Multiple cooperative interactions constrain BPV-1 E2 dependent activation of transcription

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ABSTRACT

Transcription directed by the BPV-1 long control region (LCR) is conditional upon activation by the virally encoded E2 protein. Within the 1.0 kb LCR there are five separate regions, A to E, that contain E2 responsive enhancers. The smallest functional region, A, is only 38 bp and contains two copies of the consensus sequence $ACC(N)_6GGT$ that is known to function as an E2 binding site *in vitro*. We show that a pair of these constitutes a minimal functional E2 responsive enhancer element but that the strength of enhancer activity is dramatically reduced both by increasing the spacing between them and by removing the dual elements from the proximity of other key promoter elements. Furthermore, pairs of dual elements activated transcription to varying levels depending upon their spatial arrangement and promoter proximity. We have also identified a low level constitutive enhancer in the D region which lacks an E2 consensus binding site but which can be activated by E2. We show that the activation potential of this constitutive enhancer is increased by association with a single E2 binding site suggesting some cooperation/interaction between viral and cellular enhancer proteins.

INTRODUCTION

Bovine papillomavirus gene expression is complex. There are at least three different early transcription units that initiate at different sites, P7185, P7940 and P89 within a 1.0kb non-coding region called the LCR (Figure 1). Three additional start sites are located within the early coding region, P2443, P3080 and P890 (1−3). Transcription directed by the LCR is conditional upon the expression of the virally encoded E2 protein (4). The full length E2 ORF encodes a transcriptional transactivator (4), and a transcriptional repressor function maps to the 3'portion of the E2 ORF (5). An E2/E8 fusion peptide that is probably expressed *in vivo* also has repressor activity (6). The N-terminal region is required for transcriptional activation (7). E2 has been shown to bind *in vitro*, via its C-terminus, to derivatives of the consensus sequence $ACC(N)_6GGT$ (8−10). There are ten of these sequences in the LCR. Spalholz *et al.* have defined two regions of the LCR, E2RE1 and E2RE2 (11), whilst Harrison *et al.* (12) have delineated five regions, designated A to E, that can function as E2 dependent enhancer elements (Figure 1). These all contain at least one copy of the E2 consensus binding site and maximum enhancer activity correlates with the presence of a more restricted consensus $ACCG(N)_4CGGT$ (12). The binding affinity of E2 *in vitro* is also greater to this sequence (10).

Region A is the smallest E2 dependent enhancer element; it contains two E2 consensus binding sites and can function in both orientations. Region B, also with two E2 binding motifs, functions only in the antisense orientation. Region D, like A, functions in both orientations but contains only one E2 consensus motif (12). In order to define more precisely

the mechanism of transcriptional control by the BPV-1 LCR we have now undertaken a more detailed study of several of these enhancer regions. We find that a pair of $ACC(N)_6GGT$ motifs is the minimal requirement for E2 dependent enhancer activity but that this activity is strongly distance dependent in relation to other promoter elements. The distance between single E2 binding sites also greatly affects enhancer activity. We further show that duplication of pairs of elements leads to increased transcription only if the elements are arranged in particular configurations. In addition, E2 dependent transactivation can occur in the absence of any E2 consensus motifs and this activation can be further enhanced by a single copy of the consensus E2 motif. Our analysis has suggested two modes of activation by the E2 protein and also highlighted several features of the BPV-1 LCR that may be generalised to other promoter and enhancer regions of both viral and cellular genes.

MATERIALS AND METHODS

Bacterial strains, mammalian cell lines and media:
Escherichia coli strain AKEC28 (C600,*thr*C,*leu*B6,*thy*A,*hsd*RK, *hsd*MK) was used for plasmid preparation and all cloning procedures. *E.coli* cultures were grown in Luria broth (13) containing thymidine at 50μg/ml and ampicillin at 50μg/ml when necessary.Mouse C127I cells (14) were maintained in Dulbecco's modified Eagles medium (Gibco) supplemented with 10% foetal calf serum, penicillin (100units/ml), streptomycin (100μg/ml) and glutamine (4mM).
DNA manipulations: Plasmids were prepared by the method of Clewell and Helinski (15) and purified by two caesium chloride density gradient centrifugations. Molecular cloning was performed by standard protocols. Klenow fragment of DNA polymerase I, T4 DNA ligase (BRL) and restriction enzymes (BRL, BCL, New England Biolabs) were all used according to the manufacturers instructions. BglII linkers (CAAAAGATCTTTTG) were obtained from Celltech and BamHI linkers (CCGGATCCGG) were from BRL. Oligonucleotides were synthesised using an Applied Biosystems synthesiser and purified by polyacrylamide gel electrophoresis by the method of Maniatis *et al.* (16).
Mammalian cell transfections: Mouse C127I cells were transfected by the calcium phosphate co-precipitation method (17) using 3×10^5 cells per 50 mm dish plated 24 hrs before transfection using 2μg of the CAT assay plasmid and 2μg of pCH110 as an internal control and, where appropriate, 2μg of the E2 expression plasmid pKV462 (see later). Four hours after the addition of the DNA the cells were shocked with 15% glycerol for 2 min (18), washed and incubated in fresh medium for 48hrs. Plasmid pCH110 contains the SV40 early promoter region driving the expression of the β-galactosidase gene and is used to correct for any differences in transfection efficiency of different plasmid preparations. Such differences were never more than three fold.
CAT and β-galactosidase assays: Cells were harvested and crude extracts were prepared by sonication (3×5 sec pulses at low power) in 0.25M Tris-HCl, pH 7.8. Assays for chloramphenicol acetyl transferase (CAT) activity were performed according to the method of Gorman *et al.* (19). Assays for β-galactosidase were performed as described by Hall *et al.* (20).
Plasmids:
A) BPV ORF expression plasmids: The expression vector pCMV-CAT (21) was modified by converting the unique HindIII site to a BglII site by fill-in followed by ligation with

