High and Low Levels of Cottontail Rabbit Papillomavirus E2 Protein Generate Opposite Effects on Gene Expression*

The papillomavirus E2 protein plays an important role in viral transcriptional regulation and replication. We chose to study the cottontail rabbit papillomavirus (CRPV) E2 protein as a transcriptional regulator because of the availability of an animal model for papilloma formation, which may be relevant for human papillomavirus (HPV) infection and replication. We studied the effect of expression levels of E2 on the long control region, which contains transcriptional promoter and enhancer elements, and synthetic E2-dependent artificial promoters in which the E2 was the dominant factor in the transcriptional activation. These experiments indicated that high levels of E2 were inhibitory and low levels were stimulatory for transactivation. In addition, we showed that the complex formed between CRPV E2 and the cognate binding site was less stable than the complex formed between HPV E2 and the same cognate binding site. Furthermore, we showed that CRPV E2 binding to its transcriptional regulatory sequence was stabilized by other proteins such as E1, which produced increases in transcriptional activation of E2-dependent genes. The data may be used to define conditions in which the rabbit model can be used for the screening of drugs which are inhibitory to the HPV and CRPV replication and gene expression.

The human papillomavirus (HPV) is the etiologic agent responsible for several benign epithelial proliferative lesions, including plantar warts, genital warts, and respiratory laryngeal papillomatosis (1–4). Chronic HPV infection is one of the main risk factors for the development of cancer (5). To characterize the pathogenetic mechanisms involved in the development of virus-associated cancers as well as premalignant lesions, and to develop effective strategies for their prevention and treatment, it is imperative to have a cell-free model or an animal model system to test new approaches to the treatment of HPV infections. Although papillomavirus infections are species-specific, common mechanisms of regulation of transcription and replication may exist among different species (6–15). The fact that many similarities have been identified between cottontail rabbit papillomavirus (CRPV) and HPV infections suggests that the CRPV infection may be relevant to the understanding of human disease associated with human papillomaviruses infection (16–18).

Recent studies have compared in depth the mechanisms governing the bovine papillomavirus 1 (BPV-1) and HPV infections. Three BPV-1 E2 proteins have been reported. The largest protein, E2-TA, is a transcriptional activator, and two small truncated proteins, E2-TR and E8/E2, have been reported to be transcriptional repressors (14, 19–21). In contrast, there is as yet no definitive biochemical evidence that the HPV encodes truncated E2 repressor proteins. In both species, the E2 protein regulates transcription of the viral early genes and plays an auxiliary role in replication by recruiting the E1 protein onto the origin of replication (ori) prior to the binding of the host cell DNA polymerase (22–27). E1 protein inhibits or modulates transcription of other viral genes in the BPV-1 system (28, 29). Recent studies have shown that E1 and E2 may interact not only in promoting viral replication but also in modulating the expression of other viral genes (30, 31). The E2 protein and its binding sites on the viral DNA mediate attachment of the episomal HPV to the host cell chromosomes (32, 33). The tethering may protect the HPV episomal DNA from nucleolytic digestion. Thus, both the E2 and E1 proteins are important for several steps of the viral life cycle.

Although the CRPV E2 protein has been reported as a transcriptional activator previously (34), the mechanism of transcription of the CRPV E2 protein has not been well studied in detail. We chose to study the CRPV E2 protein as a model for papillomavirus transcriptional regulatory proteins because the results in the CRPV animal model may be relevant for the understanding of HPV infection. The results of our studies suggested that high levels of CRPV E2 protein were inhibitory, whereas low levels of CRPV E2 protein were stimulatory for transactivation of reporter genes controlled by E2-dependent promoters. Furthermore, E1 may promote the CRPV E2-dependent gene expression by stabilizing E2 on its cognate recognition sites in CRPV infections. These data will be relevant to defining conditions under which the rabbit model can be used to isolate drugs which suppress HPV infection.
TRANSCRIPTIONAL REGULATION BY CRPV E2 PROTEIN

EXPERIMENTAL PROCEDURES

Cells and Transfections—Sf1Ep cells derived from rabbit epidermis and C33a cells derived from human cervical cancer were obtained from ATCC. The HPV-16 114/K clone producing CRPV E2 was introduced into the pGL3 basic vector (Promega) using the following primers: 5'-gaagatctgtccacttcgcatattaaggtga-3' and 5'-cccaag-gccgctcgagatgccgcccgtgatttgc-3'. The pCRPV-LCRwt was constructed by PCR amplification of the CRPV genome for subcloning, and PCR products were confirmed by sequencing (Yale Keck Lab). Briefly, to prepare the labeled RNA probes, the CRPV genome was grown in minimum essential medium and Dulbecco's modified Eagle's medium (Life Technologies, Inc.) containing 10% heat inactivated calf serum, respectively.

