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Linker Scanning Analysis of the BPV-1 Upstream Regulatory Region

Heedai Kim, Jaerang Rho and Joonho Choe*

Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Taejon 305-701, Korea

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Abstract: The upstream regulatory region (URR) of bovine papillomavirus type 1 (BPV) contains promoters and a conditional transcriptional enhancer that is *trans*-activated by the viral E2 protein. After deleting the 5′ and 3′ ends of BPV URR, *Bam*HI linkers were inserted into several positions of BPV URR without causing an addition or a deletion of URR sequences. Most linker scanning mutations did not show any effects on the transcription of P7940 and P89 promoters in BPV URR. However, several mutants showed reduced transcriptional activities. Based on our results we found that the AP-2 and Spl binding sites were important for basal level transcription of BPV URR in the absence of the E2 protein and that the CTF/NF-1 site is dispensable for E2 transactivation of BPV URR transcription.

Key words: bovine papillomavirus, gene expression, P2(P89) promoter.

Bovine papillomavirus type 1 (BPV-1) is associated with cutaneous fibropapillomas in cattle (Oslen, 1987) and has served as the prototype for studying the molecular biology of papillomaviruses. The BPV-1 genome consists of a circular, double-stranded DNA molecule and is 7946 base pairs (bp) in size (Chen *et al.*, 1982). Only one of the viral DNA strands is utilized as a template for transcription (Amtmann and Sauer, 1982). The early (E) region of the coding strand contains viral genes involved in cell transformation, plasmid replication, and viral mRNA transcription (Baker and Howley, 1987). The late (L) region contains two large open reading frames (ORFs) coding for the structural proteins of the virus capsid (Amtmann and Sauer, 1982). The BPV-1 genome also contains a noncoding region, referred to as an upstream regulatory region (URR), or long control region (LCR), which is located between the stop codon of L1 and the first codon of E6 (nt 7091-89). It is believed that the URR plays critical roles in the control of viral DNA replication and gene expression because of the presence of many *cis*-acting genetic elements, including several promoters, as well as constitutive and conditional enhancers (Broker and Botchan, 1986).

The regulation of the BPV-1 transcription is strongly dependent on the interactions between the two conditional enhancers, E2-responsive elements 1 and 2 (E2RE1 and E2RE2) and the E2 transactivators (E2TAs), which are encoded by the full length E2 ORF (Sowden *et al.*, 1989; Spalholz *et al.*, 1987). E2TA is a DNA-binding protein with specificity for a short palindromic sequence, ACCN$_6$GGT (Androphy *et al.*, 1987). Li *et al.* (1989) found that 17 E2 binding sites (E2BSs) are present in the BPV-1 genome and that the DNA context of nucleotides within which the recognition bases fall influences binding affinities significantly. E2RE1 is located between nt 7611 and 7806, and harbors two repeated E2BSs at each end (Spalholz *et al.*, 1987). The cooperative binding of a dimeric E2TA to the E2 BSs is required for the E2-dependent activation of the promoters P7940 and P89.

To date, a variety of studies have been carried out to identify transcriptional *cis*-acting elements in the BPV-1 URR. However, knowledge from the studies using deletion mutagenesis may be ambiguous, since not all the nucleotides within the boundaries are necessarily critical for control of the transcription. Therefore, we adopted linker scanning mutagenesis to change individual nucleotides at a target without generating gross deletion or other arrangements (McKnight and Kingsbury, 1982). In order to refine our understanding of *cis*-elements in the BPV-1 URR, double-stranded *Bam*HI linkers (5′-CGGATCCG-3′) were introduced into the region between E2RE1 and E2BS11 without changing the length of the BPV-1 URR. The linker-scanned mutants of URR fragments were inserted into a chloramphenicol acetyltransferase (CAT) expression

*To whom correspondence should be addressed.
Tel: 82-42-869-4020, Fax: 82-42-869-2610

vector and analyzed for their transcriptional enhancing activities in the presence or absence of E2TA.