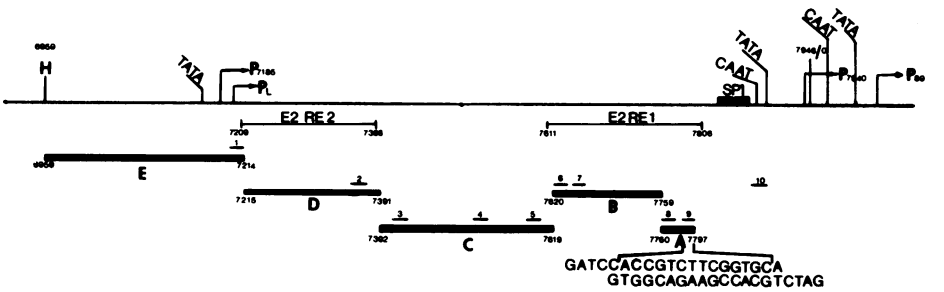

Figure 1. A summary of the cis-acting sequences in the BPV-1 LCR. The whole LCR is represented with associated RNA capsites at base pairs 7185, ~7200, 7940 and 89 and potential CAAT and TATA boxes. E2 responsive regions localised by Spalholz *et al.* (11) are labelled E2RE1 and E2RE2. Those localised by Harrison *et al.* (12) are shown as solid boxes and the E2 consensus motifs numbered. The sequence of the synthetic oligonucleotide, ES, is given below its corresponding motif.

BglII linkers to produce plasmid pKV460. The human cytomegalovirus (hCMV) promoter region and the ampicillin resistance gene and origin of replication of pBR322 was removed from this plasmid as a 2.7kb EcoRI/BglII fragment. A 1.6kb EcoRI/BglII fragment containing the SV40 polyadenylation and splice sites was removed from RSV-β-globin (22) and ligated to the former fragment to create pKV461. A 1.9kb XhoII fragment that contains the intact E2, E3, E4, and E5 open reading frames (ORFs) from pMA821 (12) was ligated into the BglII site of pKV461 to create pKV462.

B) CAT assay plasmids: The plasmid $pA_{10}CAT$ (23), which contains an enhancerless SV40 promoter linked to the CAT gene, was used to assay fragments and oligonucleotides for their enhancer activity.

Synthetic E2 binding motifs: The single-stranded oligonucleotides, 5'-GATCCACCGTCTTCGGTGCA and 5' GATCTGCACCGAAGACGGTG, were annealed to produce a double-stranded synthetic E2 binding site (ES) based on the E2 binding motif 9 (12). This was inserted into the unique BglII site of $pA_{10}CAT$ in both orientations to maintain a unique BglII site either upstream (pKV440) or downstream (pKV441) of the motif. An additional ES oligonucleotide was inserted into these plasmids at the unique BglII site to give two copies of the motifs in which the BglII site was maintained either between (ESA), upstream (ESB) or downstream (ESC) of the two motifs to produce plasmids pKV442, pKV443 and pKV444 respectively (Figure 2). A synthetic double stranded oligonucleotide corresponding to motif 2 of the D region was also inserted as a single copy into $pA_{10}CAT$ (pKV498).

RESULTS

1) Two synthetic E2 binding motifs constitute a functional enhancer element.

The five E2 responsive enhancer regions that we have identified in the BPV-1 LCR are shown in Figure 1. Region A contains two copies of the E2 binding motif and is an efficient enhancer element. Region D, too, is an effective enhancer element but contains only a single E2 binding site. To test whether a single E2 binding site was indeed the minimal requirement for activation we prepared a synthetic oligonucleotide corresponding to motif 9 (Figure 2.a). This was inserted in both orientations into $pA_{10}CAT$ and assayed for enhancer activity in the presence and absence of E2 (Figure 2.b). This synthetic oligonucleotide (ES) was completely inactive . When two copies of the oligonucleotide

