Changes in RNA Expression Pattern during the Malignant Progression of Cottontail Rabbit Papillomavirus-Induced Tumors in Rabbits

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Cottontail rabbit papillomavirus induces strictly epithelial tumors in both cottontail and domestic rabbits. A high proportion of the initial benign papillomas progress within 8 to 14 months to invasive carcinomas. With the help of mRNA-specific riboprobes for E6, E7, E1, E2, L1, and L2, we investigated by in situ hybridization the RNA expression pattern of cottontail rabbit papillomavirus in tissue sections of biopsies from different stages of tumor development. Common features of all lesions were high levels of E6 and E7 mRNAs and low levels of E1 and E2 mRNAs. In agreement with earlier reports, there was no evidence for a major mRNA class equivalent to the prominent E1⁺E4 RNA of human papillomavirus types 6/11 and 16. In cottontail rabbit papillomas, high levels of E6 and E7 mRNAs were present in the upper differentiated epithelial layers. These layers also contained most of the E1 and E2 mRNAs and the viral DNA. In contrast, papillomas of domestic rabbits revealed the opposite differentiation-dependent expression pattern for the E6 and E7 mRNAs; there were strong signals in the basal layers, and these declined with increased differentiation. Transcripts encoding the L1 mRNA were detected only in a few isolated cells of the granular layer. There was no difference between the amounts of E6, E7, E1, and E2 mRNAs present in highly dysplastic tissue and those present in adjacent normal papillomatous epithelium within a progressing papilloma. However, late transcripts and viral DNA detectable only in the upper layers of the papilloma were present throughout the thickness of the dysplastic tissue, indicating a newly acquired permissiveness of the dysplastic cells for viral DNA replication and late transcription. Carcinomas in general had the same expression patterns for E6, E7, and E1 but were dissimilar in the levels of expression of E2 and late transcripts.

Papillomaviruses have attracted special attention because substantial experimental evidence suggests that certain types, called high-cancer-risk papillomaviruses, play a role in the pathogenesis of human cancer (34). Cottontail rabbit papillomavirus (CRPV), which was the first papillomavirus identified (25), induces strictly epithelial tumors in both cottontail and domestic rabbits. The tumors are benign at first, but 8 to 14 months later carcinomas develop at the same site in 25% of the permissive hosts (the cottontail rabbits) and in 66 to 80% of the nonpermissive hosts (the domestic rabbits) (17, 30). In some animals papillomas regress spontaneously or persist as benign lesions for the entire life of the animal (30). These properties make CRPV an important model for high-cancer-risk human papillomaviruses (HPVs) in order to study the role of viral gene expression during the early steps of cancer development.

The viral transcripts in tumors of cottontail and domestic rabbits have been characterized by Northern (RNA) blotting, and three early promoters and one late promoter were identified by S1 nuclease and primer extension analyses (21, 23, 24, 32). Transcripts initiated at the three early promoters (P1 to P3) (Fig. 1) are present in both papillomas and carcinomas of domestic and cottontail rabbits as well as in the VX2 transplantable cancer line (6, 10, 22). The two major early transcripts encode a short E6 protein, initiated at the second ATG of E6, and the E7 protein. Minor transcripts encode the

full-length E6 protein, and two are polycistronic transcripts serving as mRNAs for short E6 and E2 or for E7 and E2 (1). Three of the early proteins, long E6, short E6, and E7, were shown to be transforming when tested in the rabbit epithelial cell line sf1Ep, and the simultaneous expression of all three proteins was required in order to induce papillomas (18). CRPV E5 did not transform sf1Ep cells, and its expression was dispensable for the induction of papillomas in rabbits (4, 18). E2 is a *trans*-activating protein with properties similar to those of other papillomaviruses (12).

A unique feature of virus-producing cottontail rabbit papillomas was the presence of two major transcripts of 2.6 and 4.8 kb, encoding L1 and L2, respectively, which are transcribed from the same promoter (Fig. 1, PL) located in the noncoding region, and of a third minor transcript of 1 kb which maps to the E4 region (21, 24) and could represent an E4 mRNA.

Earlier analyses of total mRNA from tumors (6, 10, 21, 22, 24, 31) did not provide an explanation for the long-established fact that papillomas of domestic rabbits progress to carcinoma more frequently than those of cottontail rabbits (17, 30), nor do they explain the recent finding of a selective increase in the antibody response to viral structural proteins with tumor progression (17). The results of the in situ hybridization presented here provide a potential explanation for both phenomena.