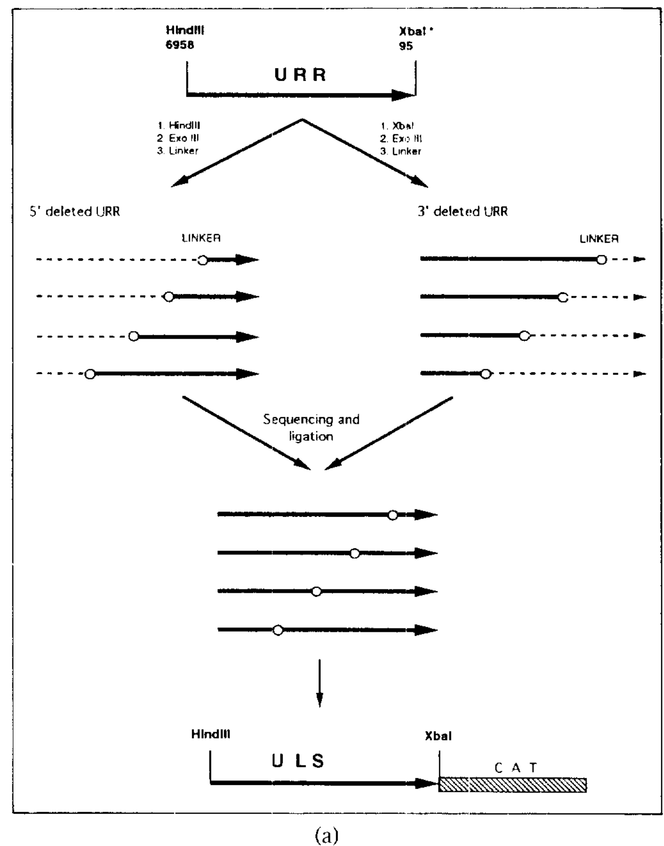
Materials and Methods

Cell culture

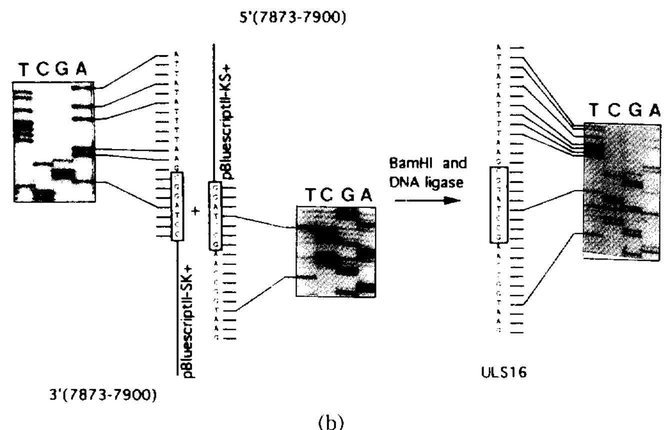
HeLa HH cells, kindly provided by Dr. J. K. Lee at Korea Research Institute of Chemical Technology, were maintained in Dubecco's modified Eagle's medium supplemented with 10% fetal bovine serum and penicillin-streptomycin.

Linker scanning mutagenesis

Plasmid pMB200 contains a 2.5 kb of *Bgl*II (nt 6945)/*Bgl*II (nt 1515) fragment of the BPV-1 genome at the *Bam*HI site. pMB200 was linearized by *Hin*dIII for constructing 5′-deleted URRs or by *XbaI for 3′*-deleted URRs as shown in Fig. 1a. ExonucleaseIII (ExoIII) was used for deleting the 5′ or 3′ end of URR. We followed the instruction of the Erase-a-Base system (Promega) on conditions for digestion of ExoIII. ExoIII-deleted URR fragments were ligated with *Bam*HI linkers (5′-CGGATCCG-3′). Then the nested 5′- and 3′-deleted URRs were subcloned into the *Bam*HI/*Xba*I-digested pBluecriptII KS+ (Stratagene, San Diego, CA.) and the *Bam*HI/*Hin*dIII-digested pBluescriptII SK+, respectively. The endpoints of the deleted mutations of the BPV-1 URRs were confirmed by the dideoxy DNA sequencing method (Sanger and Coulson, 1975). By ligating "perfect-matched" 5′- and 3′-deleted URRs at the *Bam*HI linker site, linker scanning mutants were constructed. These linker scanning mutants are called URR linker scanning (ULS) mutants hereafter. Matching, in this context, refers to two opposing deletion mutations whose deletion termini are separated by eight nucleotides, that is, the same length as a *Bam*HI linker. Therefore, the total number of nucleotides of URR and ULS were exactly the same. ULS mutants in pBluescriptII SK+ or KS+, were digested with *Hin*dIII or *Xba*I and inserted into pCAT-Basic vectors (Promega) for CAT expression. A schematic presentation for constructing linker scanning mutagenesis is shown in Fig. 1a.