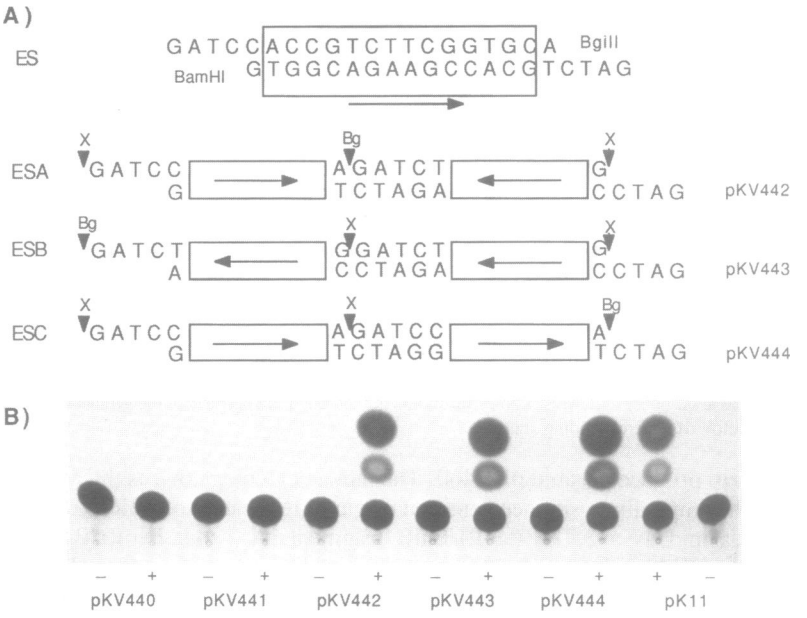

Figure 2. A) Sequence of the synthetic oligonucleotide ES that is based on motif 9 of the LCR. ESA, ESB, and ESC show the three orientations of a double insert. Restriction sites given are those created on insertion into $pA_{10}CAT$, Bg=BglII; X=XhoII. B) Autoradiograph of CAT assay illustrating the activities of ES in both orientations (pKV440 and pKV441), ESA, ESB, and ESC (pKV442, pKV443 and pKV444). pK11 is the BPV-1 LCR in SVOCAT (12). +/− indicate the presence or absence of E2.

were linked together in all possible relative orientations (ESA, ESB, ESC) high levels of enhancer activity were achieved. These data show that two copies of the synthetic binding site are essential for E2 dependent enhancer activity in $pA_{10}CAT$. A contribution by E5 to this transactivation can be excluded on the evidence of Yang *et al.* (24) and Haugen *et al.* (25) where a translational termination linker and a frameshift mutation, respectively, were introduced just downstream of the first AUG of the E5 ORF. In both studies E2 dependent transactivation of the BPV-1 LCR was retained. Similar results have recently been reported for other synthetic oligonucleotides corresponding to the E2 consensus motif (9,26).

2) A single E2 binding site is functional in association with a constitutive enhancer that is also activated by E2.

Our finding that a single E2 binding site was not an enhancer element when inserted into $pA_{10}CAT$ prompted us to ask what was responsible for the E2 dependent activation that we observed with the D region. To identify functional elements in the D region of the LCR the fragment was subcloned as follows (Figure 3.a). The 176bp RsaI fragment from the LCR in pK11 (12), coordinates 7215−7391, was inserted into the HincII site of pSP46 (27). Subsequent manipulations of this plasmid (Figure 3a) generated a 67bp fragment (W) that contains a single E2 binding site and a 150bp fragment (X) that contains no E2 binding site but which does contain a number of regions of homology with several binding sites for cellular transcription factors (Figure 3.b). Fragments W and X were inserted in

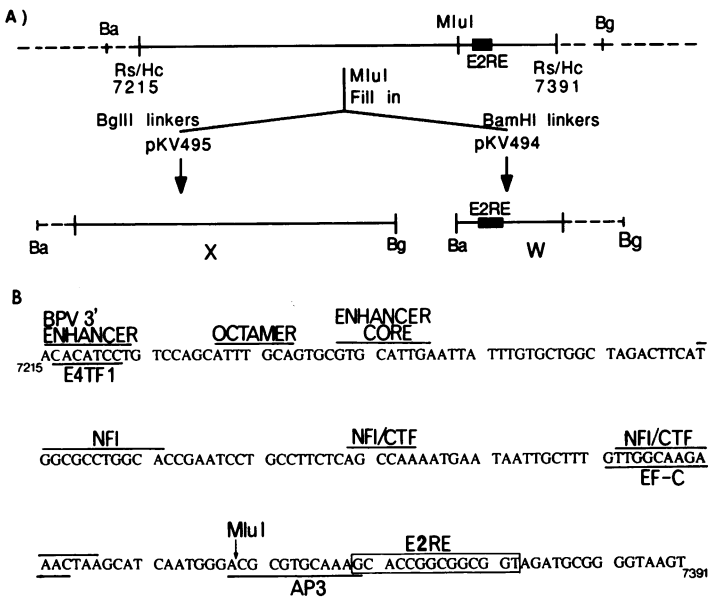

Figure 3. A) Subcloning of the BPV-1 LCR D region. Solid lines represent LCR sequences, dotted lines represent pSP64 sequences. Restriction sites are Bg=BglII; MluI=MluI; Ba=BamHI; Rs=RsaI; Hc=HincII; B) Nucleotide sequence of the BPV-1 LCR D-region. Regions of homology to previously described transcription factor binding sites are shown. BPV 3′ enhancer (40); Octamer element (45); Enhancer core (46); NF1=Nuclear factor 1 (39); CTF=CAAT box transcription factor (39); AP3=Activator protein 3 (47); EFC (48); E4TF1 (Wantabe, as cited in Jones *et al.*(49); E2RE=E2 responsive element; MluI= restriction site. All homologies are at 80% or greater.

both orientations into $pA_{10}CAT$ and assayed for enhancer activity in the presence and absence of E2.

