Positive and negative E2-independent regulatory elements in the long control region of bovine papillomavirus type 4

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The long control region (LCR) of bovine papillomavirus type 4 demonstrated enhancer activity when cloned upstream of a bacterial chloramphenicol acetyltransferase reporter gene under thymidine kinase promoter control. Deletion analysis of the LCR revealed the presence of several positive and negative control elements, all of which could function independently of the viral E2 trans-activator. Each of the three positive elements present appeared to be paired with a negative element which modulated its activity. DNase I footprinting was used to identify protein binding sites within the LCR, which might represent these control elements. The results suggest a highly complex and finely tuned control of viral gene expression.

Introduction

Infection by bovine papillomavirus type 4 (BPV-4) leads to the formation of papillomas that can progress to carcinoma in cattle grazing on bracken fern, which contains both carcinogens and immunosuppressants (Jarrett et al., 1978; Campo et al., 1980). In vitro experiments have demonstrated that the viral genome encodes protein(s) capable of transformation of established mouse fibroblasts and of cooperation with the ras oncogene to transform primary bovine cells in culture (Jaggar et al., 1990). The frequency of in vitro transformation was found to be dependent on a high level of expression of the viral E8/E7 open reading frames; transformation could be increased by co-expressing the viral trans-activator E2, as has been shown also for human papillomavirus type 16 (HPV-16; Lees et al., 1990). Thus, transcriptional control of the viral genome by viral and/or host factors may be an important determinant in progression to carcinoma. Indeed, it was proposed by zur Hausen (1987) that cancer could result from a failure of cellular control of viral genes. We have therefore been studying transcriptional control of BPV-4. The viral genome is 7265 nucleotides long and can be roughly divided into two sections, encoding the early and late viral proteins. Upstream of the early region is a long control region (LCR) which contains potential binding sites for several factors, including NF1, Sp1 and the viral trans-activator E2 (Patel et al., 1987). By analogy with BPV-1, which is able to transform mouse cells in vitro although it does not cause cancer in vivo (Broker & Botchan, 1986), both positive and negative regulation of the BPV-4 LCR might be expected to occur via the action of full-length (trans-activator) and short (repressor) E2 molecules binding at the E2 sites (Lambert et al., 1989). Although RNA mapping has shown that several transcriptional promoters are present along the papillomavirus genome (Linz & Baker, 1988; Stamps & Campo, 1988), in BPV-1 it has been demonstrated that the LCR is involved in the regulation of transcripts initiated both within and outside the LCR itself (Hermonat et al., 1988). DNase I footprinting of HPV-16 has shown the presence of 23 footprints within the 900 bp LCR (Gloss et al., 1989), suggesting an intricate array of regulatory elements although some of these sites may be involved in replication rather than transcriptional control (Lusky & Botchan, 1984). In addition to E2-independent enhancer elements identified in several papillomaviruses (Hirochika et al., 1987; Gloss et al., 1989; Cripe et al., 1987; Wu & Mounts, 1988; Chin et al., 1989), silencer elements have also been demonstrated in HPV-6 and HPV-8 (Wu & Mounts, 1988; Reh & Pfister, 1990). Thus the regulation of papillomavirus transcription by the LCR is complex, involving both host- and virus-encoded proteins. The results presented here demonstrate the existence of both positive and negative E2-independent transcriptional control elements in the BPV-4 LCR.

Methods

Transfection. CT3 cells (a line of NIH 3T3 cells obtained from Geoffrey M. Cooper) were cultured in SLM (Gibco BRL) supplemented with 10% foetal calf serum. Transfections, using 10 µg of test plasmid and 5 µg of control plasmid pHSV-βgal, were carried out by
calcium phosphate precipitation as described by Gorman (1985). Precipitates were left on the cells for 18 h, the cells were washed and refed and then harvested 30 h later. Chloramphenicol acetyltransferase (CAT) and β-galactosidase assays were performed as described by Gorman (1985). Percentage conversion of chloramphenicol to the acetylated form was quantified by scintillation counting and the values were standardized using the β-galactosidase activities.

Footprinting. CT3 nuclear extracts were prepared exactly as described by Sibbet & Campo (1990). For DNase I footprinting nuclear extract was incubated on ice for 15 min with 1 μg poly[dI·dC] in 25 mM-Tris-HCl pH 7.9, 6.25 mM-MgCl₂, 50 mM-KCl, 1 mM-EDTA, 0.5 mM-DTT in a final volume of 50 μl. DNA fragment (3 to 5 ng), end-labelled using [γ-32P]ATP and polynucleotide kinase, was added and incubation was continued for a further 15 min on ice followed by a 2 min incubation at room temperature. An equal volume of 5 mM-CaCl₂, 10 mM-MgCl₂ was then added and the samples were digested for 1 min at room temperature with between 1 and 5 μl of freshly diluted DNase I at the concentrations indicated (see Fig. 5 legend). The reactions were terminated by the addition of 100 μl 200 mM-NaCl, 20 mM-EDTA, 1% SDS, 250 μg/ml tRNA, and the samples were phenol-extracted and ethanol-precipitated prior to loading onto an 8% acrylamide sequencing gel.