(a)



(b)

Fig. 1. a) The overall scheme for condtructing linker scanning mutagenesis. b) Sequencing data for constructing ULS16 mutant. Two end points at 5′ and 3′ have same sequence of *Bam*HI linker (CGGATCCG). Two clones were digested with *Bam*HI and ligated to construct th ULS16 mutant.

CAT assay

HeLa HH cells were transfected by the calcium phosphate method (Graham and van der Eb, 1973) with modifications. Each 60-mm plate of 4×10^5 cells received 15 μg with the total amount of DNA adjusted by the addition of carrier DNA. Two μg of ULS (or URR) CAT expression plasmid was transfected to provide the CAT activity and 2 μg of NDE2, a full length E2 expression plasmid, was co-transfected for the E2-dependent transactivation. All plasmid DNAs used in this transfection were prepared by the Qiagen Plasmid Midi Kit (Qiagen Inc.) The DNA-calcium precipitate was applied to the cells for 12 h followed by 15% glycerol shock for 2 min. Cells were harvested 36 h after transfection. CAT assay was carried out as previously described (Gorman *et al.*, 1982). The amount of all extracts used for the assays was 100 μg of protein, and the assays were run for 2 h at 37℃. Radioactivities on TLC plates

were measured by Phosphor Imager (Molecular Dynamics, Sunnyvale, USA) using Image Qunat software for quantification. The results of the CAT assay were represented as the values relative to standardized values (100%) of the URR-CAT plasmid in the absence of NDE2. Each experiment was repeated at least three times.

Results and Discussion

Linker scanning mutagenesis on the BPV-1 URR

In order to understand the functions of the BPV URR *cis*-element, *Bam*HI linkers were inserted into 16 positions of BPV URR. The linker-scanned mutants of URR fragments were inserted into CAT expression vectors to construct ULS-CAT mutants. The *Hin*dIII/*Xba*I fragment of BPV URR was deleted by Exonuclease III from the 5′ or 3′ ends. After sequencing the 5′ and 3′ ends of each mutant and ligating perfectly matched 5′ and 3′ mutants, 16 ULS mutants were prepared. An example of constructing ULS16 mutants is shown in Fig. 1b. *Bam*H1 Linkers were introduced between nt 7650 and 7900 in the BPV URR. This region contains E2RE1 and 5 E2BS. E2RE1 is an important *cis*-acting element for the E2-responsive activation of the transcription from the promoters P7940 and P89 (Spalholz *et al.*, 1987). This URR region also contains the AP-2, Sp1 and CTF/NF-1 binding sites and is known as a basal element specific to the P7940 and P89 promoters (Szymanski and Stenlund, 1991). Positions of ULS mutants are indicated in Fig. 2. ULS mutants have substitute nucleotides at several important binding sites for general transcription factors. We carried out CAT assay for ULS mutants and analyzed their transcription activities.

Analysis of ULS mutants for transcriptional activity

Transcriptional activities of each of the ULS mutants in the presence of E2TA were measured by CAT assay as shown in Fig. 3. The transcriptional activities of each of the mutants in the absence of E2TA were also measured by CAT assay. CAT assays were repeated several times and quantitative analysis was performed (Fig. 4). Since the *Bam*HI linkers were inserted into random positions by ExoIII deletion, minimal changes of the BPV-1 URR context through linker scanning mutagenesis did not strongly inhibit the enhancing functions of the BPV-1 URR. The URR-CAT plasmid possesses a considerable constitutive transcriptional activity in HeLa cells. It can be further stimulated by BPV-1 E2 transactivators. We will discuss the transcriptional activities of each ULS mutant.

