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The Viral E4 Protein Is Required for the Completion of the Cottontail Rabbit Papillomavirus Productive Cycle In Vivo

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Expression of the papillomavirus E4 protein correlates with the onset of viral DNA amplification. Using a mutant cottontail rabbit papillomavirus (CRPV) genome incapable of expressing the viral E4 protein, we have shown that E4 is required for the productive stage of the CRPV life cycle in New Zealand White and cottontail rabbits. In these lesions, E4 was not required for papilloma development, but the onset of viral DNA amplification and L1 expression were abolished. Viral genome amplification was partially restored when mutant genomes able to express longer forms of E4 were used. These findings suggest that efficient amplification of the CRPV genome is dependent on the expression of a full-length CRPV E4 protein.

Papillomaviruses (PVs) are epitheliotropic viruses that can infect both cutaneous and mucosal surfaces and induce benign and malignant skin tumors (34). There are more than 100 different types of human PVs (HPVs) and many animal-specific PVs (1, 16). The life cycle of PVs can be divided into early and late phases and is dependent on the differentiation program of the infected epithelial cell (28, 49). PVs are highly host and tissue specific. Infections at different histological sites trigger a series of viral life cycle events that are conserved among different virus types (35, 39). The early stages begin in the actively proliferating cells in the basal and parabasal layers, where expression of E6 and E7 (the viral transforming proteins), as well as E1 and E2 (the viral replication proteins), induces papilloma formation and maintains the episomal viral genome at a low copy number (19, 48, 49, 53). The late stage of the virus life cycle is triggered when the infected cells further differentiate during their migration toward the epithelial surface (28, 49). Although the exact mechanism(s) by which the late events are initiated remains unclear, a change in promoter activity occurs as the virus enters its productive cycle in the upper epithelial layers (2, 22). In these differentiated cells, E4 protein expression and the onset of viral DNA amplification begin, and these processes are followed by the expression of the viral capsid proteins (L1 and L2) and the assembly of infectious virions (35, 39).

In PV-infected regions, the normal differentiation program of the epithelium is disturbed, and alterations in tissue morphology are apparent. Some of the changes are characteristic of PV infections, including acanthosis (thickening of the epithelium), the presence of koilocytes, and parakeratosis (retention of the nuclei in the cornified layers) (34). These changes are partly the consequence of the expression of the viral E6 and E7 proteins in the lower layers of the epithelium (48, 53). E6 and E7 act via different mechanisms to stimulate cell cycle progression, to increase the number of nucleated amplifying cells in the differentiating layers, and to delay keratinocyte terminal differentiation (51, 53). Since PVs rely to a large extent on the host cell machinery to replicate their genomes, the expression of E6 and E7 is crucial to maintain the cells in S phase so that the replication of their genomes can be supported even in the cells that are undergoing differentiation (which would otherwise occur predominantly in G_1 phase) or in the terminally differentiated layers (upper layers) of the epidermis (which are normally in G_0 phase) (11). Based on immunostaining and in situ hybridization results, it has been suggested that amplification of viral DNA occurs in a region in the differentiated layers, where the expressions of early and late genes overlap (35, 39).

It has previously been shown that the viral events triggered during infection of cottontail (CT) and New Zealand White (NZW) rabbits by CT rabbit PV (CRPV) follow a conserved pattern and that this pattern is preserved in infections caused by other PV types (39). By using specific E4 antibodies raised in rats, the expression of the CRPV E4 protein was apparent in infected rabbit tissues and was shown to occur in the intermediate layers of the epidermis. In CT rabbit papillomas, E4 protein was detected in the cytoplasm as well as the nuclei of cells that appear to support virus genome amplification. Using specific antibodies, L1 expression was detected in the terminally differentiated layers following a spatial gap of one to two cell layers from the first appearance of E4.

Little is known about the role of the E4 protein in the PV life cycle. E4 is a small (10- to 20-kDa) phosphoprotein that is expressed from a spliced E1∧E4 mRNA initiated from the differentiation-dependent promoter (12, 17, 27, 38). Immunodetection of E4 in different virus-infected tissue samples showed that the expression of high levels of E4 protein corre-

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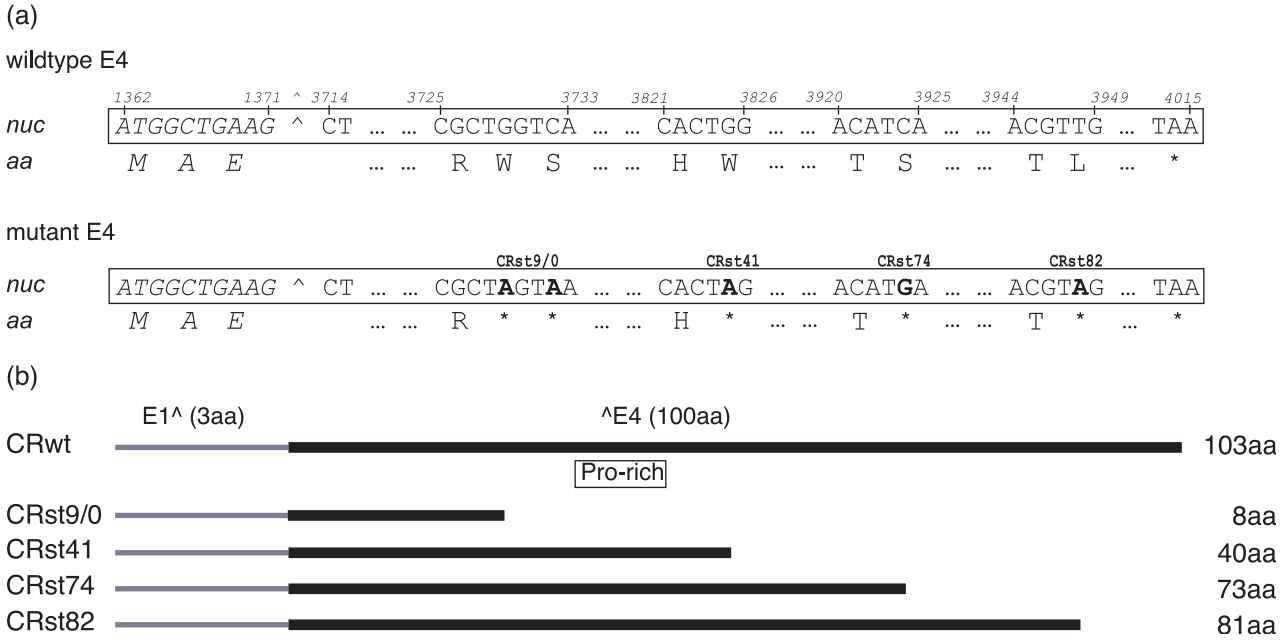