MATERIALS AND METHODS

Specimens. Cottontail rabbits were inoculated with CRPV by intradermal injection, and New Zealand White rabbits were inoculated by light scarification or, in one case (D. PAP-1), by

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FIG. 1. Genetic and transcriptional maps of CRPV and localization of mRNA-specific probes 1 to 9. The ORFs (open boxes) and the nucleotide positions of the probe boundaries (top) correspond to the published sequence (11). Vertical lines within the ORFs represent the translational start codons. The general transcription map with the major transcripts (a to e) is depicted in the lower part (31). Dotted lines represent alternative splicing patterns, and dashed lines represent unknown 5' ends. The early promoters are P1, P2, and P3, and the late promoter is PL.

infection with a pedo jet injector as previously described (3, 27). One DNA-induced papilloma was obtained by intradermal inoculation with recombinant DNA (pLAI-CRPV Δ XbaI) as described previously (20). All investigated carcinomas developed after 8 to 14 months without further treatment. Biopsy specimens were snap frozen after surgical excision and stored at -70°C until required.

Construction of probes for in situ hybridization analysis. To construct expression vectors for the synthesis of mRNA-specific riboprobes, small subgenomic fragments of CRPV were created by restriction enzyme digestions, purified by agarose gel electrophoresis, and cloned, together with one larger fragment (nucleotides [nt] 4571 to 6991), into the vector Bluescribe M13+ (Vector Cloning Systems, San Diego, Calif.). The map positions (nucleotide numbers are according to reference 11) of the 11 small subgenomic CRPV DNA fragments are illustrated in Fig. 1. The identity of each clone and the orientation of the insert were confirmed by restriction enzyme digestions and additionally for probe 7 by sequence analysis. All plasmids were linearized prior to transcription. Depending on the choice of the promoter, the in vitro-synthesized RNAs were either in the same polarity as mRNA (sense orientation) or in the opposite polarity (antisense orientation). In vitro RNA synthesis in the presence of ^{35}S -UTP, resulting in probes with a specific activity of 3.5×10^8 cpm/ μg , was followed by alkaline hydrolysis to reduce the probe length to approximately 200 nt (5). The quality of the transcribed RNAs was tested by polyacrylamide gel electrophoresis. The shortest probe (probe 2a, with a complexity of 123 nt) was applied at a concentration of 3×10^7 cpm/ml of

hybridization mixture, and the concentrations of the others were normalized in relation to the probe lengths.

In situ hybridization. For RNA in situ hybridization, serial cryostat sections of biopsy material mounted on aminopropylsilyl-coated slides were fixed in 4% paraformaldehyde in phosphate-buffered saline and then dehydrated through graded ethanols. The dry sections then were acetylated in 0.1 M triethanolamine-0.25% acetic anhydride for 10 min, washed in $0.2\times$ SSC ($1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and preincubated for 2 h at 42°C covered by solution I, which contained 45% formamide, 0.6 M NaCl, $2.5\times$ Denhardt's solution, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1% sodium dodecyl sulfate, and 0.15 mg of tRNA per ml. After removal of the prehybridization solution, the tissue sections were incubated for 16 h at 42°C with ^{35}S -labelled antisense riboprobes in solution I containing 10% dextran sulfate. The incubated slides were washed in 50% formamide- $1\times$ SSC at 55°C , treated with RNase A (10 $\mu\text{g}/\text{ml}$), washed twice for 2 h each in $0.1\times$ SSC at 60°C , dehydrated, and finally coated with Kodak NTB-2 emulsion. The conditions for the DNA-hybridization with a sense strand riboprobe for CRPV (nt 4571 to 6991) have been described previously (33). All slides from one experiment were developed after the same exposure time (10 to 12 days) to allow the direct comparison of signals obtained with different probes. Hematoxylin-and-eosine (H&E)-counterstained sections were evaluated and photographed with a Zeiss Axiophot microscope with a dark- or bright-field condenser. The level of background signals for RNA hybridization was determined by using sense riboprobes

FIG. 2. The upper part shows the genetic map and the localization of mRNA-specific riboprobes 1 to 9 used in this study. The lower part gives the intensities of the in situ hybridization signals detected with the CRPV-specific riboprobes for each individual case. +, weak; ++, moderate; +++, strong; +++++, very strong; nd, not determined; up, upper layers of the epithelium; lo, lower layers of the epithelium; C., cottontail rabbit; D., domestic rabbit; PAP, papilloma; DYSPL, dysplastic epithelium; CA, carcinoma; MET, metastasis. Signals just above the level of background hybridization are indicated by +/- . a, signals only in a few single cells; b, signals only in some areas of the tissue section; c, well-differentiated carcinoma; d, moderately differentiated carcinoma; e, poorly differentiated carcinoma.

and by pretreatment of the tissue with DNase-free RNase before hybridization.