ULS1: The ULS1 mutant (nt 7654-7661) disrupted the AP-2 site and the first inverted repeat in E2RE1.

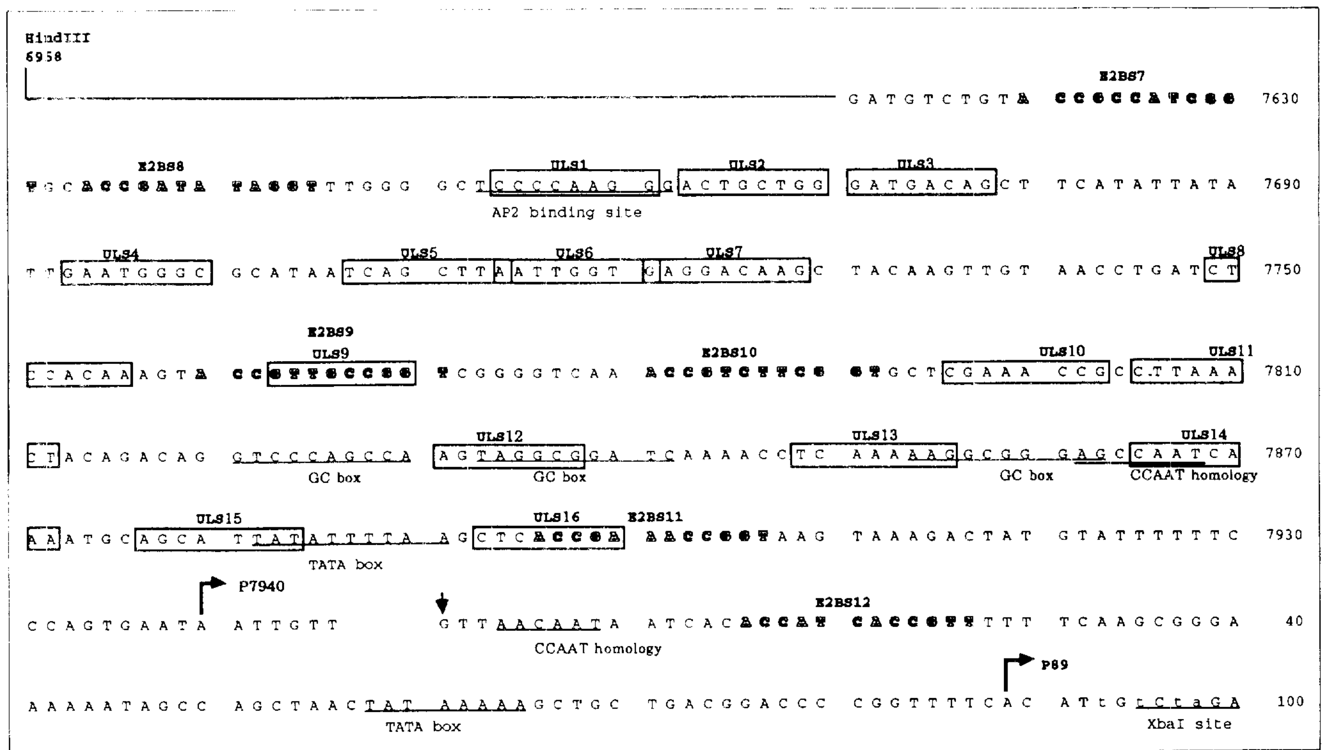


Fig. 2. Nucleotide sequence of URR region. Nucleotide residues replaced by *Bam*HI linker (CGGATCCG) are surrounded by boxes. E2 binding sites (E2BS) are written as bold cases. The binding sites for transcription factors are underlined. The broken arrows indicate the start site of transcription.