Results

Enhancer activity of the BPV-4 LCR

From the DNA sequence of BPV-4 a number of consensus sites for transcription factors, including three E2 binding sites, were identified in the LCR between nucleotides 7050 to 280 (see Fig. 4). This region of DNA does not contain the major transcriptional promoter identified by Stamps & Campo (1988) and has negligible transcriptional activity in bovine palate or mouse fibroblasts (data not shown). Therefore the LCR was subcloned in both orientations into the enhancer test plasmid p41X (Fig. 1) as an 865 bp NalIII fragment (nucleotides 6710 to 310), to yield plasmids p41XLCR+ and p41XLCR− (Fig. 1). Each of these plasmids was transfected into CT3 mouse fibroblast cells using the calcium phosphate technique and assayed for CAT activity 48 h after transfection. All CAT assays were standardized by cotransfection with plasmid pHSV-βgal, which carries the Escherichia coli β-galactosidase gene under the control of the herpes simplex virus immediate early 4 promoter. From Fig. 1 it can be seen that the presence of the BPV-4 LCR upstream of the thymidine kinase (TK) promoter increased CAT activity irrespective of orientation; thus, the LCR displays the characteristics of a transcriptional enhancer in this assay.

Assay of BPV-4 LCR deletion mutants for enhancer activity

Two series of unidirectional deletions were made in the LCR by Bal 31 digestion and ligation into vector p41X, followed by DNA sequencing to determine the extent of each deletion. The recombinant clones are identified by the letters A and B, representing deletions from the 5' and 3' end of the LCR respectively, and by numbers representing the nucleotide present at the deletion endpoint (Fig. 2). The enhancer activity of each deleted clone, in the '+' orientation with respect to the TK promoter, was determined by transfection and CAT assay in CT3 cells. The results (Fig. 2), which are expressed as percentages of the complete LCR enhancer activity, show that deletion of DNA between nucleotides 6710 and 7130 had little effect on enhancer activity. However, the additional deletion of nucleotides 7131 to 7211 resulted in a reduction in enhancer activity to 16% of that of the complete LCR, suggesting that a major positive element had been wholly or partially removed. The remaining deletions in series A all possessed between 4 and 8% of the complete LCR activity, with the exception of A-20 which had 34% activity. The greater activity of A-20 is apparently associated with the removal of DNA including a GC box, thought to be a potential Spl binding site, and is discussed further below.

Of the series B deletions, B-246 possessed 88% of the activity of the complete LCR. However, when a further 42 nucleotides were deleted (B-204) the enhancer activity of the LCR was increased sixfold. The next deletion in the series, B-167 had only 64% of the complete LCR activity. To eliminate the possibility that the increased enhancer activity of B-204 was due to a positional effect (i.e. moving certain LCR elements into optimal configurations with respect to TK promoter elements), the orientations of the BPV-4 LCR fragments of B-246, B-204 and B-167 were reversed with respect to the TK promoter. The enhancer activities of the reversed clones were found to correlate well with the original values, with B-204 possessing a fourfold greater activity than B-246.
Thus the DNA between nucleotides 204 and 246 appears to encode at least part of a negative regulatory element (negative regulator 3; NR3), removal of which allows a four- to sixfold increase in activity. Nucleotides 167 to 204 seem to encode (part of) a positive regulatory element (control element 3; CE3) because deletion B-167 had enhancer activity approximately eightfold less than that of B-204 in either orientation.

Deletions B-152, B-118, B-99, B-66, B-21, B-7261 and B-7226 all retained approximately 30 to 50% activity. However, deletion B-7130 possessed only 10% of the activity of the complete LCR. Putting these results together with those of the series A deletions it appeared that a major positive element (CE1) was present between nucleotides 7130 and 7226.