Virus Construction—To generate the pCMV-CRPV2E, XhoI and BamHI sites were introduced upstream and downstream of the CRPV E2 gene by PCR for subcloning, and PCR products were digested with enzymes prior to their introduction into the pCMV vector driven by a CMV promoter (35). The primers for PCR were 5'-gtcatgatagctgct-3' and 5'-cgggatccgcatacgttataaggtga-3'. The CRPV-pLAII extract was then centrifuged for 10 min at 4 °C, and the supernatant was frozen in aliquots. The protein concentration was determined by a BCA protein assay kit (Pierce).

Preparation of Nuclear Extracts—Sf1Ep cells in 100-mm dishes were transfected with varying ratios of the E2 and the backbone vector DNA. In each transfection mixture, the amount of the DNA vector was constant. We modified the preparation of nuclear extracts as described previously (37). Briefly, at 48-h posttransfection, cells were scrapped off the surface of the culture dish with a reporter lysis buffer (Promega) containing 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 1 μg/ml pepstatin. Cells were incubated on ice for 30 min, then 25 μl of 10% Nonidet P-40 was added. After centrifugation, the nuclear extract was resuspended in 50 μl of buffer containing 50 mM HEPES (pH 7.6), 420 mM KCl, 0.1 mM EDTA, 5 mM MgCl2, and 20 μg/ml glycerol and incubated on ice for 20 min. The nuclear extract was then centrifuged for 10 min at 4 °C, and the supernatant was frozen in aliquots. The protein concentration was determined by a BCA protein assay kit (Pierce).

Expression and Purification of His Tag Protein—The pSET-CRPV E2DBD and the pSET-HPV16DBD were transformed into Escherichia coli BL21(DE3)(plysS) (Novagen). The isopropyl-β-thiogalactosidase (IPTG) was added to a final concentration of 1 mM at 28 °C for 3 h. Bacterial pellets were collected and washed and PBS and stored at −80 °C. Pellets were resuspended in sonication buffer containing 20 mM Tris-HCl (pH 8.0). After shearing with an 18-gauge needle, bacteria were sonicated and subjected to ultracentrifugation at 90,000 × g at 4 °C for 1 h. The supernatant was recovered and added to the Talon resin (CLONTECH). After incubation at 4 °C for 3 h, the resin was washed three times with sonication buffer prior to elution of the His tag proteins with sonication buffer containing 10 mM imidazole. The eluted His tag protein was dialyzed against dialyzed buffer containing 20 mM Tris-HCl (pH 8.0), 500 mM NaCl, and 10% glycerol.

Electromobility Shift Assay—A double-stranded oligonucleotide DNA was end-labeled by Klenow fill-in with [32P]dCTP and used as a probe. The reaction mixture was incubated at 37 °C for 2 h in a final volume containing 10 mM HEPES (pH 7.6), 50 mM KCl, 1 mM EDTA, 5 mM MgCl2, 10% glycerol, 1 mM dithiothreitol, 0.7 mM phenylmethylsulfonyl fluoride, 4 μg/ml leupeptin, and 160 ng of dAdT. The reaction mixture containing 10 μg of protein of total cell extracts was preincubated in the presence or absence of the cold competitor DNA on ice for 10 min. After addition of the radiolabeled DNA probe, samples were further incubated at 22 °C and 24 °C for 20 min. The samples were then loaded on a 5% polyacrylamide gel and run for 2 h at room temperature.