RESULTS

The following specimens were chosen for in situ hybridization analysis: two papillomas from cottontail rabbits (Fig. 2, C. PAP-1 and C. PAP-2), one early papilloma of a domestic rabbit (Fig. 2, D. PAP-1) excised 3 weeks after infection with virus, and three other papillomas of domestic rabbits (Fig. 2, D. PAP-2, -3, and -4) taken at later times. One of the papillomas (Fig. 2, D. PAP-4) contained a small region of highly dysplastic epithelium (Fig. 2, D. DYSPL) exhibiting a loss of the regular arrangement of the epithelial layers and microinvasion into the connective tissue. Another papilloma (Fig. 2, D. PAP-X) was induced by infection with a mutant DNA of CRPV with a large deletion within the L2 ORF (pLAI-CRPV Δ XbaI). Two of five malignant tumors could be classified histologically as well-differentiated keratinizing squamous cell carcinomas containing nests of neoplastic cells with smooth borders (Fig. 2, D. CA-3 and D. CA-5), one of the carcinomas was moderately differentiated (Fig. 2, D. CA-4), and two other carcinomas consisted mainly of poorly differentiated cells (Fig. 2, D. CA-1

and D. CA-2). We further included in our analysis two lung metastases (Fig. 2, D. MET-1/2) and the VX2 transplantable cancer line (6, 15). Biopsy specimens D. PAP-3/4 and D. CA-1 as well as D. PAP-2 and D. MET-1 were from the same animals.

To assess the presence of different mRNA species in tumor tissues, appropriate CRPV fragments were cloned into the Bluescribe M13+ RNA expression vector. The orientations of the inserts were determined, and ³⁵S-labelled antisense and negative control sense riboprobes were synthesized in vitro. The hybridization specificities of the different probes for the mapped RNA species are as follows. Probe 1 is specific for the long E6 and short E6 mRNAs starting at promoters P1 and P2 (Fig. 1), respectively. Probe 2 additionally recognizes the E7 mRNA initiated at promoter P3 as well as the mRNAs for L1 and L2 (Fig. 1). Since late transcripts are not detectable on Northern blots of domestic rabbit tumors (21, 24), a higher level of hybridization with probe 2 than with probe 1 in domestic rabbit tumors would indicate the presence of E7 mRNA. Probes 3 and 4 identify potential transcripts covering the open reading frame (ORF) E1 but do not hybridize to mRNAs that were processed by cleavage at the splice donor site 1371 (6). The lack of an E1 mRNA in the transcription

map of CRPV in Fig. 1 is due to the fact that the original studies (31) could not distinguish whether the weak hybridization of probes colinear to ORF E1 with polyadenylated RNA isolated from rabbit tumors represented nuclear precursors of the major transcripts or specific E1 mRNA (31a). Probe 5 hybridizes to mRNA species with the coding capacity for a full-length E2 protein. Since E1 mRNA has not been mapped and the structure of an E4 mRNA is still unclear, it is possible that probe 5 might also hybridize to E1 mRNA and E4 mRNA in addition to a full-length E2 mRNA. Probe 6 hybridizes to the major early transcripts and to the minor E2 mRNA as well as to the L2 mRNA and a potential E4 mRNA. Evidence for a putative E4 mRNA (21), however, could be obtained only if that mRNA was present in epithelial layers that contain small amounts of major early transcripts and L2 mRNA. To evaluate further the existence of an E1⁺E4 RNA and potentially provide information about the location of its cap site, two small probes, 2a and 2b, were prepared (Fig. 1). Stronger signals with probe 2b than with probe 2a could indicate a start site within E7, in analogy to the E1⁺E4 RNA of HPV6/11 and HPV16 (2, 7, 13, 19, 26). Probe 7 is unique for the L2 mRNA, and probes 8 and 9 hybridize to both major late transcripts.