A further positive element was identified between nucleotides 20 and 66 (CE2). Deletions in this region led to a reduction in enhancer activity from 34% to 4% in series A (compare A-20 with A-44) and from 51% to 27% in series B (compare B-66 with B-21). Between nucleotides 20 and 66 there is a sequence with homology to the BPV-1 5' enhancer (Spalholz et al., 1985) which may well be an active element within CE2. With the series A deletions, removal of region NR2 led to a fivefold increase in enhancer activity (compare A-20 with A-7259); no increase in enhancer activity was seen with the series B deletions when this region was deleted (compare B-21 with B-7261). These results suggest that NR2 may be a negative regulator of the adjacent CE2 and that NR2 does not affect the activity of CE1.

Nucleotides 7135 to 7226 act as an enhancer (CE1)

The 91 bp fragment between nucleotides 7135 and 7226 was subcloned into p41X in both orientations (Fig. 3). In the ‘+’ orientation with respect to the TK promoter fragment this fragment showed 83% enhancer activity and in the ‘−’ orientation 144% enhancer activity relative to the activity obtained for the complete LCR. These results confirm that nucleotides 7135 to 7226 do encode an enhancer element (CE1). It is interesting that the enhancer activity for the 91 bp fragment encoding
Fig. 3. Enhancer activity of the 91 bp fragment between nucleotides 7135 and 7226, cloned in both orientations in p41X. The enhancer activity for each clone relative to the complete LCR fragment is expressed as described in the legend to Fig. 2. Enhancer activities for deletions A-7131, A-7212, B-7130 and B-7226 are shown for comparison. C1, CE1; N1, NR1.

CE1 alone is more than twice that observed for deletion B-7226, encoding nucleotides 6710 to 7226. This suggests the presence of sequences upstream of the enhancer which negatively modulate its activity; these sequences have been termed NR1.

Footprinting of the BPV4 LCR

To identify protein binding sites within the LCR, DNase I footprinting was carried out using CT3 nuclear extract. Between nucleotides 7000 and 310, 16 footprints were observed (Fig. 4 and 5). Of these footprints, three (FP9, FP10 and FP11) were found within fragment 7135 to 7226, which contains the enhancer element CE1, and two further footprints (FP12 and FP13) mapped immediately upstream of this region; they could represent the NR1 activity as suggested above. Footprint FP8 lies adjacent to CE1, but removal of this protein binding site had little effect on enhancer activity in CT3 cells (compare A-7261 with A-7226).

In the region identified as CE3, two footprints were found, FP2b and FP2c. FP2c coincides with an E2 consensus sequence and the CT3 nuclear factor binding to this site may well compete with the viral E2 protein when both proteins are present. Between nucleotides 204 and 246, containing a putative negative regulatory element (NR3), two footprints were found, FP1b and FP2a. Although there is only a single band involved in FP1b, this band does decrease significantly in intensity in relation to the bands on either side. FP1b and FP2a map to either side of a region showing homology to a region of the simian virus 40 (SV40) late promoter (Brady et al., 1982), but no footprint was found directly over this region. FP1a lies adjacent to an E2 motif, but no significant changes in enhancer activity were associated with the removal of this region.

Discussion

Using an enhancer assay we have demonstrated the presence in the BPV-4 LCR of multiple positive and negative regulatory elements which are able to act independently of E2 products. Since the BPV-4 LCR has negligible transcriptional promoter activity in bovine or mouse fibroblasts and because results obtained with the LCR and its derivatives in either orientation with respect to the TK promoter are comparable, we are confident that transcription initiates within the TK promoter itself.

The first regulatory element, CE1, has been cloned as a 91 bp fragment and shown to possess greater activity in this form than when cloned together with upstream sequences (83% compared to 35%; Fig. 3). The upstream sequences (NR1) thus appear to negatively regulate CE1. Blessing et al. (1987) identified an octamer sequence, AARCCCAA, present in the upstream region of epithelially expressed genes, including cytokeratins. This
motif is present at the border of the CE1 element, albeit in the reversed orientation with respect to the direction of transcription (see Fig. 4). Such an inverted octamer element has also been found within a constitutive enhancer element of HPV-11 (Chin et al., 1989) and in the keratinocyte-dependent enhancer of HPV-16 (Cripe et al., 1987).

The activity of positive element CE2 appears to be negatively regulated by an adjacent element, NR2. A GC box, present within the limits of NR2 defined by the deletion analysis, was initially identified as a potential binding site for the trans-activator Sp1 (Patel et al., 1987). However, Kageyama & Pastan (1989) have identified a transcriptional repressor, GCF, in human cells which binds to GC-rich sequences, the consensus binding sequence being GCGGGGC. In addition, Hata et al. (1989) reported a sequence, GG(C/G)CCGT(C/G), reiterated four times upstream of the human calcium-dependent protease large subunit gene, which correlates with a negative regulatory activity. The GC box in BPV-4 NR2 in the reverse orientation shows similarity (six of seven and six of eight bases, respectively) to both of these consensus sequences (Table 1). Thus we infer that the BPV-4 GC box within NR2 may well be the binding site for a repressor protein which down-regulates the adjacent CE2, although its presence appears to have little effect on CE1.