For affinity studies, purified E2 proteins were added to the reaction mixture containing 0.15 mg/ml bovine serum albumin with various amounts of cold competitor oligo DNAs. The dissociation constants (Kd) were estimated by mixing [32P]-labeled oligonucleotide probe (5 fmol) with increasing concentration of unlabeled oligonucleotide in binding mixtures prior to the addition of a 10-fold molar excess of E2 DBD protein as reported before (21, 38, 39). The Kd values were equal to the unlabeled competitor concentrations that depleted the bound complex formation by 50%. The off-rate ratio was determined by adding cold competitor to each reaction mixture prior to running the gel after binding purified proteins to the [32P]-radiolabeled oligonucleotide probe. Dissociation half-time (τoff) represents the time required to exchange 50% of labeled, bound oligonucleotide DNA. The gels were dried and scanned using a PhosphorImager TM (Molecular Dynamics).

Transient Replication Assays—The pCRPV-LCRLucWT or the pCRPV-LCRLucMut was cotransfected with both/either the pRSV-CRPV and/or the pCMV-CRPV1E vector in Sf1Ep cells. Eighty-eight hours after transfection, the cells were harvested and subjected to the transfection lysis buffer (Promega) followed by DynaBeads isolation (24). The pCRPV-LCRLucWT vector was linearized with XhoI digestion, and the probe was generated by a Prime II-IT (Stratagene). Hybridizations were carried out at 65 °C in 6 × SSC, 5 × Denhardt's solution, 0.5% SDS, and 200 μg/ml salmon sperm DNA for 8 h. The filters were washed in 2 × SSC and 0.1% SDS at 65 °C for 30 min, and then the filters were exposed to the Kodak X-Omat AR film at −80 °C for 3 days.
are also shown.

the E2 and E1 expression vectors in which the CMV and RSV promoters

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the original two E2 binding sites of 2E2luc vector. The pCRPV-LCRluc-

contains four E2 binding sites, which were separated by 55 bp (//) from

4//2E2luc contains four E2 binding sites as indicated. The 2//2E2luc

CRPV-LCR (pCRPV-LCRlucWT) was cloned into the pGL3 basic vector.

arrows

binding sites are indicated.

BPV-1 (43), HPV16 (38, 39), and putative CRPV E2 binding sites: the

ments (data not shown). Thus we conclude that the particular

PCR nor E6/E7/E2 transcript by RT-PCR after six passages of

workers had reported that the CRPV genome was maintained

a RSV Promoter on the CRPV LCR—

Since the level of E2 expression is known to be critical for

function. The nuclear sequence shows the 2E2 and Sp1 cassette

us to assemble the E2-dependent promoters. The two E2 binding

sites are shown in bold letters, and the Sp1 binding sites are

undertlined (21). The two E2 and two Sp1 cassette (2E2luc) or the

CRPV-LCR (pCRPV-LCRlucWT) was cloned into the pGL3 basic vector.

The 4E2luc contains four E2 binding sites as indicated. The 2/2E2luc contains

four E2 binding sites, which were separated by 55 bp (//) from the

original two E2 binding sites of 2E2luc vector. 4//2E2luc contains

additional four E2 binding sites, which were separated by 55 bp (//) from

the original two E2 binding sites of 2E2luc vector. The pCRPV-LCRluc-

Mut contained the mutation in the CRPV LCR ori to abolish the func-

tional nature of the origin of replication (28, 31). The designations for

the E2 and E1 expression vectors in which the CMV and RSV promoters

are also shown. B, comparison of E2 binding sites present in the LCR of

BPV-1 (43), HPV16 (38, 39), and putative CRPV E2 binding sites: the

E2 (arrows) and E1 (circles) binding sites are indicated.

RESULTS

Opposite Effects of the CRPV E2 Protein Driven by a CMV or

a RSV Promoter on the CRPV LCR—Despite the fact that other

workers had reported that the CRPV genome was maintained as an episome in Sf1Ep cells (40), we found no CRPV genome by PCR nor E6/E7/E2 transcript by RT-PCR after six passages of Sf1Ep cells, which were used in our laboratory for our experiments (data not shown). Thus we conclude that the particular clone of the Sf1Ep cells, which is being used in our laboratory, is appropriate for the transactivation studies outlined below.