In all specimens investigated we observed high levels of E6 and E7 mRNAs and low levels of E1 and E2 mRNAs. In none of the tumors was there evidence for a major mRNA class homologous to the prominent E1⁺E4 RNA of HPV6/11 and HPV16 (2, 7, 13, 19, 26). No difference was found between the intensities of the hybridization signals of probes 2a and 2b (Fig. 1), which were designed to detect prominent transcripts initiating within ORF E7, or between those of a probe located within ORF E7 and one located within ORF E4. This clearly indicated the absence of a prominent E1⁺E4 mRNA.

With papillomas of two cottontail rabbits (Fig. 2, C. PAP-1 and -2), we observed for all riboprobes an increase in the intensity of the hybridization signal in the more differentiated layers of the epithelium. The high content of pigmented granules, which were present only in cottontail rabbit warts, resulted in artificial signals in dark-field observation (Fig. 3H). These granules, however, could be clearly distinguished from specific silver grains under higher magnification with bright-field optics (Fig. 3H, inset). Hybridization with probe 1 (specific for E6 mRNAs) and with probe 5 (identifying mRNAs with a coding capacity for a full-length E2 protein) showed in addition weak signals in the lower epithelium (Fig. 3B and E, respectively). The comparison of the hybridization signals of probes 1, 2, and 8 revealed a fair amount of E7-specific transcripts in the less-differentiated keratinocytes and basal cells (Fig. 3C). Strong signals were observed after hybridization with late-mRNA-specific probes 8 (Fig. 3G) and 9 exclusively in the granular cell layer, whereas weak L2-specific signals (probe 7; data not shown) could be detected in only one papilloma (Fig. 2, C. PAP-2).

An early papilloma of a domestic rabbit (Fig. 2, D. PAP-1), which developed 3 weeks after infection with virus particles, showed a histology reminiscent of that of an inverted condyloma; it projected into the corium and grew laterally to come to underlie the normal epithelium. The granular layer was thickened, and beginning keratinization could be observed. Hybridization with riboprobes 1 and 2 revealed high levels of transcripts encoding E6 and E7 (data not shown); however, the differentiation-dependent expression pattern was opposite to that in cottontail rabbit papillomas (Fig. 3B and C). This was even more pronounced in an older papilloma with deeper papillae (Fig. 2, D. PAP-2), in which E6 and E7 mRNA levels were rather high within the lowest layers of the epithelium and gradually declined along with the differentiation of the kerati-

nocytes (Fig. 4). In all papillomas of the domestic rabbits, a weak signal was observed throughout the epithelium with riboprobes 3 (Fig. 5D) and 4 (data not shown), which cover the E1 region, and a moderate signal was observed with an E2-specific probe (Fig. 5E). In addition, the E1 and E2 probes showed, in some areas, clustered, mostly nuclear signals in the upper third. None of the domestic rabbit papillomas revealed transcripts encoding the minor capsid protein L2. Signals specific for L1 were detected only in a few isolated spots of more highly differentiated cells in the granular layer (Fig. 5G, inset), with the exception of one papilloma (Fig. 2, D. PAP-X), which was induced by infection with a mutant DNA of CRPV with a large deletion within the L2 ORF (pLAII-CRPV Δ XbaI). In this mutant DNA, however, the L1 ORF is separated from the early transcription unit by the insertion of pLAII vector DNA sequences, which may be responsible for the unusual expression of the L1 gene. The transcription patterns of all other mRNAs and the replication of the viral DNA for this wart and for virus-induced papillomas showed no further difference. Overall, the patterns of viral gene expression related to epithelial differentiation were clearly different for papillomas of cottontail and domestic rabbits.

One papilloma of a domestic rabbit (Fig. 2, D. PAP-4) contained a small region of highly dysplastic epithelium (Fig. 2, D. DYSPL; Fig. 6A) exhibiting a complete lack of epithelial differentiation, the loss of a sharp border against the connective tissue (Fig. 6B), and microinvasion into the connective tissue of single cells that were positive by *in situ* hybridization (Fig. 6C). Interestingly, the *in situ* hybridization with all early riboprobes showed no difference in the amounts of E6, E7, E1, and E2 mRNAs for the highly dysplastic and adjacent papillomatous tissue (Fig. 5B, C, D, E, and F). However, a clear difference could be observed in the expression patterns for late transcripts and in the permissiveness of the cells for the replication of the viral DNA (Fig. 5H). Whereas signals specific for L1 were detectable only in a few isolated spots of more highly differentiated cells in the papilloma (Fig. 5G, inset), a weak signal was observed after hybridization with probe 8 (Fig. 5G) and probes 7 and 9 throughout the whole dysplastic epithelium. The same change was true for the distribution of viral DNA signals, which were strongest in the granular layer of the papilloma but were present in all cells of dysplastic origin (Fig. 5H).