From the footprint analysis, CE1 and CE2 appear to share a common protein binding site, identified by footprints FP9 and FP6. As shown in Table 1, these footprints occupy over a 12 bp sequence, underlined in Fig. 4, differing by only two bases between CE1 and CE2. This sequence also bears some similarity to the OCT-1 binding site in its 5' half (Table 1). OCT-1 has been implicated in the transcriptional activation of many cellular and viral genes (reviewed in Goding & O'Hare, 1989) and this nuclear factor or a related one may be involved also in control of BPV-4 transcription.

Two footprints were observed in the region of the BPV-4 LCR identified as CE3. Either or both of these sites might be responsible for the observed enhancer activity but in cells expressing viral trans-activator E2, competition would be expected to occur between E2 and the host FP2c factor. Immediately downstream of CE3 is NR3. In HPV-6g, a motif, ACTGT, has been identified as being important for silencer activity because its replacement in HPV-6e by the sequence TAGTGTTA leads to loss of the negative effect (Wu & Mounts, 1988). BPV-4 footprint FP2a, which maps to the NR3 region, lies over the sequence AAGTGTTG, which has a six bp core homology to the HPV-6e sequence. The significance of this is unclear because this sequence in HPV-6e is associated with the loss of a negative effect and in BPV-4 with the presence of a silencer.

Three footprints (FP3, FP4 and FP5; Fig. 4) were observed between CE2 and CE3, over sequences displaying various degrees of identity with the NF1 consensus sequence (Table 1), and in each case the strength of the footprint correlated with the relatedness to NF1 (Fig. 4 and 5; Table 1). NF1-like sites are a feature of the LCRs of other papillomaviruses (Gloss et al., 1989; Sowden et al., 1989; Sibbet & Campo, 1990), although the actual protein binding to these sites has not yet been identified.

BPV-4 is unusual among the papillomaviruses in that it does not possess paired E2 consensus sites at the 3' end of the LCR (Cole & Danos, 1987). However, adjacent to the E2 site between nucleotides 267 and 278 there is a degenerate version of the E2 consensus sequence (nucleotides 252 to 263; ATCNGGCT). This degenerate...
Fig. 5. Footprinting analysis of the BPV-4 LCR. DNase I footprinting was carried out as described in Methods. (g) Probes used in each of the panels (a) to (f); * denotes the position of the 32P label. Lanes 1, marker lanes in which the probe has been subjected to G-track sequencing; lanes 2, 0 µg CT3 nuclear extract, 1 µl of 5 ng/µl DNase I; lanes 3 and 4, 20 µg CT3 nuclear extract, 1 and 5 µl of 5 ng/µl DNase I; lanes 5 to 7, 50 µg CT3 nuclear extract, 1 and 5 µl of 5 ng/µl DNase I, and 1 µl of 100 ng/µl DNase I; lanes 8 to 10, 125 µg CT3 nuclear extract, 1 and 5 µl of 5 ng/µl DNase I, and 1 µl of 100 ng/µl DNase I, respectively. The numbers to the right of each panel indicate footprints.

E2 site (dE2) is not a cloning artefact, as shown (M. E. Jackson & M. S. Campo, unpublished results) by direct sequencing of polymerase chain reaction products from two independent, naturally occurring bovine oesophageal papillomas and from a bovine papilloma experimentally induced in nude mice (Gaukroger et al., 1989). FP1a maps to dE2 and, because it has been demonstrated that E2 trans-activator binding at some sites interferes with the binding of cellular transcription factors (Thierry & Yaniv, 1987; Stenlund & Botchan, 1990), it may be that during the evolution of the virus, mutation of the E2 site between nucleotides 252 and 263 permitted more efficient action of the cellular factor(s) binding at this site.

The results discussed above demonstrate the existence of a major constitutive enhancer element, CE1, and two additional positive elements, CE2 and CE3, within the BPV-4 LCR. Each of these elements was found to be associated with a negative regulator. Similar results were obtained using bovine palate fibroblasts (data not shown), except that the differentials between the activities of the different clones were smaller. Additional E2-dependent positive and negative regulation of transcription would also be expected to occur, allowing a highly complex and finely tuned control of viral gene expression. A breakdown in this control circuitry, either in the viral or cellular constituents, could allow increased transcription of the viral transforming genes and thus progression to carcinoma, as proposed by zur Hausen (1987).

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References


Transcriptional regulation of BPV4 LCR


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