Since the level of E2 expression is known to be critical for transactivation (41, 42) and has also been reported to be important for BPV-1 replication (30), we compared the effects in the two systems of varying the level of the E2 expression in cells in which the level of the E2 dependent reporter gene expression was being studied. To accomplish this (see Fig. 1), we used two different promoters to sustain the level of the expression of E2 protein: the RSV promoter, which is known to produce relatively low levels of expression of a heterologous transgene, and the CMV promoter, which is known to produce a high level of the transgene expression (42). We measured the levels of luciferase activity expressed by a luciferase transcriptional regulatory vector, in which the expression of the luciferase gene was driven by the CRPV LCR, after the cotransfection with the CRPV E2 expression vector. The activity as a promoter of CRPV LCR depended on the level of the E2 protein. The CRPV E2 protein was expressed from the expression vector driven by the CMV or RSV promoter. Thus, we measured the influence of CRPV E2 protein on its LCR activity. As shown in panel 1 of Fig. 2, no increase of luciferase activity above that seen in control experiments in the absence of any E2 expression vector was observed with E2 input vector DNA amounts less than 0.1 ng (data not shown). Interestingly, at levels of the input E2 expression vector DNA greater than 1 ng, there was a decrease in the level of expression of luciferase, which reached a minimum of the luciferase activity at 1200 ng of DNA. We also obtained the same results using C33a cells and 293 cells (data not shown).

In contrast, when we cotransfected the pRSV-CRPVE2 vector with the pCRPV-LCRlucWT reporter vector into Sf1Ep cells, we found a pattern of initial dose-dependent activation of the luciferase reporter gene expression (2.5–3-fold) as the amount of the E2 vector DNA increased from 10 to 100 ng and then a decrement in luciferase expression as the amount of the E2 vector DNA increased from 200 to 1200 ng. These results showed the opposite effects of CRPV E2 protein driven by the CMV or the RSV promoter on the CRPV LCR.

Use of Simplified Promoters for Luciferase Assay to Study the Function of E2—As shown in Fig. 1B, there are differences in the organization of the number of E2 binding sites in the LCR of papillomaviruses among different species. The HPV16 has only four E2 binding sites, whereas the BPV-1 has 12 E2 binding sites in the LCR (43). Since many host and viral proteins interact to regulate transcriptional activation in the LCR, we created simplified promoters for the expression of luciferase gene which were E2 dependent. To investigate how the number and specific distribution of the E2 binding sites contribute to transcriptional activation, we constructed reporter gene promoters with different numbers of E2 binding sites and different spacing among E2 binding sites (Fig. 1). The BPV-1 E2 is unable to activate the expression of reporter genes driven by E2 dependent promoters that contain only E2 binding sites and a TATA box. This can be overcome by the addition of Sp1 binding sites to the E2 binding sites and the TATA box transcriptional element (44). The Sp1 element is known to be associated with the E2 protein in the native LCR element. Thus, our vectors included Sp1 binding sites as well as E2 binding sites next to
assess reproducibility. Each luciferase vector (and a series of artificial luciferase vectors, are indicated as shown by pRSV-CRPVE2 expression vector (see Fig. 3, (4//2 E2luc, 2//2 E2luc, and 4 E2luc) at 200 ng or greater of the 14–16-fold activation of all of the luciferase reporter constructs DNA increased from 200 to 1200 ng. In contrast, there was a reduction in luciferase expression as the amount of the E2 vector DNA was decreased with the artificial reporter vectors, the 4//2 E2 luciferase, and the 4E2 luciferase vectors into Sf1Ep cells transfected with the E2 expression vector driven by the CMV or the RSV promoters (see panel 2). The intensity of the signal generated by the protected E2 mRNA extracted from Sf1Ep cells transfected with the E2 expression vector driven by the RSV vector was much lower than found in extracts of Sf1Ep cells transfected with equal amounts of the E2 driven by the CMV expression vector (lanes 6–10 of panel 2). The amount of CRPV E2 mRNA reached a plateau in the presence of 800–1200 ng of RSV E2 expression vectors (lanes 4 and 5 in panel 3), which was equal to the amount of mRNA level in the presence of 200 ng of the CMV E2 expression vector (lanes 3, 4, and 7 in panel 3). It should be noted that the CRPV E2 transactivated the luciferase gene driven by the artificial promoters in Fig. 3 along with 800–1200 ng of the pRSV-CRPV E2 vector or 200 ng of the pCMV-CRPV E2 vector.

Taken together, these results suggest that lower levels of the E2 expression were generated in Sf1Ep cells transfected with the pRSV-CRPV E2 vector than was the case with the pCMV-CRPV E2 expression vector. These results further support the contention that high doses of CRPV E2 can be inhibitory to expression of reporter genes governed by E2-dependent promoters (the artificial promoters presented in Fig. 3 or the native rabbit LCR in Fig. 2), whereas low doses of CRPV E2 resulted in transactivation.