FIG. 1. CRPV E4 C-terminal truncation mutant sequences. (a) Wild-type and mutant E4 nucleotide sequences *(nuc)* and positions are represented in their respective diagrams. Nucleotide positions where mutations were introduced are indicated by stars. The insertions of termination codons in the E4 ORF are also shown in amino acids (aa). (b) The full-length CRPV E1∧E4 protein consists of 103 amino acids. E4 mutants CRst9/0, CRst41, CRst74, and CRst89 are predicted to express 8, 40, 73, and 81 amino acids of E1∧E4, respectively. Pro-rich, a proline-rich region in the E4 protein sequence.

lated exactly with the occurrence of vegetative viral genome amplification (18, 35, 39), and recent work has suggested that HPV type 1 (HPV-1) E4 protein may be involved in the replication of the virus in the differentiated cells (15, 41). In this study, we have investigated the in vivo role of the E4 protein in the context of the life cycle of CRPV in its natural host (the CT rabbit) as well as in the experimental host (the NZW domestic rabbit), and we have shown that E4 protein expression is required for the onset of the late stage of the virus life cycle in both rabbit species.

CRPV DNA capable of expressing different forms of C-terminally truncated E4 proteins were generated in the backbone sequence of the Washington B strain of the CRPV genome (GenBank accession number U09496). Four E4 mutant CRPV genomes, namely, CRst9/0, CRst41, CRst74, and CRst82, were constructed (Fig. 1). These mutants were predicted to express 8, 40, 73, and 81 amino acids of the 103-amino-acid CRPV E1∧E4 protein sequence, respectively. The mutants were constructed by introducing stop codons at specific positions along the E4 open reading frame (ORF) by using the QuikChange site-directed mutagenesis method (Stratagene, Amsterdam, The Netherlands), according to the manufacturer's protocol (Fig. 1). The primer pairs used to generate each E4 mutant genome were as follows: for CRst9/0, CAG GGG CAC TCA CTA GCG GCT GGG and CCC AGC CGC TAG TGA GTG CCC CTG; for CRst41, ACC TTC GTC TGC CTA GTG TAC GCG and CCA CGC GTA CAC TAG GCA GAC GAA GG; for CRst74, GAG CAG GCG GTC TTA TGT CCC CGG and CCG GGG ACA TAA GAC CGC CTG CTC; for CRst82, TAG TTC TTC GTC CTA CGT CCT CTG and CAG AGG ACG TAG GAC GAA GAA CTA. Since the E4 gene lies entirely within the E2 gene, each mutation point was chosen so that stop codons were introduced into the E4 ORF, while the translated E2 sequence remained unaffected. Each mutant genome was sequenced (in both directions) across the entire E4 gene to ensure that no additional base change was present (sequence data not shown). Although the complete abolition of E4 protein expression was not possible (as the first three N-terminal amino acids of the E1∧E4 protein are derived from the E1 ORF), the CRst9/0 E4 mutant was predicted to act as an E4 knockout genome, since the eight-amino-acid E1∧E4 peptide expressed from this mutant genome was expected to be rapidly degraded by cellular proteases in vivo. Based on known splice site positions and consensus sequences, none of the mutants described here are predicted to affect CRPV splicing (42, 43).

A loss of E4 did not affect the ability of the virus to induce papilloma development. Four outbred NZW domestic rabbits (designated Rb 1, Rb 2, Rb 3, and Rb 4) were each inoculated with the recircularized form of the wild-type genome (CRwt), CRst9/0, CRst41, CRst74, and CRst82, as well as the plasmid CRPV-pLAII, which contains the CRPV genome in the vector pLAII (52). The CRPV genome was recircularized in a ligation reaction containing 0.65 μg of CRPV DNA/μl that was linearized with SalI (Roche Diagnostics Ltd., East Sussex, United Kingdom) and 20 U of T4 ligase (New England Biolabs Ltd., Hertfordshire, United Kingdom) and incubated at 14°C overnight. Recircularized DNA was purified with a QIAQuick gel extraction kit (QIAGEN Ltd., West Sussex, United Kingdom), according to the manufacturer's protocol. Viral DNA was delivered intraepithelially onto the shaven backs of the NZW rabbits with a helium-driven gold particle-mediated DNA de-

TABLE 1. Papilloma formation during the course of the experiment[a]

CRPV DNA	NZW rabbits				Wild CT rabbits		
	Rb 1	Rb 2	Rb 3	Rb 4	CTR 1	CTR 2	CTR 3
CRwt	5→4 (5)	7→4 (5)	0→0 (5)	9→0 (5)	2 (2)	2 (2)	0 (2)
CRst9/0	2→18 (5)	5→7 (5)	1→5 (5)	3→0 (5)	3 (3)	2 (4)	4 (3)
CRst41	2→12 (5)	9→8 (5)	0→4 (5)	2→0 (5)	ND	ND	ND
CRst74	4→12 (5)	6→6 (5)	0→1 (5)	3→0 (5)	ND	ND	ND
CRst82	6→3 (5)	18→16 (5)	0→2 (5)	3→0 (5)	ND	ND	ND