As shown in Figure 4, lanes 11,12,13 and 14, fragment W was inactive. This confirmed that a different variant of the E2 consensus (motif 2) was also inactive in a single copy. A synthetic oligonucleotide corresponding to this motif was also inactive (lanes 15 and 16). The X fragment had low level enhancer activity and this was increased significantly in the presence of E2 (lanes 7 and 9). This fragment contains sequences that have been recognised as either constitutive or conditional functional elements in other promoters. Clearly they are barely active in $pA_{10}CAT$ in the absence of E2 (see tracks 4,6,8 & 10 in Figure 4). In the presence of E2 they are active (tracks 3,5,7 & 9). This activity is however less than the activity of the full length D fragment (compare tracks 3 & 5 with tracks 7 & 9) suggesting that the E2 binding site may cooperate with these enhancer elements to produce the full E2 dependent enhancer activity. There was also some orientation preference observed for the D region; the greater enhancer activity being in the antisense orientation.

These data indicate that a single E2 motif is inactive. Furthermore, they show that the activity of a constitutive enhancer is augmented by E2 and that a single E2 binding site can cooperate with cellular enhancer elements.

3) Separation of E2 binding sites beyond 100bp dramatically reduces transcription.

As two synthetic E2 binding sites are required to achieve enhancer activity we wished to determine if their spatial relationship was critical. It has been suggested that for efficient

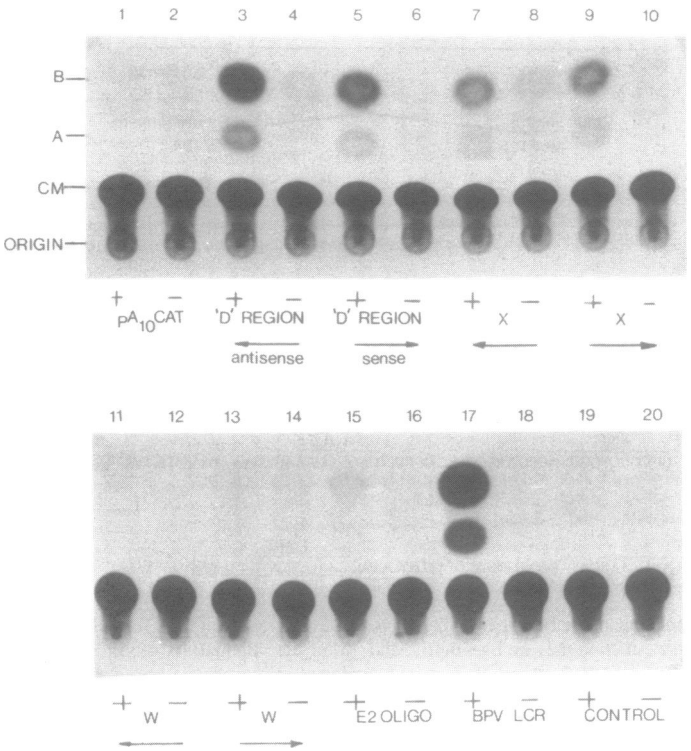

Figure 4. Autoradiograph of CAT assay illustrating activities of the D region and its constituents. The 'D'region inserted into $pA_{10}CAT$ in the antisense and sense orientations constitutes plasmids pKV491 and pKV492 respectively; fragment X (contains the regions of homology with binding sites for cellular transcription factors) inserted into $pA_{10}CAT$ in the antisense and sense orientations constitutes plasmids pKV496 and pKV496a respectively; fragment W (contains the E2 binding site) inserted into $pA_{10}CAT$ in the antisense and sense orientations constitutes plasmids pKV497 and pKV497a respectively. The sequence of the E2 oligo is shown boxed in Figure 3B. Sense and antisense refers to the orientation of the fragment in $pA_{10}CAT$ relative to its orientation in the BPV LCR. The BPV-1 LCR in plasmid pK11 is described elsewhere (12).

protein−protein interactions to occur all the transcription factors must be aligned on the same face of the helix or they must be sufficiently separated to allow the DNA to loop and twist and thus juxtapose the factors. For example the lambda-phage repressor (28), the heat shock transcription factor of *Drosophila melanogaster* (29), the early gene promoter of simian virus 40 (30) and the *E.coli ara*BAD operon (31). In the BPV-1 LCR there are two pairs of tandem E2 binding sites; one pair is located in region B and one in region A and both are enhancer elements when assayed in $pA_{10}CAT$. The centres of the palindromes are separated by 14bp and 21bp respectively. In the synthetic double oligonucleotide ESA the centres are separated by 22bp. In the A region and the synthetic double oligonucleotide the palindrome centres are therefore 2 helix turns apart and in the B region the centres are 1.5 turns apart, given that one turn of the B form of the DNA helix is 10.5bp. These observations suggest that there is no absolute requirement for alignment on the helix but we wished to test whether there was any significant quantitative effect. To do this we altered the spacing in the synthetic double E2 binding motif, ESA, in pKV442.

