the original promoter (Wasylyk *et al.*, 1983); the spatial arrangement of the various URR promoters relative to the enhancer elements could therefore affect the degree to which the promoters are activated. In addition, the elements with the minimum $ACCN_6GGT$ sequence do not appear to have as high an affinity for the E2 protein (at least *in vitro*), as do the elements with the $ACCGN_4CGGT$ sequence, suggesting that differential promoter activation might also be regulated by the preferential binding of the E2 activator protein to a subset of the motifs. Third, these elements probably also function as binding sites for a transcriptional repressor encoded by the c-terminal half of the E2 open reading frame (Lambert *et al.*, 1987). It remains to be determined whether the activator and repressor forms of the E2 protein [which share the DNA-binding domain of the protein (our unpublished observation)] have differences in their affinities.

Some of the motif pairs are separated by multiple whole turns of the helix. For example, motifs 8 and 9 are separated by exacty two complete turns and 2 and 3 are separated by four turns (using 10.5 nt per turn). Other motifs are separated by fractions of turns; for instance motifs 6 and 7 are within a turn and a half of each other. E2 molecules bound to elements that are whole turns apart would presumably sit on the same face of the helix, an arrangement that appears to be important for the activity of other *trans*-activating proteins (Takahashi *et al.*, 1986), raising the possibility that binding to certain pairs of the motif could lead to enhancement while binding to others pairs might inhibit the process. In the experiments described here, the oligonucleotides used to construct the E2-dependent enhancer were 21 bp in length so corresponding sequences of tandem motifs were separated by complete helical turns. We are currently investigating if changes in the spacing of the binding motifs influences their enhancing activity.

Our results suggest that E2 is the only protein that is required to bind directly to the enhancer for *trans*-activation. The oligonucleotide used to construct the functional enhancer contained only the minimum sequence required for specific E2 binding, as defined in the primer extension immunoprecipitation assay, plus one half of a restriction site on each end. Since the equivalent of this entire sequence was protected by the bacterial E2 in the previously reported DNaseI protection assay (Moskaluk and Bastia, 1987), it is unlikely that another protein could interact with the motif when it is bound by E2. However, we do imagine that the interaction of E2 with other proteins, perhaps ones associated with an initiation complex at the promoter, may be critical for E2 *trans*-activation.

Since the E2-dependent enhancer is simply two or more copies of the E2 binding site, and the E2 protein appears to be the only binding factor required for enhancement, this protein with its binding site may now provide a simple well-defined model system for studying the basic mechanism of transcriptional enhancement. Using the bacterial derived full-length E2 protein (or an E2 produced in animal cells by an expression vector if needed) and the clones with the minimum sequences required for *in vivo* enhancement, it may be possible to develop an *in vitro* E2-dependent enhancement assay and, using the E2-specific antisera, perhaps to identify and characterize E2 associated cellular factors that are involved in the process.

Materials and methods

Primer extension DNA immunoprecipitation assay

Sanger dideoxy DNA sequencing reactions were performed on single-stranded M13 clones using an Amersham kit and [^{32}P]dATP according to the manufacturer's instructions. 5 μl of each sequencing reaction was added to 100 μl of 3 × S1 buffer (150 mM Na acetate, pH 4.7, 900 mM NaCl, and 30 mM Zn acetate) containing 100 units of S1 nuclease (Bethesda Research Laboratories) and incubated for 10 min at room temperature. The reactions were stopped with 10 μl of 1 M Tris−HCl (pH 9.0) and extracted with neutral phenol:$CHCl_3$ (1:1). After addition of 0.5 μg of carrier DNA (pML2d or pBR322), the DNA was ethanol precipitated and the precipitates were resuspended in 200 μl of DIB [20 mM Hepes, 150 mM NaCl, 5 mM EDTA, 1 mM DTT, 0.05% NP−40, 100 μg/ml BSA, and 20 μg/ml Aprotinin (Boehringer Mannheim)]. Immune complexes were formed by incubating 1 μl of anti-E2 serum (Androphy *et al.*, 1987) with ~250 ng (1 μl in 4 M urea) of bacterially synthesized E2 protein in 100 μl DIB for 1 h on ice. 400 μl of DIB and 100 μl Protein A−Sepharose (20% slurry in DIB) were then added and the suspension rotated for 1 h at 4°C. After four 1-ml washes with DIB, the pellets were resuspended in the 200 μl DNA solutions described above and rotated overnight at 4°C. The complexes were washed four times with 1 ml of DIB, and the bound DNA was dissociated by incubating the pelleted complexes in 100 μl of 50 mM EDTA (pH 8.0) containing 1% SDS for 10 min at 65°C. After phenol-$CHCl_3$ extraction and addition of 0.5 μg of carrier DNA, the DNA was ethanol precipitated and the precipitated DNA was resuspended in 8 μl of 1 × loading buffer (Amersham) and, after incubation at 100°C for 10 min, the samples were subjected to electrophoresis in a standard denaturing DNA sequencing gel. As controls, 1 μl of each of the initial sequencing reactions was diluted in 50 μl of the loading buffer and 2 μl of the diluted reaction was run. After drying, the gels were exposed to Kodak X-AR5 film for 1 to 4 days at −70°C.