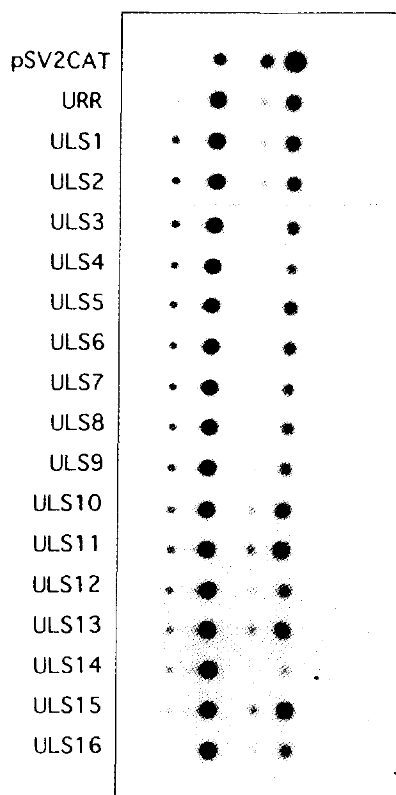


Fig. 3. Transcriptional activities of ULS mutants. CAT assay was performed in the presence of E2TA.

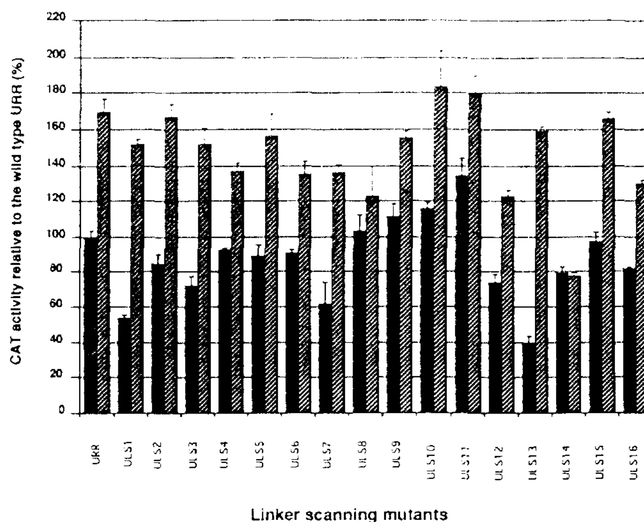


Fig. 4. Relative transcriptional activities of ULS mutants with or without E2TA. Each value represents the relative one to the arbitrarily standardized value (100%) of wild type URR-CAT plasmid without E2TA. ■ : No NDE2. ▥ : with 2 μg of DNE2.

In the absence of E2TA, the ULS1 mutant showed very low transcriptional activity (52% CAT activity compared to wild type BPV URR), whereas it has normal transcriptional activity in the presence of E2TA. The DNA loops formed by the association of DNA-bound E2 dimers can mediate full activation for themselves and may provide no space for the binding of AP-2 and/or a transcription factor bound to the first inverted repeat (Knight *et al.*, 1991). Therefore, in the presence of E2TA, the AP-2 factor may not be necessary for activation of transcription in BPV URR.

By making point mutations at nt 7649 and 7650, Spalholz *et al.* (1988) found that this region had no dramatic effect on enhancer activity in African green monkey (CV-1) cells. This mutation does not change the AP-2 site. However, the ULS1 mutant completely changed the AP-2 site. Our result clearly indicates that the AP-2 site is very important for basal level transcription of BPV URR without E2TA. AP-2 and/or a transcription factor bound to the first inverted sequence may have some positive effects on the noninduced transcriptional processes occurring at the P89 promoter elements. It is known that a mutation of AP-2 motif in SV40 enhancer results in a severe loss of transcriptional activity indicating its absolute requirement for transcription (Zenke *et al.*, 1986).

ULS2 to ULS8 : These ULS mutants, where *Bam*HI linkers were laid at the region internal to the paired E2 binding sites, showed negligible effects on transcriptional activity in the presence or absence of E2TA. This result was consistent with previous results (Spalholz *et al.*, 1987 and 1988) and indicated that the internal region between the paired E2BSs was not required for constitutive and induced transcriptional activities. ULS7 showed a reduced transcriptional activity in the absence of E2TA. At this point, it is difficult to explain why ULS7 had lower promoter activity compared to wild type.