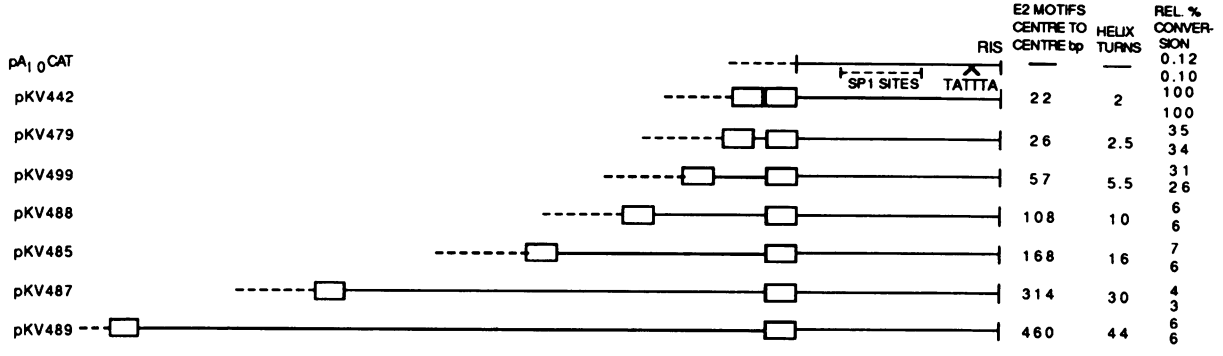

Figure 5. Analysis of the spatial requirements for an E2 dependent enhancer element: distance between E2 binding motifs. ▭ represents a single E2 binding site. Quoted figures for relative % conversions are from duplicate experiments. The value for pKV442 is taken as 100% and the others are scaled accordingly.

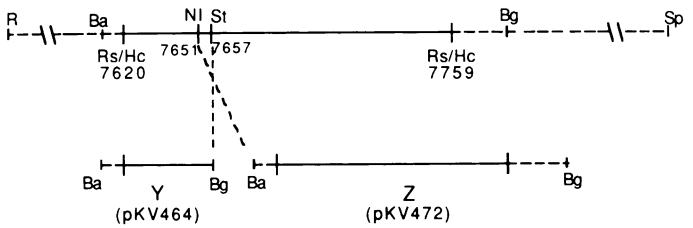

Figure 6. Subcloning of the BPV-1 LCR B region.Solid lines represent LCR sequences, dotted lines represent pSP64 sequences. Restriction sites are: Sp=SphI; St=StyI; Nl=NlaIV; R=EcoRI.

The plasmid pKV442 (Figure 2.a) was cut with BglII and a 35bp fragment derived from pSP46 (the BamHI−BglII polylinker sequence) was inserted to produce pKV499. A four base pair insertion was introduced by filling in the BglII site of pKV442 before religation to produce pKV479.

The modified synthetic enhancer fragments were assayed in $pA_{10}CAT$ and the results are shown in Figure 5. The maximum activity is obtained when the two motifs are separated by two turns and, therefore, theoretically aligned on the same face. Separation by 2.5 and 5.5 turns reduced the activity to one third. This indicated that the efficiency of activation was reduced by altering the topological relationship suggesting that interaction between adjacent E2 proteins is important.

It has been suggested that such interactions can still occur when transcription factor binding sites are sufficiently separated to allow the DNA to loop and/or twist. We therefore separated the motifs further apart by the insertion of the following fragments into the BglII site of pKV442: an 86bp fragment derived from pBR322 to create pKV488 (the 51bp Hae III fragment was inserted into the HincII site of pSP46 and removed as a 86bp BamHI−BglII fragment),and a 146bp fragment (IFN-C) derived from the coding region of the human interferon alpha-2 gene to create pKV485. IFN-C, was generated by the insertion of the 111bp AluI fragment (coordinates 578−689) of the human interferon alpha-2 gene (32) into the HincII site of pSP46 and the subsequent removal of a 146bp BglII/BamHI fragment. Multiples of the 146bp IFN fragment were also inserted to produce pKV487 and pKV489. When the spacing between the two motifs was increased by 108bp to 460bp the activity was dramatically reduced. As 460bp is about 44 helix turns this is sufficient separation to allow the DNA to loop and rotate to align the E2 proteins. These data show that the binding motifs must be close i.e 14 to 60bp to allow significant activation. As the activity is much reduced when the motifs are separated this suggests that if the activity is indeed mediated by the juxtaposition of sites by DNA looping and twisting then this is not an efficient process in this case.

4) Efficient E2 dependent activation requires close proximity of E2 binding motifs to promoter elements.

The synthetic double E2 binding motif ESA and the BPV-1 A region both function in either orientation in $pA_{10}CAT$. The B region of the LCR (coordinates 7620 to 7759) was however only active in the 'antisense' orientation. We considered three explanations for this observation; 1) the enhancer activity of motifs 6 and 7 is inherently orientation specific; 2) a transcriptional silencer is located in the B region which when interposed between the E2 binding sites and other promoter elements blocks activation, or 3) the ability of the E2 binding sites to activate is distance dependent.