Chloramphenicol trans-acetylase (CAT) assay

Cotransfections of African green monkey kidney V-1 cells were performed according to Graham and van der Eb (1973), using 5 × 10^5 cells per 60-cm dish plated 24 h prior to transfection. Two to 11 μg of plasmid DNA (described in figure legends) was coprecipitated with calcium phosphate for 30 min and then incubated on the cells for 6 h. Cells were washed and fed fresh media each day, and harvested at 48−72 h post-transfection. After lysis by repeated freezing and thawing, the cytosols were assayed for CAT activity according to Gorman *et al.* (1982). Incubations were performed with 10−75 μl cytosol for 15 to 60 min (adjusted to result in acetylation of less than 50% of the total added chloramphenicol in each assay) at 37°C in 0.25 M Tris pH 8.0, 0.5 μCi of [^{14}C]chloramphenicol (NEN) and 0.44 mM acetyl CoA (Pharmacia) in a total volume of 180 μl. Ethyl acetate-extracted chloramphenicol was separated by ascending chromatography in 95:5 chloroform:methanol on plastic-backed silica gel plates (Baker). Spots were visualized by autoradiography, cut and counted in Econofluor.

Trans-activation inhibition experiment

Double-stranded DNA fragments of 23 bp, synthesized *in vitro*, were cloned into the *Hinc*II site of pUC12. The oligonucleotide that contained the consensus motif had the sequence GGTCAAACCGTCTTCGGTGCTCG and the derivative that bound E2 inefficiently *in vitro* had the sequence GGTCAAACCGTCTTCcGTGCTCG (where c indicates the changed nucleotide). The pUC12 derivative containing three copies of the consensus or mutated oligonucleotide (10 μg) was cotransfected with p407 (1 μg) and pRSVE2E5 (0, 10 or 50 ng) and the transfected cells were assayed for CAT activity.

Enhancer activity of the E2-binding palindromes

One to three copies of the consensus element

$$\left(\begin{array}{l}\text{GATCTAACCGTCTTCGGTGCG}\\ \text{GATTGGCAGAAGCCACGCTAG}\end{array}\right)$$

or the derivative

$$\left(\begin{array}{l}\text{GATCTAACaGTCTTCcGTGCG}\\ \text{GATTGtCAGAAGgCACGCTAG}\end{array}\right)$$

was cloned into the *Bgl*II or *Bam*HI site of the enhancerless expression vector pA10CAT (Laimins *et al.*, 1982; Figure 7a) and the CAT activity expressed by each was assayed after cotransfection of 1 μg of each DNA with 0.2 μg of pRSVE2E5 and 0.8 μg of pUC12 carrier DNA.

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References

Androphy,E.J., Lowy,D.R. and Schiller,J.T. (1987) *Nature*, **325**, 70–73.
Angel,P., Imagawa,M., Chiu,R., Stein,B., Imbra,R.J., Rahmsdorf,H.J., Jonat,C., Herrich,P. and Karin,M. (1987) *Cell*, **49**, 729–739.
Baker,C.C. and Howley,P.M. (1987) *EMBO J.*, **6**, 1027–1036.
Dartman,K., Schwarz,E., Gisslann,L. and zur Hausen,H. (1986) *Virology*, **151**, 124–130.
Devare,S.G., Shatzman,A., Robbins,K.C., Rosenberg,M. and Aaronson, S.A. (1984) *Cell*, **36**, 43–49.
Gorman,C.M., Moffat,L.F. and Howard,B.H. (1982) *Mol. Cell. Biol.*, **2**, 1044–1051.
Graham,F.L. and van der Eb,A.J. (1973) *Virology*, **52**, 456–461.
Haugen,T.H., Cripe,T.P., Ginder,G.D., Karin,M. and Turek,L. (1987) *EMBO J.*, **6**, 145–152.
Herr,W. and Clarke,J. (1986) *Cell*, **45**, 461–470.
Hirohiko,H., Broker,T.r. and Chow,L.T. (1987) *J. Virol.*, **61**, 2599–2606.
Laimins,L.A., Khoury,G., Gorman,C., Howard,B. and Gruss,P. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 6453–6457.
Lambert,P., Spalholz,B.A. and Howley,P.M. (1987) *Cell*, **50**, 69–78.
McKay,R.D.G. (1981) *J. Mol. Biol.*, **145**, 471–488.
Moskaluk,C. and Bastia,D. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 1215–1218.
Phelps,W.C. and Howley,P.M. (1987) *J. Virol.*, **61**, 1630–1638.
Spalholz,B.A., Lambert,P.F., Yee,C.L. and Howley,P.M. (1987) *J. Virol.*, **361**, 2128–2137.
Spalholz,B.A., Yang,Y.-C. and Howley,P.M. (1985) *Cell*, **42**, 183–191.
Takahashi,K., Vigneron,M., Matthes,H., Wildeman,A., Zenke,M. and Chambon,P. (1986) *Nature*, **313**, 121–126.
Veldman,G.M., Lupton,S. and Kamen,R. (1985) *Mol. Cell. Biol.*, **5**, 649–658.
Wasylyk,B., Wasylyk,C., Augereau,P. and Chambon,P. (1983) *Cell*, **32**, 503–514.
Zenke,M., Grundstom,T., Matthes,H., Wintzerith,M., Schatz,C., Wildeman,A. and Chambon,P. (1986) *EMBO J.*, **5**, 387–397.

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