Comparison between the CRPV E2 and the HPV16 E2 Proteins for Transactivation—We also conducted experiments on the luciferase gene expression driven by either the tk core promoter or the SV40 promoter along with pCMV-HPV16E2 vector. Luciferase activity showed a 2- or 3-fold increase using the tk core promoter, whereas a 20-fold increase was seen using the SV40 promoter (p2 × 2xE2BS-luc), which contains two pairs of two E2 binding sites separated by 35 base pairs, originally obtained from HPV6b as reported (kindly provided by Dr. Robert Kovelman) (42). Interestingly, the luciferase gene driven by the SV40 promoter showed much higher activity than the tk core promoter independent of the number of E2 binding sites in our assay (data not shown). It must be noted that the SV40 promoter contains six GC-rich repeats that bind to Sp1, which might lead to enhancement or effacement of the subtle fine tuning of transactivation by E2 protein.

As shown in Fig. 5, panel 2, we found that the luciferase activity increased at all levels (200–1200 ng) of the pCMV-HPV16E2 expression vector when the reporter gene was driven by the following promoters: the 4/2 E2 luciferase or the 2/2 E2 luciferase expression vectors. In contrast, no activation of ex-
Quantified by densitometric image analysis. The numbers on the bottom and 18 S ribosomal RNA (top position of E2 mRNA (intensity of the protected E2 mRNA adjusted with 18 S RNA was indicated by the triangles above the lanes. The amount of input DNA for transfection was normalized with each backbone vector. The oligonucleotide DNAs, in which the E2 binding site was underlined, were annealed and filled-in with 32P dCTP (panel 4). The 32P-labeled oligonucleotide in the absence of the extract is in the lane to the extreme left side of the gel (F). The lane at the extreme right side of the gel contains the nuclear extract to which unlabeled oligonucleotide was added first before addition of the 32P-labeled oligonucleotides (C). Bars on the right were E2 protein and oligonucleotide DNA complex (top) and the free probe (bottom). Panel 2, RNase protection assay to estimate the level of the E2 mRNA in cells transfected with E2 expression vectors driven by the RSV promoter and by the CMV promoter. Sf1Ep cells were transfected with various concentrations of the E2 expression vectors driven either by the CMV promoter (right lanes) or the RSV promoter (left lanes). The nuclear proteins were extracted and subjected to electromobility shift assay. The concentration of the E2 expression vector driven by the RSV promoter in the left four lanes and by the CMV promoter in the right four lanes was indicated by the triangles above the lanes. The amount of input DNA for transfection was normalized with each backbone vector. The oligonucleotide DNAs, in which the E2 binding site was underlined, were annealed and filled-in with 32P dCTP (panel 4). The 32P-labeled oligonucleotide in the absence of the extract is in the lane to the extreme left side of the gel (F). The lane at the extreme right side of the gel contains the nuclear extract to which unlabeled oligonucleotide was added first before addition of the 32P-labeled oligonucleotides (C). Bars on the right were E2 protein and oligonucleotide DNA complex (top) and the free probe (bottom). Panel 3, the intensity of the protected E2 mRNA adjusted with 18 S RNA was quantified by densitometric image analysis. The numbers on the x axis correspond to those in panel 2. AU = arbitrary units of absorbance (y axis).

**Fig. 4. Expression of E2 proteins in transfected cells.** Panel 1, electromobility shift assay to detect the levels of E2 expression with CMV E2 and RSV E2 expression vectors. Sf1Ep cells were transfected with various concentrations of the E2 expression vectors driven either by the CMV promoter (right lanes) or the RSV promoter (left lanes). The nuclear proteins were extracted and subjected to electromobility shift assay. The concentration of the E2 expression vector driven by the RSV promoter in the left four lanes and by the CMV promoter in the right four lanes was indicated by the triangles above the lanes. The amount of input DNA for transfection was normalized with each backbone vector. The oligonucleotide DNAs, in which the E2 binding site was underlined, were annealed and filled-in with 32P dCTP (panel 4). The 32P-labeled oligonucleotide in the absence of the extract is in the lane to the extreme left side of the gel (F). The lane at the extreme right side of the gel contains the nuclear extract to which unlabeled oligonucleotide was added first before addition of the 32P-labeled oligonucleotides (C). Bars on the right were E2 protein and oligonucleotide DNA complex (top) and the free probe (bottom). Panel 2, RNase protection assay to estimate the level of the E2 mRNA in cells transfected with E2 expression vectors driven by the RSV promoter and by the CMV promoter. Sf1Ep cells were transfected with various concentrations of the E2 expression vector.