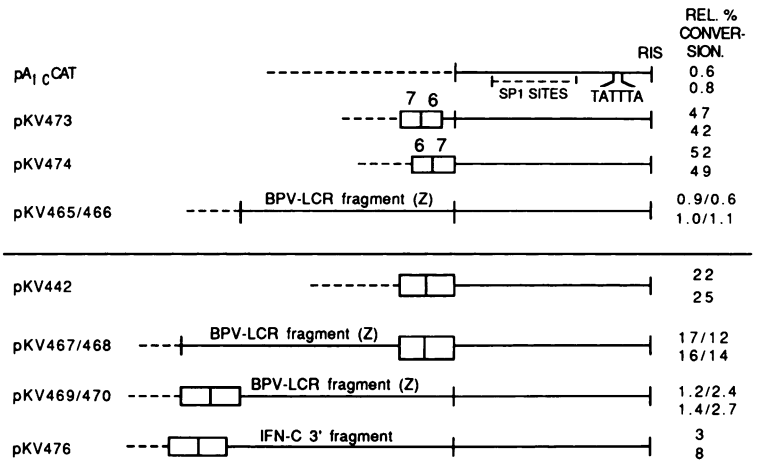

Figure 7. Analysis of the spatial requirements for an E2 dependent enhancer element: distance of E2 motifs from promoter elements. Where two pKV numbers are given this indicates two orientations of the inserted fragment. Quoted figures for relative % conversions are from duplicate experiments. A representative CAT assay for pKVs 442, 469 and 476 is shown in figure 8B.

To test these hypotheses we subdivided the B region into two fragments by cleavage at the StyI and NlaIV restriction sites (Figure 6). The B region was inserted into pSP46 to produce pSP46B (Figure 6). pSP46B was linearised at the unique StyI site, filled in and religated in the presence of BglII linkers to create pKV472. A 47bp BglII – BamHI fragment (Y) from this plasmid contains the two E2 responsive motifs, 6 and 7. The remainder of the B region was subcloned by the isolation of a 623bp SphI/EcoRI fragment from pSP46B and its subsequent digestion with BglII and NlaIV. The desired 133bp BglII/NlaIV fragment was ligated to HincII/BglII digested pSP46. BamHI/BglII digestion of this plasmid, pKV464, generated a 143bp fragment, (Z). Fragment Y contains the two E2 binding sites and fragment Z contains no related sites. The enhancer activity of the two subfragments was assessed by inserting them in both orientations into $pA_{10}CAT$. The ability of the Z fragment to block transcriptional activation was tested by inserting it into the BglII site of derivatives of $pA_{10}CAT$ that contained the synthetic double E2 oligonucleotide (Figure 2). These are plasmid pKV444 to place the Z fragment between the enhancer and the mRNA start site and pKV443 to place it upstream of the enhancer. In addition a control fragment of the same size as the Z fragment derived from within the coding region of the human interferon alpha-2 gene (IFN-C, see above) was used to test the specificity of any effect.

As shown in Figure 7, fragment Y was an efficient E2 dependent activator in both orientations and fragment Z had no activity. Clearly motifs 6 & 7 are not inherently orientation specific.

The Z fragment blocked activation by the synthetic double oligonucleotides in both orientations but only when placed downstream of the binding sites. An identical result was, however, obtained with the control IFN-C fragment. These data show that the enhancer activity of the E2 binding sites can be reduced but that this is not a specific effect of a BPV-1 LCR fragment and is therefore unlikely to be mediated by a specific transcriptional silencer. The most likely explanation is that the enhancer activity of motifs 6 and 7 is strongly dependent upon their distance from other promoter components and that the observed effects are a consequence of the increase in this distance.