ULS9 : This mutant disrupted the E2BS9 which is one of two repeated E2BSs at the 3′ end of E2RE1. The effect on transcriptional activity of ULS9 was negligible, because E2BS9 is less important in enhancement of E2RE1 to E2BS10 (Spalholz *et al.*, 1988) and only one nucleotide of the E2 binding motif was changed (ACCN$_6$G̲GT to ACCN$_6$C̲GT). It may be possible that the internal 4 nucleotides, which were changed transversionally (TTGA to GGAT), influenced E2 bind-affinity. But these 4 bases are known not to enhance or decrease E2 binding affinity in missing contact probing analysis (Li *et al.*, 1987).

ULS10 and ULS11 : These ULS mutants had a slight positive effect on transcription from P7940 and P89 promoters regardless of E2TA. From these results, it may be suggested that this region (nt 7796 to 7812) acts as a negative element or silencer for transcription of P7940 and P89 promoters. The negative regulatory element has been identified as 23 nucleotides (nt 7849-14) of HPV18 P105 promoter (Bauknecht *et al.*,

1992). This element has previously been shown to be the target of the transcriptional repressor YY1 (Shi *et al.*, 1991). However, there is no homology between BPV (nt 7796-7812) and the HPV18 (nt 7849-14) sequence of this region. The supposed negative element of BPV URR may be different from the negative element of HPV18 in its mechanism of repressing transcription of P7940 and P89 promoters.

ULS12 to ULS14: There are three Spl binding sites (GC-box) and a CCAAT element from nt 7821 to 7870 as shown in Fig. 2. Considering the importance of Spl and CTF/NF1 on promoter activity, we could easily assume that the mutations in this region might have strong effects on the transcription initiated from the P7940 and P89 promoters. Direct interaction between Spl and the BPV-1 E2 protein mediates synergistic activation of transcription (Li *et al.*, 1991). Spl functions in concert with CTF/NF1 to facilitate expression; that is, both of these factors appear to be required for mRNA synthesis, yet neither alone is sufficient for proper expression (McKinght and Tjian, 1986). The ULS12 mutant (nt 7831 to 7838) destroyed the second GC box (nt 7833 to 7842) and the transcription activity of this mutant was not seriously affected in the presence or absence of E2TA. However, the transcription activity of the ULS13 mutant (nt 7849 to 7856), which modified the third GC box (nt 7854 to 7863), was reduced to 40% of wild type in the absence of E2TA. In the presence of E2TA, the transcriptional activity of the ULS13 mutant was not affected. It is not surprising that a mutation at a GC box lowered the transcription efficiency in the absence of E2 transactivator because Spl is a general transcription factor for transcription. However, it is difficult to explain why a mutation in one GC box does not affect transcriptional activity but a mutation in the other GC box does. The GC box at nt 7854 to 7863 may be more important for transcriptional activation than the other GC box (nt 7833 to 7842) because it is located near the CTF/NF-1 binding site at nt 7861 to 7868. Another interesting result was observed in the ULS14 mutant (nt 7865 to 7872). This mutant destroyed a CTF/NF-1 binding site (nt 7861 to 7868). The transcriptional activity of this mutant was severely reduced in the presence of E2TA. In fact, this is the only mutant which has reduced transcriptional activity in the presence of E2TA in our studies. From the above result, it may be suggested that CTF/NF-1 may be required for E2 transactivation of P7940 and P89 promoters. Disruption of the CTF/NF1 binding site at nt 7864 to 7867 may result in no E2 transactivation through Spl.

ULS15: The ULS15 mutant (nt 7877 to 7884) destroyed a TATA box (nt 7882 to 7891), which has been described by Baker and Howley (1987). The transcriptional expression of this mutant was not changed compared to the wild type. It may suggest that the TATA sequence at nt 7882 is not functional and that some other TATA homologous sequence (nt 7906 to 7914) or a TATA sequence (nt 58 to 65) may replace its function.

Based on the results described in this paper, we confirmed that the AP-2 and Spl binding sites are important for a basal level of BPV URR transcription in the absence of E2TA. A negative regulatory element may exist in the nt 7796-7812 region. Our data also suggest that CTF/NF-1 is indispensable for E2TA activation of BPV URR transcription. In order to confirm whether CTF/NF-1 is indeed important for E2TA activation of URR transcription, more experiments should be carried out.

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