**Fig. 5. Transcriptional regulation of a series of artificial reporter vectors by HPV16 and CRPV E2 expression vectors in C33a cells.** C33a cells (panels 1 and 2) in six-well plates were transfected with the amounts of nanograms of E2 expression vectors DNA shown on the x axis; the pCMV-CRPVE2 (panel 1) and the pCMV-HPV16E2 (panel 2) along with a β-gal vector and a series of artificial reporter vectors indicated as below. Luciferase activity was indicated as one in the absence of the E2 expression vector (y axis). Luciferase activity was normalized by a β-gal activity. This experiment was performed at least three times to assess reproducibility.

Expression was seen when the luciferase gene was controlled with the 4E2 or the 2E2 luciferase expression vector. In contrast, when CRPV E2 expression vectors were cotransfected with the synthetic E2-dependent luciferase reporter vectors, a dose-dependent inhibition of the luciferase expression was seen following transfection of 400 ng or greater of E2 expression vector DNA, although an initial activation of the luciferase gene expression was seen when 200 ng of the E2 expression vector DNA was used for the transfection. Interestingly, the 4/2 E2 luciferase, the 2/2 E2 luciferase, and the 4E2 luciferase vectors all showed a similar -fold increase between 200 and 800 ng of the CMV expression vector, whereas the 2 E2 luciferase reporter vector showed much lower levels of expression. These data suggest that the number of E2 binding sites within the promoter of the reporter gene is more important than the space separating these E2 binding sites in terms of CRPV E2. In addition, these data suggest that there might exist some intrinsic differences between the HPV16 and the CRPV E2 protein.

Comparison of the Affinity and Stability of the CRPV and HPV16 E2 Proteins to the Cognate Sites—We assumed the differences between the result obtained with the E2 proteins of the two species (human and rabbit) of E2 transcriptional activity was due to differences in the levels of affinity of the E2 protein for the cognate E2 binding sites. We therefore investigated the Kd value of E2 binding to the cognate sites by mobility shift assay. The E2 binding site presented here was referred to as a high affinity binder previously (21) and identical as those used in artificial promoters in Fig. 1. As shown in panel 1 of Fig. 6, we determined that Kd values of the HPV16 E2 and the CRPV E2 protein for the E2 binding site was 13 and 20 nM, respectively, suggesting that affinity of the E2 proteins derived from both species was similar.