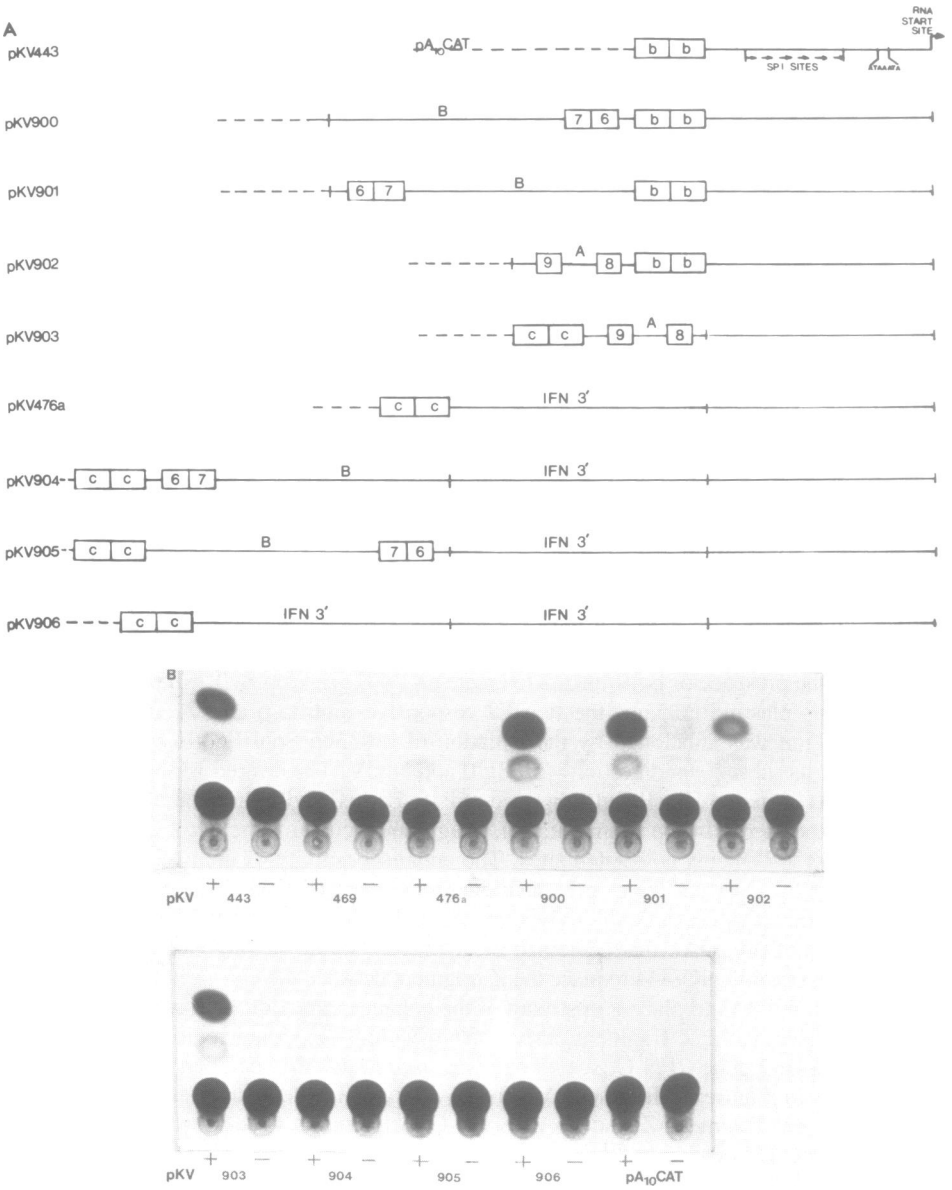

Figure 8. A) Analysis of the organisation of E2 enhancer. ▭ represents a single E2 motif. b and c are the synthetic motifs ESB and ESC. 7 and 8 refer to the E2 motifs of the LCR. A and B represent the A and B regions of the LCR and IFN 3′ is the 146bp IFN-C fragment (see text). B) Autoradiograph of CAT assay showing the activities of the constructs detailed in figure 8A. +/− indicates the presence or absence of E2.

5) The BPV-1 LCR is composed of multiple functional enhancer elements.

It has been demonstrated that the BPV-1 LCR has a complex structure and consists of multiple functional elements (11,12). The demonstration that two response motifs in

particular arrangements constitute an E2 dependent enhancer raises the question as to the significance of the number and arrangement of the E2 binding motifs in the LCR. Other enhancers have been shown to contain multiple functional elements; the best characterised of these being that of SV40. By analogy to the study of Zenke *et al.* (33) it could be argued that each E2 dependent enhancer region contributes incrementally to the enhancer activity of the full LCR. In this way, cooperation between the functional elements may serve to compensate for the distance dependence of pairs of E2 binding motifs. For example, the analysis of Herr and Clarke (34) of the SV40 enhancer in which three enhancer elements, A, B and C, were identified which although inactive alone can cooperate with one another to generate a functional enhancer. Recently Ondek *et al.*(35) have suggested that each of these elements consists of two subunits called enhansons, each of which corresponds to a transcription factor binding site. These enhansons which are inactive alone can be combined to generate enhancer elements although their separation is critical.

We therefore wished to analyse whether E2 response motifs have similar characteristics. To this end the molecules shown in Figure 8 were constructed. The parental molecules used were pKV443, pKV444, and pKV476. pKVs900−906 were created by inserting the BPV-1 LCR A region (subcloned as a 73bp BglII−BamHI fragment via SP46), the B region (subcloned as a 174bp BglII−BamHI fragment via SP46) and the 146bp IFN-C fragment described earlier in various combinations and orientations into the unique BglII site in the parental molecules.

The results show that the duplication of pairs of E2 binding motifs increases enhancer activity, compare pKV443 with pKV900 and pKV904 with pKV906. This increase in enhancer activity is, however, dependent upon the configuration and specific combination of single E2 response motifs that have different sequences, compare pKV900, pKV901, pKV902 and pKV903. Comparison of pKV900 with pKV904 indicates that even multiple E2 response motifs are strongly distance dependent with respect to enhancer activity. pKV906 is included to show that distance independence is not restored to a pair of tandem E2 response motifs at a particular distance from the promoter.