[a] For the NZW rabbit experiment, the data are shown as $a \rightarrow b$ (c), where a represents the number of papillomas present at week 5, b represents the number of papillomas present at the end of the experiment (week 13), and c represents the number of inoculations delivered per rabbit. For the CT rabbit experiment, the data correspond to b (c) as described for the NZW rabbit. ND, not done. Note that it is not unusual that the number of papillomas harvested is greater than the number of inoculations.

livery device (a gene gun) (PowderJect Vaccines, Madison, Wis.) at a concentration of 1 μg per site as described previously (54). Each rabbit was inoculated at five sites with each DNA type at a pressure of 350 lb/in^2. Papilloma development was monitored weekly from day 24 postinoculation by measuring their volumes and numbers, and papillomas were harvested at week 13 postinoculation. The numbers of papillomas present at the end of the experiment are shown in Table 1. By week 4 postinoculation, papillomas had appeared on all four NZW rabbits at the sites inoculated with the wild-type and the E4 mutant genomes. Papillomas persisted on Rb 1, Rb 2, and Rb 3 throughout the next 9 weeks of the experiment. Unfortunately, total regression of the papillomas was observed on Rb 4 by week 6 (Table 1). Papilloma regression was also evident on Rb 3 during weeks 8 and 9 at the sites inoculated with the recircularized CRPV DNA preparations but not the plasmid CRPV genome (data not shown). Rb 3 showed a longer latent period (when compared to Rb 1 and Rb 2) (Table 1) and a quicker regression pattern during the course of the experiment (data not shown). Papillomas on Rb 1 and Rb 2 continued to grow until the time of harvest and showed no obvious signs of regression. Genetic variation among the rabbits was thought to have contributed to differences in the course of the CRPV infection, and indeed, major histocompatibility complex class II genotypes have previously been shown to play a significant role in determining the outcome of CRPV infections in rabbits (23, 24).

Interestingly, a second wave of papilloma formation was seen at week 9 on Rb 1, Rb 2, and Rb 3; the reason for this wave was not pursued. Despite the use of a standard protocol for the inoculation of DNA, the number of papillomas present at the end of the experiment was unexpectedly greater at the sites inoculated with the E4 mutant genomes than the sites inoculated with the wild-type genome in both Rb 1 and Rb 2 (Table 1). Even though the loss of E4 protein expression did not affect the ability of the virus to induce papilloma growth, it is possible that E4 may play an inhibitory role in the initial proliferation phase of virus infection. This effect has also been observed in experiments carried out in differentiating raft cultures using similar E4 mutants prepared in the context of the HPV-16 genome (S. Southern, personal communications).

The E4 knockout experiment was also carried out separately in the natural host for CRPV infection. Three wild CT rabbits (designated CTR 1, CTR 2, and CTR 3) were used, and each rabbit was inoculated with the wild-type and CRst9/0 E4 sequences constructed in the CRPV plasmid genome (CRPV-pLAII). A grid comprised of two columns and four rows was drawn on the shaven backs of the rabbits, and 1 μg of DNA was inoculated into each grid space at a pressure of 300 lb/in^2 with a gene gun (Bio-Rad Laboratories). Each rabbit received two shots of the wild-type DNA in the top row, three shots of the CRst9/0 E4 mutant DNA in the second to fourth rows (right column), and three shots of a CRPV genome containing an E8 ATG mutation described previously (29) in the second to fourth rows (left column). In the CT rabbit hosts, as in the NZW rabbit hosts, the E4 knockout genomes were also capable of inducing papilloma growth following viral DNA inoculations (Table 1). Interestingly, the number of papillomas obtained from the inoculation of the CRst9/0 mutant genome was also greater than that obtained from the wild-type mutant genome from the same rabbit.

The sizes of the papillomas harvested at the end of the experiments from the NZW and CT rabbits were variable. However, the difference in papilloma volume was not thought to be a consequence of mutations in E4, as there was no obvious difference between wild-type- and E4 mutant-induced papillomas that developed during the same period of time on each rabbit (data not shown).

CRPV DNA was maintained as an extrachromosomal genome in the wild-type- and E4 mutant-induced NZW rabbit papillomas. Ten to 20 mg of frozen tissue from CRwt-, CRst9/0-, and CRst41-induced NZW papillomas was minced and resuspended in buffer (50 mM Tris-HCl [pH 7.5], 10 mM EDTA, 100 mM NaCl) containing 50 μg of RNase (Roche Diagnostics Ltd.) per ml. The tissues were digested with 200 μg of proteinase K (QIAGEN Ltd.) per ml at 55°C for 2 h and then dissolved in an alkaline lysis buffer (50 mM Tris-HCl [pH 12.6], 10 mM EDTA, 100 mM NaCl, 0.5% sodium dodecyl sulfate) overnight at 4°C. DNA was precipitated with ethanol from the supernatant and resuspended in 50 μl of Tris-EDTA buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA). The DNA was then digested with the restriction enzymes SalI and HindIII (Roche Diagnostics Ltd.) in the manufacturer's recommended buffers at 37°C for at least 4 h. The restriction digests, as well as nondigested DNA, were separated on a 0.7% agarose gel and analyzed after Southern blotting with a phosphorus-32-labeled CRPV probe (Amersham Ready-To-Go DNA labeling reactions). Radioactivity was exposed on a phosphoscreen, and data were collected with a Storm860 scanner (Amersham Biosciences, Bucks, United Kingdom) and ImageQuant 5.0 analysis software (Amersham Biosciences). Similar DNA species were present in the nondigested and enzyme-