We then measured the stability of E2 proteins to the cognate recognition site by increasing the amount of excess cold competitors following the formation of the complex between the E2
amounts of unlabeled oligonucleotide (cold competitor) at a concentration of 375 nM. The cold competitor was added prior to addition of 32P-labeled dissociation binding mixtures in lanes 2–7 proteins and the 32P-labeled oligonucleotide DNA containing one E2 binding site (see Fig. 4, a high affinity binder (38, 39) for binding studies. Its which was located upstream of the HPV16 LCR and reported as stable than that of the CRPV E2 protein. We also tested the HPV16 E2 protein with the E2 DNA binding site was more was greater than 90 min, indicating that the complex of the CRPV E2 from the complex was 11 min and that of the HPV E2 were examined by electromobility shift assay in the presence of increasing concentrations of the unlabeled oligonucleotide (cold competitor): 0 nM (lanes 1 and 7), 1.6 nM (lanes 2 and 8), 8 nM (lanes 3 and 9), 40 nM (lanes 4 and 10), 120 nM (lanes 5 and 11), and 600 nM (lanes 6 and 12). The top bar corresponds to complex formed between the DNA binding domain of HPV16 or CRPV E2 proteins and the 32P-labeled oligonucleotide DNA containing one E2 binding site (see Fig. 4, panel 1). The bottom bar corresponds to the free probe. The $K_d$ values represented the average of triplicate assays. Panel 2a, the off rates of HPV16 or CRPV E2 DBD proteins and the 32P-labeled oligonucleotide DNA containing the E2 binding site in the presence of the excess cold competitor: the HPV 16 E2 DBD (lanes 8–14), and no protein (lane 15) were first incubated with the 32P-labeled oligonucleotide probe for 30 min at room temperature. These binding mixtures in lanes 2–7 and in lanes 9–14 were added to the sample well of a running gel 3, 5, 10, 30, 60, and 90 min after addition of excess amounts of unlabeled oligonucleotide (cold competitor) at a concentration of 375 nM. The cold competitor was added prior to addition of 32P-labeled probe in lanes 1 and 8. Panels 2b and 2c, the intensity of the free probe and DNA-protein complex was measured by an Image analyzer. The dissociation $t_{1/2}$ of HPV16 E2 (panel 2b) and CRPV E2 (panel 2c) was measured at least three times and one of the representative experiments is shown. The dissociation $t_{1/2}$ was 11 min for CRPV and >90 min for HPV 16, respectively. Arbitrary units of intensity was indicated in the y axis. The excess amount of the cold competitor (C) corresponds to $t_{1/2}$ for dissociation of the complex formed between the DNA binding domain of HPV16 or CRPV E2. Since the CRPV LCR vector contains an origin of replication, it is possible that the increased expression of luciferase seen in the presence of E1 (Fig. 7, panel 1) is due to an increased copy number of the luciferase-LCR vector. To test if this is the case, we compared the luciferase activity in cells cotransfected with E1 and E2 expression vectors with either the CRPV LCR-luciferase vector or the luciferase vector in which the CRPV LCR was mutated so as to inactive its ability to promote the replication of vectors containing the LCR-CRPV (28). As shown in panel 2 of Fig. 7, there was no difference in the activation seen in the replication defective versus the wild type LCR luciferase reporter vector in the presence of both E1 and E2, although the wild type LCR luciferase vector replicated in the presence of E1 and E2, whereas the mutant CRPV LCR (CRPV-LCRmut) did not (panel 3, Fig. 7). These data suggests that the CRPV E1 protein enhances the E2-dependent transcriptional activation of reporter genes governed by E2-dependent promoters, perhaps by stabilizing the binding of the CRPV E2 protein on the CRPV E2 binding site of the CRPV.

**DISCUSSION**

Importance of the Level of the E2 Protein as to whether Activation or Inhibition Occurs in the Presence of E2—According to the previously proposed model for the binding of E2 to the papillomavirus LCR, low levels of E2 bind to the higher affinity upstream E2 binding sites, thereby activating transcription, whereas the lower affinity-proximal E2 binding sites, which are proximal to the TATA box of HPV16 P97 promoter, are occupied at high levels of E2 protein, leading to transcriptional repression (38, 39). Since the number of binding sites are
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quite different between CRPV and HPV, we investigated how the CRPV E2 protein worked as a transcriptional regulator. In addition to using the native LCR to drive the expression of a luciferase reporter gene, we have utilized a series of artificial promoters composed of two Sp1 sites, the tk core promoter, and a varying number of CRPV E2 binding sites in which the number and spatial organization of these sites have been varied. These synthetic promoters were included in the experiment to study the interaction of the E1 and E2 proteins in isolation of many other host cell proteins which may interact with the native LCR regulatory elements.

We documented, by use of the mobility shift assay (for the protein level) and the RNase protection assay (for the mRNA level), that the experiments involving E2 expression vectors driven by the CMV promoter generated much higher levels of E2 than was the case in experiments with E2 expression vectors driven by the RSV promoter. The dose-dependent decrement of the luciferase expression by the authentic CRPV LCR was demonstrated by the cotransfection with the CMV promoter-dependent CRPV E2 expression vector, whereas the activation of luciferase expression was demonstrated with the RSV promoter-dependent CRPV E2 expression vector. Similar results were obtained by the use of E2 expression vectors cotransfected with the artificial E2 promoter-dependent luciferase gene. This suggested that the CRPV E2 worked as a transcriptional activator when the concentration of expressed E2 proteins are low in cells and high concentration of E2 results in switching off the transcription.