The investigation of five invasive carcinomas, two metastases, and the transplantable VX2 cancer line revealed in general the same expression pattern for E6, E7, and E1 but dissimilarities in the expression levels of E2 and late transcripts. In two well-differentiated carcinomas (D. CA-3 and -5), two lung metastases (D. MET-1 and -2), and the VX2 tumor, we observed low levels of E2 transcripts (D. CA-3 is shown in Fig. 7D), whereas two poorly differentiated carcinomas (D. CA-1 and -2; Fig. 8E) and one moderately differentiated carcinoma (Fig. 2, D. CA-4) revealed higher levels of transcripts with a coding capacity for a full-length E2 protein. One of the well-differentiated carcinomas (D. CA-3) exhibited moderate levels of L1 (Fig. 7E) and low levels of L2 (Fig. 7F), whereas most of the other tumors contained barely detectable late transcripts. The predominant transcripts in the VX2 line and the lung metastases encoded the E6 and E7 ORFs; all other early genes were expressed only at a low level. Hybridization with probes 7 to 9, which are specific for late transcripts, resulted in no signal above the background level. In the VX2 cancer line, viral DNA was present at a low copy number, in contrast to the case for most other tumors investigated (Fig. 2).

FIG. 3. In situ hybridization of adjacent tissue sections of a cottontail rabbit papilloma (Fig. 2, C, PAP-2). (A) Histopathology observed by bright-field microscopy after staining with H&E stain. Individual adjacent tissue sections after hybridization with riboprobes 1 (B), 2 (C), 3 (D), 5 (E), 6 (F), and 8 (G) and with probe 7 (H) in the sense orientation as a negative control are shown as observed by dark-field microscopy. The silver grains generated in the film emulsion after exposure to the ^{35}S -labelled probes are seen as white grains under dark-field illumination. The high content of pigmented granules in the lower part of the epithelium of cottontail rabbit warts (see bright-field inset in panel H) can be mistaken for a hybridization signal when evaluated by dark-field observation. Panel H demonstrates the level of artificial signal caused by those pigmented granules (irregularly shaped large grains) together with the level of background signal after hybridization with a sense probe (small grains) as seen in dark-field observation.

FIG. 4. In situ hybridization of adjacent tissue sections of a domestic rabbit papilloma (Fig. 2, D, PAP-2) after hybridization with riboprobes 1 (A) and 2 (B).

DISCUSSION

Gross analysis of transcripts encoding early genes by Northern blot, S1 nuclease, and primer extension analyses revealed no major quantitative or qualitative differences between papillomas of cottontail and domestic rabbits or between benign tumors and carcinomas (21, 24, 31). To gain more insight into the roles of individual genes in the viral life cycle and in the progression of virus-induced lesions, it was necessary to study their expression in the immediate context of tissue morphology with the help of in situ hybridization.

There was a major difference in the expression patterns of the E6 and E7 genes between papillomas of cottontail and domestic rabbits. Warts of the cottontail rabbit had the highest levels of E6 and E7 mRNAs in the upper third of the epithelium, and this was also true for all other viral transcripts. This transcription pattern has some similarities to that observed with HPV16-infected low-grade intraepithelial lesions (2, 9, 13, 28), which were shown to contain the majority of the viral transcripts in the more highly differentiated epithelial layers. In contrast, in papillomas of domestic rabbits, expression of E6 and E7 was restricted to the lower third of the epithelium, an expression pattern more reminiscent of HPV6-induced condylomata acuminata (14). An additional difference in the expression of the E6 and E7 genes was the relative amounts of E6 and E7 transcripts in cottontail versus domestic rabbits. In cottontail rabbit papillomas, E6 expression in the less-differentiated epithelial layers was lower than E7 expression, while they were about equal in domestic rabbit papillomas. The high level of expression of both viral oncogenes in the undifferentiated epithelium of domestic rabbit papillomas may provide an explanation for the high frequency of tumor progression. It has been shown for HPV16 that increased levels

of E6 and E7 in undifferentiated cells may be an essential step to evoke full transformation of human keratinocytes (8).