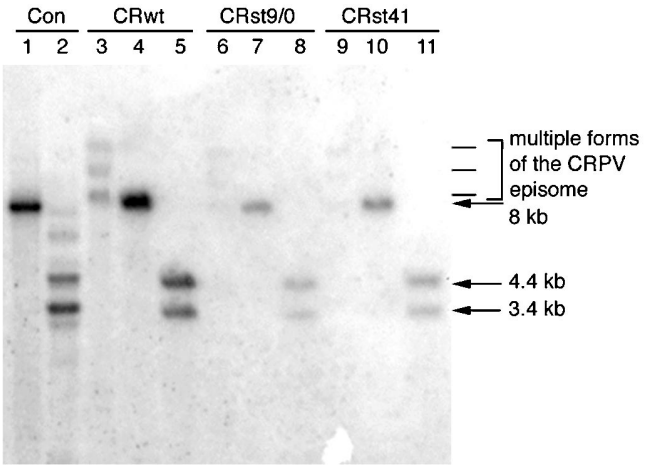


FIG. 2. Southern blot analysis of the CRPV genome from NZW rabbit papilloma tissues. DNA extractions from CRwt (lanes 3 to 5)-, CRst9/0 (lanes 6 to 8)-, and CRst41 (lanes 9 to 11)-induced papilloma samples were analyzed together with a religated CRPV DNA preparation (control lanes 1 and 2). Lanes 3, 6, and 9, nondigested DNA; lanes 1, 4, 7, and 10, SalI-digested DNA; lanes 2, 5, 8, and 11, HindIII-digested DNA. The nondigested CRPV DNA migrated as three bands (lanes 3, 6, and 9), which were likely to represent different conformations of the viral DNA. These bands are very faint in the lanes representing extractions from mutants, probably due to the absence of genome amplification in the tissues. Quantitative analysis between the DNA levels in the wild-type and mutant lanes could not be done since it was not possible to distinguish a productive region from a nonproductive region in the papilloma biopsy samples. A single band of linearized CRPV DNA (approximately 8 kb) was detected in the control and tissue extraction lanes following digestion with SalI (lanes 1, 4, 7, and 10). Two strong bands of approximately 3.4 and 4.4 kb were present in the control and tissue extraction lanes following HindIII DNA digestion (lanes 2, 5, 8, and 11).

digested CRwt, CRst9/0, and CRst41-induced papilloma samples (Fig. 2). A single band of approximately 8 kb was detected in the wild-type- and mutant-induced samples following digestion with SalI (Fig. 2, lanes 4, 7, and 10). As expected, two strong bands (estimated masses of 3.4 and 4 kb) were detected in the HindIII-digested samples (Fig. 2, lanes 5, 8, and 11). These data indicated that CRPV DNA may be maintained in the episomal form even in the absence of E4 expression.

Papilloma histology was preserved in lesions caused by E4 mutant DNA. The histological features of the infected tissue sections were examined following hematoxylin and eosin counterstaining. All papillomas induced by the wild-type and E4 mutant genomes showed evidence of acanthosis, papilloma formation, the presence of koilocytes, an increase in the number of dark granules in the differentiated layers, and parakeratosis. Although there were some variations among lesions, these were generally attributed to differences in lesion maturity. From our examination, the development of pathological features typical of CRPV infection was similar between the papillomas induced by wild-type and E4 mutant DNA (Fig. 3).

The early stage of the CRPV life cycle was not disrupted by the expression of truncated E4 proteins. PCNA and cyclin E are surrogate markers for the expression of E6 and E7 proteins in the lower layers of PV-infected lesions, and their presence identifies cells that are supporting the early stage of the virus life cycle (46, 47). PCNA was detected in formalin-fixed, paraffin-embedded tissue sections by using the mouse monoclonal antibody PC10 (Neomarkers) following heat denaturation in citrate buffer by microwave treatment for 15 min. High levels of PCNA expression were detected in the basal to intermediate layers of the epidermis in both CRwt and the CRst9/0 and CRst41 DNA-induced papillomas from NZW and CT rabbits (Fig. 4). Cyclin E was detected by using mouse monoclonal antibody 13A3 (Novocastra Laboratories), after which tissue sections were used for fluorescent in situ hybridization (FISH) to detect the presence of viral genome amplification using digoxigenin-labeled CRPV probes as described previously (39). Cyclin E staining was detected in the parabasal to intermediate layers but not in the uppermost layers of the epidermis (Fig. 4). In the NZW and CT rabbit papillomas induced by the E4 mutant DNA, the cyclin E immunostaining patterns were similar to those observed in the wild-type-genome-induced papillomas.