Kovelman et al. (42) reported that the space between cognate binding sites was important for transactivation by the HPV16E2 protein. The spacing between E2 binding sites did not appear to affect on the outcome of the CRPV E2 protein, suggesting the spacing is less important for transactivation in the rabbit E2 protein, perhaps because of many binding sites present in the authentic CRPV LCR. Furthermore, we recognized that transcriptional activation of the HPV-16 E2 reached a plateau with the input DNA for transfection (see panel 1 of Fig. 5). Our experiments showed that the affinities of the DNA binding domains of the human and rabbit E2 were similar, but that the stability of the complexes differed markedly, with the CRPV E2 and the HPV16 E2 having a $t_{1/2}$ of dissociation of 11 min and more than 90 min, respectively. This difference may be consistent with the much greater number of E2 binding sites in the CRPV LCR than in are present in the HPV LCR. One possible model that would explain the observed activation of E2-dependent promoters at low levels of the CRPV E2 expression and inhibition of E2-dependent promoters at high levels of the CRPV E2, in view of the instability of the complex between E2 proteins and its cognate recognition binding sites, is that there are other proteins of host or viral origin which bind to E2 and to the E2-dependent promoters, thereby stabilizing the complex between the CRPV E2 protein and its promoter. This action of the additional protein enhances the expression of the E2-dependent promoters. Because of the greater stability of the HPV E2 protein for its E2 binding sites, the role of the accessory protein in transcriptional activation may not be as great as is the case with the CRPV E2 protein. The greater number of E2 binding sites in the CRPV LCR may partially compensate at lower E2 concentrations for the decreased binding stability of the CRPV E2 protein for cognate E2 binding sites. However, at higher levels of the E2 protein, the interactions of the multiple E2 protein with each other and with other proteins may alter the overall conformation of the LCR and its protein complexes. It is possible that an excess amount of the CRPV E2 protein results in the conformational change of the LCR (8, 45, 46) and thereby eventually decreases the transactivation. Another possible model that could explain the decreased transcriptional activation at higher levels is that the E2 protein could have a squelching effect on the binding of other proteins which promote transcriptional activation by binding both to the E2 protein and to the LCR. In other words, if there are proteins other than E2, which directly promote the transcriptional activation by binding to the LCR, but which also bind to E2, it is possible to imagine that the binding of the other protein to the LCR is reduced. This would only happen if the affinity of the E2 protein for the other protein is as great or greater than the affinity of the other proteins for the LCR. In contrast, the HPV16 E2 protein binds at a much higher stability to cognate E2 binding sites in the LCR and therefore does not need the other protein to bind to the LCR compared as the CRPV E2 protein. Therefore, there is no substantial squelching effect for the HPV16 E2 protein at higher E2 concentrations in our experiment.
Transcriptional Regulation by CRPV E2 Protein

The experiments with the CRPV with the mutant ori shows that increased expression of the luciferase gene is not due to activation of replication of the E2-dependent reporter construct as an episome. Another possibility is that the E2 protein recruits the E1 protein to bind nonspecifically to DNA sequence, followed by a conformational change in the transcription initiation complex as it is being assembled. The results presented in this paper indicate that a similar situation may also be the case for the HPV-16 (47) or the BPV-1 (28). Le Moal et al. (28) reported that low concentrations of the E1 vector enhance the activity of E2-dependent promoters, but that high concentrations of the E1 vector showed repression. We did not find the repression at high concentrations of the E1 vector in our system. It is difficult to compare two systems, however, since any effect may be species-specific. It is also known that cellular transcriptional regulatory proteins such as TFIID, TFIIB, USF, and SP1 are associated with the E2 protein and modulate transcriptional activation (10, 48–50). We believe that not only host proteins, but also viral proteins, modulate transcriptional activity in a species-specific manner. The identification of the other transcriptional factors, which participate along with E2 proteins in the regulation of the other HPV genes will provide the information needed to understand more fully how the papillomaviruses interact with the cellular proteins to replicate.

Here we show that different mechanisms of transcriptional regulation may be involved in CRPV and HPV. These mechanisms may arise both from the organization of E2 binding sites, which are different in the CRPV and HPV LCR, and as well from the intrinsic differences in the properties of the HPV and the CRPV E2 proteins. We have shown that the CRPV E2 protein functions at low levels of expression of E2 protein as a transactivator and at high levels of E2 expression as a repressor substantially. These results may be useful for assembling a comprehensive picture of the complex interactions occurring between papillomavirus proteins and host proteins and will be useful as well for the identification of compounds that can be used for the inhibition of papillomavirus gene expression or replication.

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