E1 and E2 mRNAs were in general much less abundant than mRNAs encoding E6 and E7. In productive cottontail rabbit papillomas and in nonproductive domestic rabbit papillomas, E1-specific transcripts were observed in the same epithelial layers where viral DNA also accumulated, and this would be in line with the function described for the E1 protein of papillomaviruses (reviewed in reference 16). In addition to detecting cytoplasmic signals with E1 and E2 probes, we observed as an unusual phenomenon the presence of strong nuclear signals. Similar observations have been made previously for HPV-infected condylomas, for intraepithelial lesions, and for carcinomas (14, 28, 29). The nuclear signals may represent introns of the unspliced major transcripts as suggested previously (13, 28), or they could also indicate an accumulation of E1- and E2-specific mRNAs in the nucleus.

In contrast to the results obtained with HPVs, in situ hybridization with a probe containing sequences of ORF E4 (Fig. 1, probe 6) did not result in a signal higher than that expected for the hybridization to the major early transcripts. This result, of course, does not exclude the presence of an E4 mRNA as a minor species, as suggested by the Northern blot analysis of cottontail rabbit papilloma RNA (21, 24).

There was no fundamental difference in the expression of early gene transcripts between papillomas and carcinomas of domestic rabbits. High levels of E6 and E7 mRNAs were present in the whole spectrum of lesions, from a 3-week-old papilloma to the VX2 cancer line, which has been continuously maintained in rabbits for more than 50 years (15). In contrast, there was a striking difference in permissiveness for late gene expression and viral DNA replication between domestic pap-

FIG. 5. In situ hybridization of adjacent tissue sections of a progressing domestic rabbit papilloma (Fig. 2, D. PAP-4) containing a highly dysplastic region (Fig. 2, D. DYSPL). Histopathology is shown by light microscopy after staining with H&E stain (A). An enlargement of papillomateous epithelium is given as an inset in the upper right of each photography, displaying individual adjacent tissue sections after hybridization with riboprobes 1 (B), 2 (C), 3 (D), 5 (E), 6 (F), and 8 (G) and with a sense probe (nt 4571 to 6991) for DNA detection (H) in dark-field illumination.

FIG. 6. (A) Histopathology of a progressing domestic rabbit papilloma (Fig. 2, D. PAP-4). (B) Higher magnification of the papillomatous epithelium (left) and the highly dysplastic area (right). (C) In situ hybridization of epithelial cells invading into the connective tissue after hybridizing with probe 1, shown in dark-field illumination. Arrows denote the epithelial basement membrane.

FIG. 7. In situ hybridization of a well-differentiated domestic rabbit carcinoma (Fig. 2, D, CA-3). Individual adjacent tissue sections after hybridization with riboprobes 1 (A), 2 (B), 3 (C), 5 (D), 8 (E), and 7 (F) are shown in dark-field illumination. Histopathology is shown by light microscopy after staining with H&E stain (G).

FIG. 8. In situ hybridization of a poorly differentiated domestic rabbit carcinoma (Fig. 2, D, CA-1). Histopathology is shown by light microscopy after staining with H&E stain (A). Individual adjacent tissue sections after hybridization with riboprobes 1 (B), 2 (C), 3 (D), and 5 (E) and with a sense-oriented probe for DNA detection (F) are shown in dark-field illumination.

illoma cells and dysplastic cells. In papillomas, transcription of structural genes was confined to the most-differentiated cells, while carcinomas, containing mainly less-differentiated cells, were permissive for late transcription. The detection of signals specific for the late exon (probe 9) clearly indicated that the late promoter was active in some carcinomas. The level of late transcription in carcinomas was quite variable and is probably related to the highly variable viral DNA content of domestic rabbit tumors, which varies from 10 to a few hundred copies per diploid-cell DNA equivalent (27). Since a large majority of carcinoma-bearing rabbits (90%) have a strong antibody response to L1 (17), the detection of L1 transcripts in less than half of the carcinomas analyzed may reflect a sensitivity limit for in situ hybridization. A likely reason for the increased

antibody response to L1 in carcinoma- compared with papilloma-bearing rabbits is the change in the location of the L1 transcript. In papillomas, L1 is expressed only in the uppermost layers of the epithelium, which does not allow efficient recognition by the immune system, while in carcinomas, expression is observed throughout the thickness of the whole tumor, which is a much more favorable situation for immune stimulation.

Overall, the results of the in situ hybridization experiments revealed a basic difference in the differentiation-specific expression of the viral transforming genes and have provided a possible explanation for the increased frequency of tumor progression in domestic compared with cottontail rabbits. Furthermore, the loss of differentiation-dependent expression

of structural genes with tumor progression can explain the selectively increased immune response to structural proteins in carcinoma-bearing rabbits.

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