DISCUSSION

We have shown that a single BPV-1 LCR E2 binding site (i.e motif 2) or synthetic oligonucleotides corresponding to motifs 9 and 2 are not E2 dependent enhancer elements, although two motifs in all combinations, when promoter proximal, are functional. Similar results have recently been reported by Hawley-Nelson *et al.* and Hirochika *et al.* (9,26). McBride *et al.* (10) have suggested that the C-terminus of the E2 gene product represses E2 transactivation by competitive binding to the same *cis* DNA target elements required for E2 transactivator binding. As E2 dependent activation via binding sites requires at least two molecules of the protein any significant production of repressor could abolish expression by the formation of mixed and therefore inactive complexes. A fine balance on the activity of elements 8 and 9 could therefore be acheived by repressor concentration. The single sites could also act as targets for repression and the activity of the D region for example could be stepped down by repressor binding. In this respect it is also interesting that it has been recently shown that E2 binding at motif 1 (7203−7216) inhibits the expression of the P7185 transcription by interfering with the binding of a cellular factor (36). Similarly, the presence of the E2 binding sites adjacent to the 2443 and 3080 capsites maybe important in the autoregulation of the expression of E2 and the repressor.

Our data show that although two E2 motifs are required activation can still occur when the E2 sites are on opposite faces of the helix; a similar situation is seen for the GAL4 protein of yeast (37). This observation is unlike the periodicity seen in the cooperative binding of the lambda-phage and *lac* repressors where maximal effect requires a unique rotational alignment to form essential contacts between the proteins bound to two separate DNA elements; the energy required to both loop and twist the DNA over such a short distance being prohibitive. We suggest that the relatively long, proline rich, and therefore flexible, 'hinge' region of E2 proposed by Giri and Yaniv (7) allows the E2 protein to wrap around the DNA such that significant activation can still occur when the two binding sites are not aligned.

Our finding that activation is however dramatically reduced when the two binding sites are separated by anything greater than 60bp was unexpected. Previous studies with the SV40 promoter (30) and the heat shock transcription factor of *Drosophila* (29) indicated that although activation was spatially constrained over short distances this was relieved over long distances and this was explained by the ability of the DNA to loop and twist. It could be that the interactions between the E2 proteins are not sufficiently strong to stabilise a loop. Alternatively, the proteins may not have sites that directly contact each other.

We have also identified an additional spatial constraint upon E2 dependent activation. Activation only occurs when the E2 binding sites are close to promoter elements in $pA_{10}CAT$. These data extend recent observations made by Spalholz *et al.* (38) that also show E2 motifs must be close to the SV40 promoter for optimum activity. This suggests that additional protein−protein interactions are required. Possible sites for this interaction are at the GC boxes or the TATA box with Sp1 or TFIID respectively. The more likely is with TFIID as a TATA box precedes each of the RNA initiation sites in the LCR i.e. P7185, P7940 and P89. The distance dependence may also indicate that each RNA initiation site is independently regulated and this is partially achieved by differential interaction between the various initiation elements and E2 dependent enhancer elements. We have shown that the addition of another pair of elements increases the efficiency of action at a distance but does not restore full activity.

Our analysis of the D fragment has revealed an additional mode of activation by the E2 protein. The region from coordinates 7215 to 7353 contained within the D region only has significant enhancer activity in the presence of E2. This fragment contains an octamer element and sites for the transcription factors NFI/CTF (39) and the 'enhancer core' element that was identified in the SV40 promoter in addition to the BPV 3′ enhancer (40). It is possible that E2 interacts directly with one or more of these enhancer elements via a DNA binding motif different to that used to bind to $ACC(N)_6GGT$ motifs but this seems unlikely. We favour the idea that like the adenovirus E1a protein, E2 has the ability to affect DNA indirectly through a host cellular factor (41,42). This discovery that E2 can increase the activity of other enhancers that lack E2 response motifs may be the same phenomenon as described in observations that the activity of heterologous promoters can be enhanced by E2 and by an N-terminal domain of E2 that lacks the DNA binding domain (11,25,43). The finding that the response of heterologous promoters to E2 can be further increased by the presence of a single E2 binding site, which is in itself inactive, suggests some form of interaction between E2 and other enhancer binding proteins. This could effectively present the same surface for further protein interactions as presented by two E2 proteins/dimers binding to adjacent E2 consensus binding sites. Alternatively the E2 binding site may serve simply to concentrate more transcription factors at the promoter.

Nucleotide sequence comparisons reveal that all papilloma-viruses have a similar genetic organisation (44). Like BPV-1, all human papillomaviruses (HPVs) have an LCR situated upstream from the early region that contains the E2 consensus motif $ACC(N)_6GGT$. It has been shown that the E2 gene product from HPV-1, HPV-11, or BPV-1 can cross activate and repress the LCRs of BPV-1 and HPV-16. An examination of different strains of HPV highlights a different number and arrangement of E2 binding motifs and RNA start sites. Our analysis of the BPV-1 LCR thus sheds light onto the potential for differential control of these RNA start sites via the different combinations and spacing of both cellular and viral enhancers. The specific configuration of these elements in the papillomavirus LCRs may determine the different clinical manifestations of different HPV strains.

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