The onset of viral late events was inhibited to various extents with the expression of the different E4 mutants. Immunodetection of the viral late proteins E4 and L1 and the detection of viral DNA amplification were carried out as described previously (39). In CT rabbits, E4 expression was detected in papillomas caused by the CRwt and E8 mutant genomes but not in papillomas induced by the CRst9/0 mutant genome. In adjacent sections of wild-type-induced papillomas, viral genome amplification and L1 expression were detected in regions similar to those where E4 expression was found. As expected, E4 expression and the amplification of the viral genome correlated closely, with the expression of L1 being detected in the more differentiated cell layers (Fig. 5a). Viral genome amplification, as well as the expression of E4 and L1, could not be detected by the same methods in any of the CRst9/0-induced CT rabbit papilloma sections (Fig. 5a), indicating that the late stage of the CRPV life cycle is severely disrupted by the loss of E4 protein expression. Only results obtained from Rb 1 and Rb 2 were fully analyzed from the NZW rabbit data, since no papillomas were obtained from Rb 3 at the CRwt genome-inoculated sites (Table 1). Virus genome amplification was detected in all the CRwt DNA-induced NZW rabbit papillomas from Rb 1 and Rb 2 (Table 2; Fig. 5b). In contrast, no viral DNA amplification was detected in any of the CRst9/0 E4 mutant-induced papillomas (Table 2; Fig. 5b). Interestingly, infection with the CRst41 DNA produced different results for Rb 1 and Rb 2. Low levels of CRPV DNA could be detected in a subset of CRst41 DNA-induced papillomas from Rb 1, whereas viral DNA was undetectable in papillomas from Rb 2 (Table 2). Using a standard FISH method, the level of staining obtained from the CRst41 DNA-induced papillomas was considerably lower than that seen in the CRwt DNA-induced papillomas (Fig. 5c). This finding suggests that the virus may have initiated its productive life cycle in these cells but that amplification of its genomic DNA is not efficiently carried out. This hypothesis is further supported by the analysis of papillomas induced by CRst74 and CRst82. From the data obtained from these E4 mutants, it appears that viral DNA amplification is partially restored in mutants expressing longer versions of the C-terminally truncated E4. Among the E4 mutant-induced papillomas, the number of papillomas as well as the population of cells supporting viral DNA amplification was highest with CRst82 (Table 2; Fig.

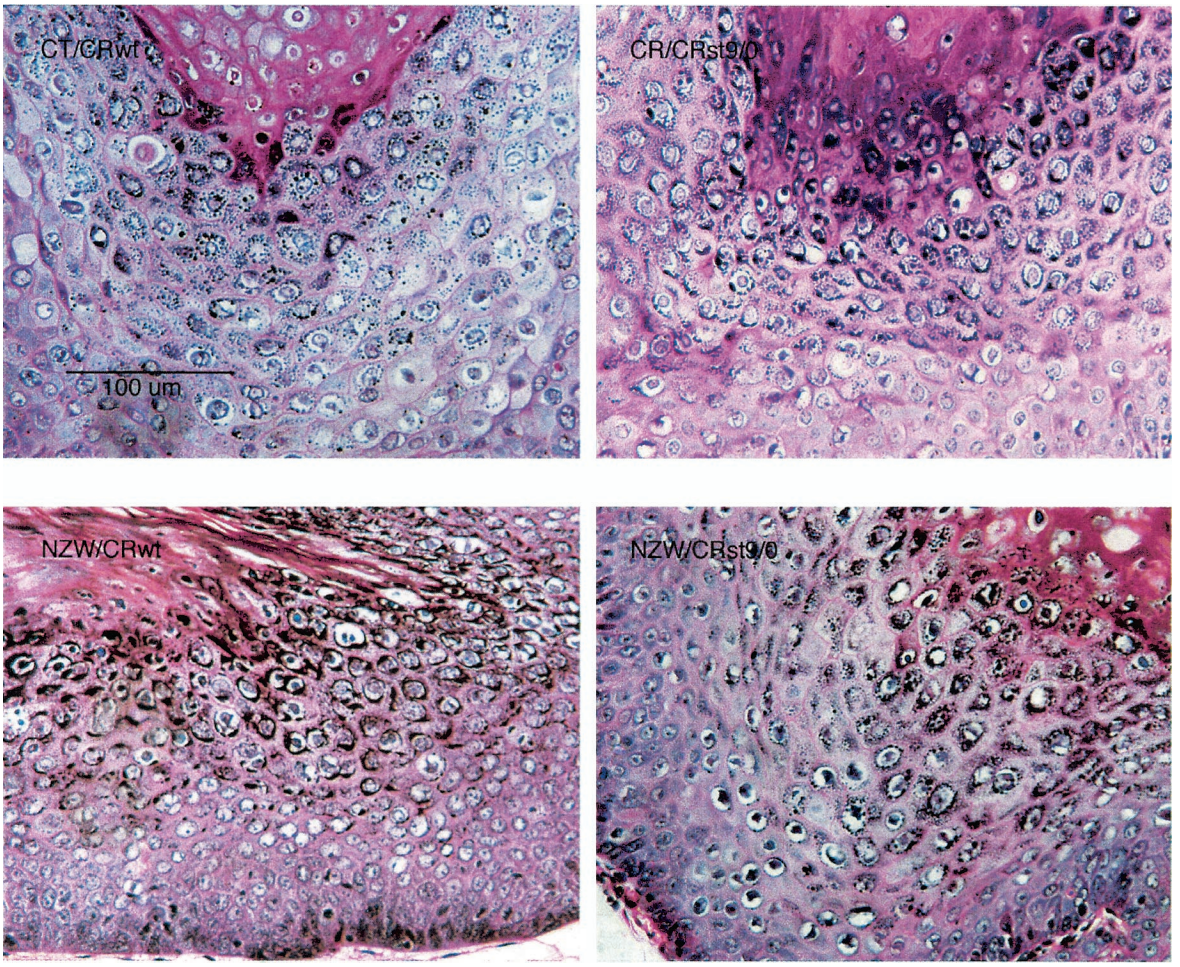


FIG. 3. Comparison of histologies of CRwt and CRst9/0 DNA-induced CT and NZW rabbit papilloma tissues. Characteristic features of papilloma development, such as acanthosis, parakeratosis, the presence of koilocytes, and an increase in pigmented granules, were present in both the CRwt and CRst9/0 DNA-induced CT (upper panels) and NZW (lower panels) rabbit papilloma tissues.

5c). From the papillomas collected from Rb 2, low levels of viral DNA amplification were first detected in the CRst74 DNA-induced tissues (Table 2). Since disease progression and outcome are influenced by the genetic variations among different hosts, Rb 1 may be genetically more susceptible to CRPV infection than Rb 2. It has previously been found that the detection of E4 expression in NZW rabbit papillomas is highly variable when compared to that of the CT rabbit papillomas (39). When E4 staining could be detected in the NZW rabbit tissue sections, viral DNA amplification was also found to occur in the same regions of the papillomas (Fig. 5b). Unfortunately, L1 expression could not be investigated in the NZW rabbit tissues since CRPV infections in this rabbit species usually result in nonproductive infections.

Experimental infections with CRPV have shown that the virus can infect a variety of different rabbit species, including domestic NZW rabbits, although infectious viruses have only rarely been recovered from these animals (3, 32, 44, 45). Studies comparing CRPV infections in both CT and NZW rabbits have found that the courses of infection in the two rabbit species were influenced by similar factors, such as virus dose and potency, possession of circulating antibodies, and local tissue and cell conditions (21, 31). However, the main differences between CRPV infections in CT and NZW rabbits is that CRPV produces abortive infections in NZW rabbits and is three times more likely to induce malignancy in NZW rabbits than in CT rabbits (50). It is still not clear if the lack of (or poor) late promoter activity contributes to abortive infections and the high rate of carcinoma development in the domestic rabbits (37, 55). Nonetheless, the CRPV-NZW rabbit animal model is widely used in studies of viral gene functions and vaccine development and for the identification of host factors that influence disease outcome (4–7, 23–26, 30, 53). Studies of the early viral transcription patterns have shown that the expressions of viral transcripts in the lower layers of CT and NZW rabbit papillomas were similar (40), and we have also shown that CRPV infection in rabbits results in a pattern of life cycle events which is comparable to that seen in other PV infections (39).

Previous efforts to isolate a major late transcript containing the E1∧E4 ORF from CRPV-infected CT and NZW rabbit tissues were not successful. However, transcripts which contain the E1∧E4 splice junction were detected in the VX2 cell line (a transplantable rabbit carcinoma derived from a CRPV-in-

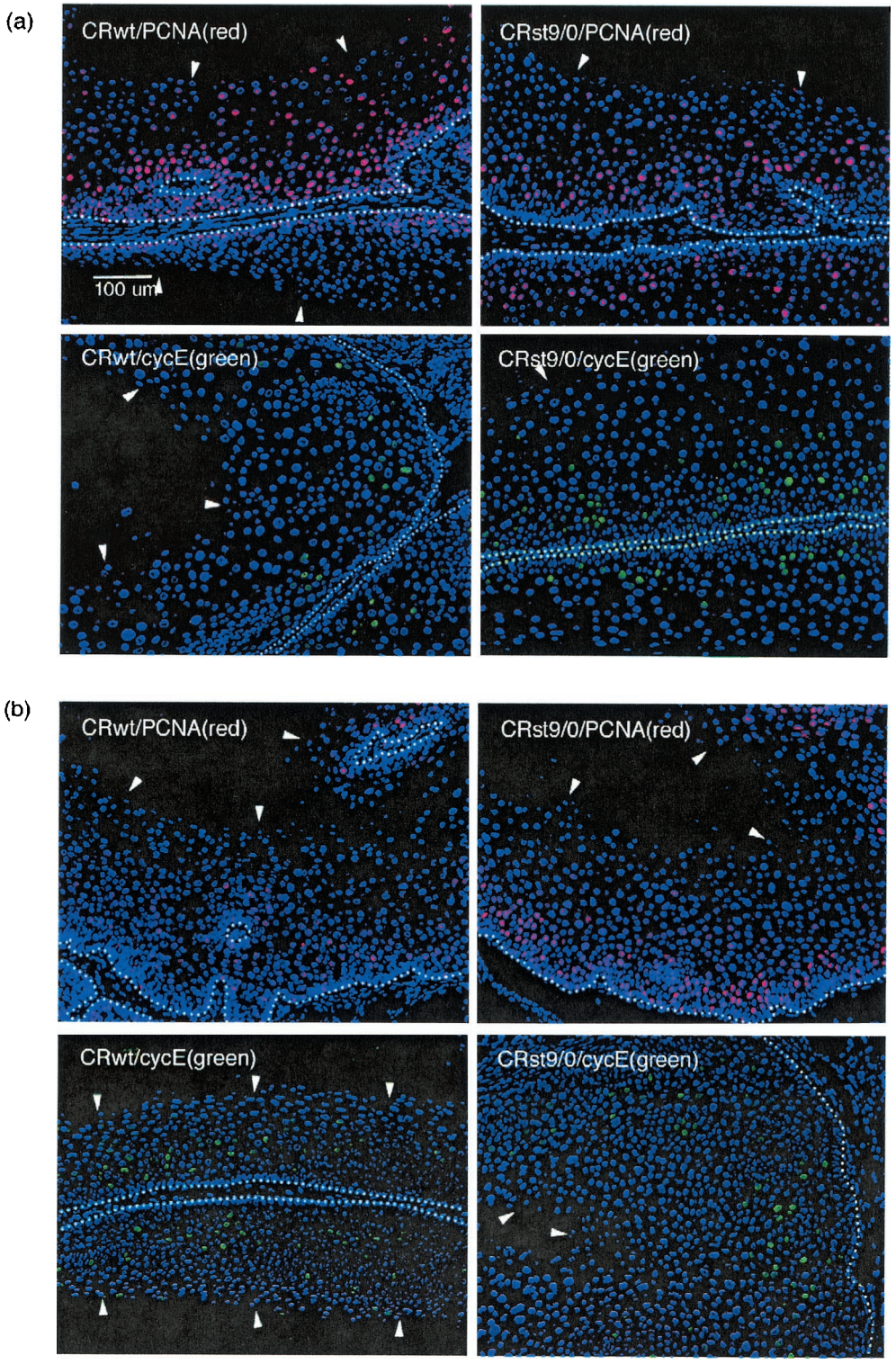
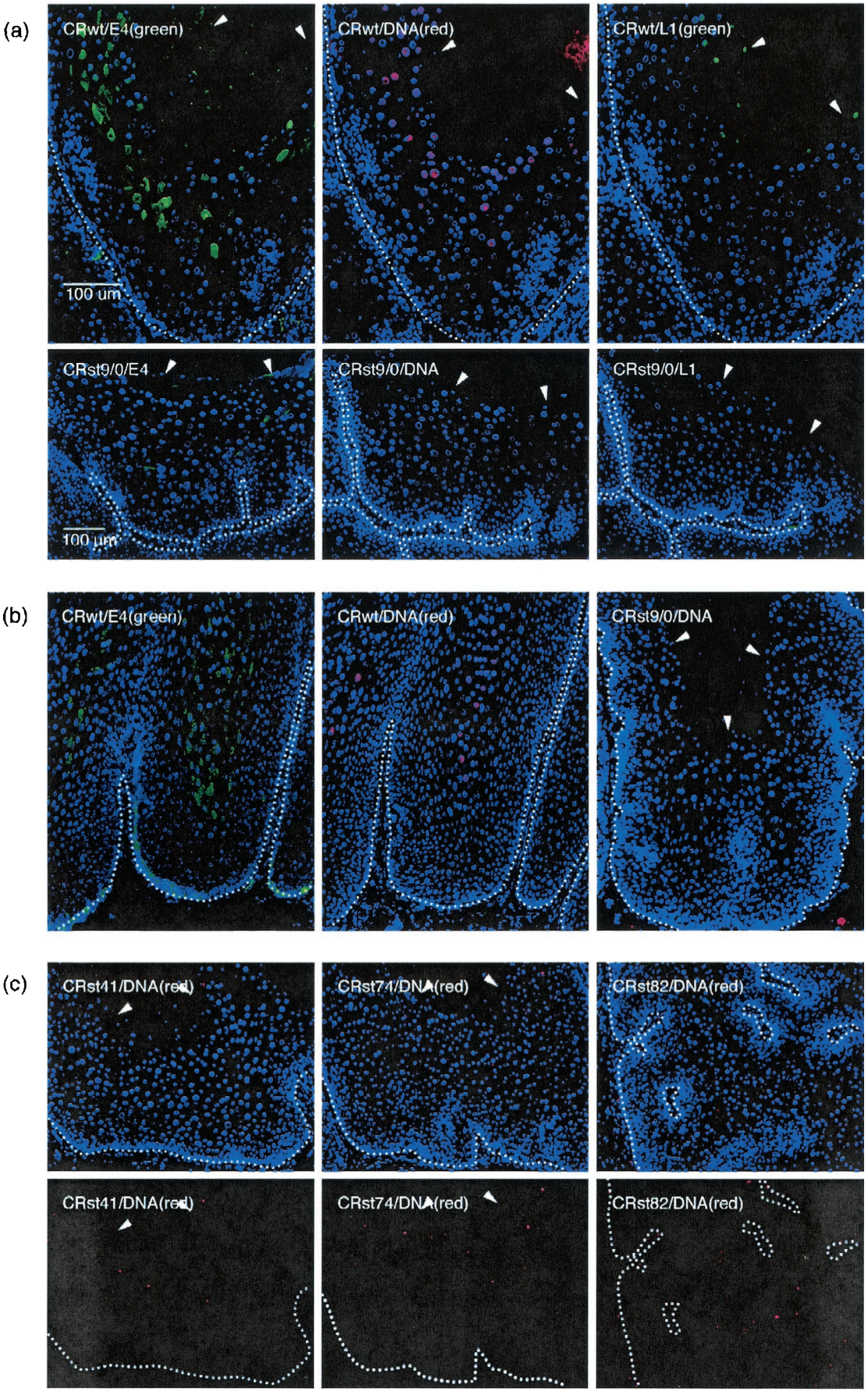


FIG. 4. Immunodetection of surrogate markers of E6 and E7 expression (PCNA and cyclin E) in CT and NZW rabbit papillomas. (a) CT rabbit papillomas. Up-regulation of PCNA expression from the basal to the differentiated layers of the epithelia was detected in both the CRwt and CRst9/0 DNA-induced papillomas. Similarly, cyclin E (cycE) was detected from the suprabasal cells to the intermediate layers of the epithelia in the wild-type and E4 knockout papillomas. (b) NZW rabbit papillomas. Up-regulation of PCNA and cyclin E were evident in the CRwt and CRst9/0 DNA-induced papillomas. There was no obvious difference in the staining patterns between the wild-type and the E4 knockout papillomas. Blue indicates the nuclear counterstain. Dotted lines indicate the epithelial basal layers. Arrowheads indicate the tops of the lesions. The staining patterns shown are representative of the results obtained in repeated experiments.


(a)
CRwt/E4(green)
CRwt/DNA(red)
CRwt/L1(green)
100 um
CRst9/0/E4
CRst9/0/DNA
CRst9/0/L1
100 um
(b)
CRwt/E4(green)
CRwt/DNA(red)
CRst9/0/DNA
(c)
CRst41/DNA(red)
CRst74/DNA(red)
CRst82/DNA(red)
CRst41/DNA(red)
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duced papilloma) (13, 33) as well as in an in vitro study using the recombinant retrovirus system (14). This result is unlike that with the E1∧E4 transcript, which is found to be a major late transcript expressed in HPV-1-induced verrucas (17). HPV-1, like CRPV, is a member of the E supergroup of PVs (10). Using CRPV E1∧E4-specific antibodies, we have detected CRPV E4 protein in formalin-fixed, paraffin-embedded CT rabbit papillomas. Unfortunately, the detection of the E4 protein in NZW rabbit lesions by use of the same antibodies produced inconsistent results and high-background staining patterns. Possible explanations for this poor staining include low-level E4 expression as well as epitope masking, possibly as a result of protein-protein interaction or formalin fixation, which could not be effectively overcome by using various specific antigen exposure methods (9, 20). Despite these difficulties, previous immunostaining data (39) as well as results described here strongly suggest that the CRPV E4 protein is expressed during virus infections in both NZW and CT rabbits and that E4 plays an important role in the productive stage of the CRPV life cycle.

Although E4 has been regarded as a viral late protein and is expressed in abundance in the differentiated layers of the infected epithelia, we also suggest a possible role for CRPV E4 in the initial stages of PV infection. Viral transcripts containing the E4 ORF have been detected in CRPV DNA- and HPV DNA-immortalized and undifferentiated cells in culture. Therefore, the expression of very low levels of E4 early on in the virus life cycle and below the sensitivity of immunodetection methods cannot be dismissed. Here, we have shown that the expression of CRPV E4 is not required for papilloma formation or for the E6- and E7-induced events during the early stages of the virus life cycle in vivo. However, it is possible that E4 may be involved in negatively regulating cell proliferation during the early formation of the rabbit papillomas, as the number of E4 mutant-derived papillomas was consistently higher (up to four times) than the number obtained with the wild-type DNA (Table 1).

Using E4 mutant CRPV genomes, we observed a direct effect of the loss of E4 expression on the productive (late) stage of the CRPV life cycle in vivo. This observation is particularly interesting since earlier reports had shown a close correlation between the occurrence of E4 expression and viral DNA amplification in PV-infected tissues (18, 35, 39). In our study, lesions produced by CRst9/0 DNA were predicted to be unable to express a functional E4 protein. A loss in the initiation of the viral late events following the inoculation of the CRst9/0 mutant was demonstrated in both the natural (CT rabbit) and experimental (NZW rabbit) hosts for CRPV infection. CRPV E4 protein is likely to have a common role in the late stage of the virus life cycle in the two rabbit species even though the outcome of CRPV infections may be different.

TABLE 2. No. of NZW papillomas with positive viral DNA amplification

CRPV DNA	Rb 1		Rb 2	
	No. of papillomas with DNA amplification[a]	Levels of DNA amplification[b]	No. of papillomas with DNA amplification	Levels of DNA amplification
CRwt	4/4	++++	3/3	+++
CRst9/0	0/11	−	0/4	−
CRst41	9/12	±	0/8	−
CRst74	10/14	+	2/6	±
CRst82	5/5	+	7/11	+

[a] Data are shown as *a*/*b*, where *a* represents the number of tissues with positive FISH signals and *b* represents the number of papillomas that were prepared in formalin-fixed, paraffin- embedded blocks and examined by FISH.

[b] Levels indicated by FISH signal: +, signal present; ++++, very strong immunofluorescence in a large number of cells; ±, very faint immunofluorescence in one or a few cells; −, complete absence of genome amplification. The results shown are typical of what was found following the analysis of lesions in each group.

Several hypotheses regarding the function of PV E4 have pointed towards a role in the late stage of the virus life cycle, particularly during the amplification of the virus genome. Two different groups have recently provided evidence to show that the expression of HPV-16 and HPV-18 E4 causes a G_2 cell cycle arrest in both yeast and mammalian cells (15, 36). By inhibiting mitosis and by maintaining expression of S-phase proteins, the virus may utilize the cellular replication machinery for efficient amplification of its genome. Although HPV-16 E4 does not appear to associate with promyelocytic leukemia protein (PML) components (P. Laskey, personal communication), others have shown that the E4 protein of a different virus (HPV-1) causes PML to redistribute to nuclear E4 inclusions (41). Roberts et al. (41) suggest that this process may be necessary for the efficient amplification of the PV genome, since virus replication and PML association have previously been shown to occur in other DNA virus infections, such as those caused by Epstein-Barr virus and cytomegalovirus. HPV-11 E4 can also cause a G_2 cell cycle arrest (15) and, in addition, may be involved in the release of infectious virus particles (8). The association between HPV-11 E4 and the cornified cell envelope was shown to be critical for weakening the epidermal cell barrier in order to enable membrane rupture and the spread of infectious virions. Thus, there is mounting evidence that the PV E4 protein may have an essential role, or possibly multiple roles, in the productive stage of the virus life cycle, in agree-

FIG. 5. Detection of viral DNA amplification and E4 and L1 expression in CT and NZW rabbit papillomas. (a) CT rabbit papillomas. E4 expression (left panel; green), DNA amplification (middle panel; red), and L1 expression (right panel; green) were detected in the differentiated layers of the CRwt DNA-induced CT papilloma tissues but not in any CRst9/0 DNA-induced CT papilloma tissues. It should be noted that the weak green staining in the CRst9/0/E4 panel is nonspecific staining of cornified (dead) cells that folded onto the tissue section during coverslip mounting. (b) NZW rabbit papillomas. DNA amplification (middle panel; red) was detected in the CRwt DNA-induced papillomas but was not detectable in any CRst9/0 DNA-induced papillomas (right panel). In CRwt tissue sections, numerous areas that contained cells supporting viral DNA amplification were found. E4 expression was restricted to areas that were shown to support viral DNA amplification (left panel; green). (c) NZW rabbit papillomas. Low levels of viral DNA amplification (red) were apparent in the papillomas induced by E4 mutants CRst41, CRst74, and CRst82. Blue indicates the nuclear counterstain. Dotted lines indicate the epithelial basal layers. Arrowheads indicate the tops of the lesions. The staining patterns shown are representative of the results obtained in repeated experiments.

ment with the work presented here. It appears from our work that the full-length CRPV E4 protein is necessary for the completion of the virus life cycle in rabbits and may be involved in inducing a suitable environment for the efficient amplification of the virus genome.

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