Papillomas and Carcinomas in Transgenic Rabbits Carrying EJ-*ras* DNA and Cottontail Rabbit Papillomavirus DNA

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Two transgenic rabbits (TRI and TRIII) that carried cottontail rabbit papillomavirus (CRPV) DNA alone were identified; another (TRII) carried both CRPV DNA and EJ-*ras*. TRI and TRIII developed extensive skin papillomas at about 1 month of age, and transcripts of CRPV DNA were detectable only in skin and/or papillomas. TRII developed extensive squamous carcinomas of the skin at a very early age. Transcription of both CRPV DNA and EJ-*ras* was found in the skin cancers. Thus, the tissue specificity of CRPV DNA expression in transgenic rabbits was the same as in virion-infected animals. The expression of EJ-*ras* could be dependent on the expression of certain CRPV genes and may be a critical cofactor of CRPV DNA in the progression of carcinomas.

Papillomaviruses are a group of small DNA viruses which have long been known to be associated with benign and malignant lesions of the skin and other epithelia in some animal species and humans (5, 12). More than 65 types of human papillomaviruses (HPVs) have been identified, most of which are associated with benign lesions. However, several types are usually found in lesions which have a high frequency of carcinomatous progression (7). The DNAs of HPV type 16 (HPV-16), HPV-18, and HPV-33 are detectable in most cervical carcinomas (26), and the E6 and E7 early genes are selectively retained and transcribed in cervical cancer cells (21). These results strongly support the hypothesis that certain types of HPV play an essential role in the development of cervical cancers. However, studies suggest that oncogenic HPV infection alone is not sufficient to cause malignant changes in cervical epithelium (14, 18) because these viral DNAs are also found in mild cervical intraepithelial neoplasia (22) and some activated oncogenes, such as *c-Ha-ras* and *c-myc*, are detectable in papillomavirus DNA-involved cervical cancers (20). Transfection studies in vitro have shown that HPV-16 alone initiates nontumorigenic immortalization of normal keratinocytes (4, 19, 24, 25), while subsequent transfection of the immortalized cells with EJ-*ras* causes tumorigenic transformation (3). Therefore, it is possible that malignant neoplasia involving oncogenic HPV DNAs is the result of a multistep process in which certain HPV genomes are essential but not sufficient for progression to malignancy. However, the complex mechanisms which operate are still not understood.

Studies of the mechanism by which papillomaviruses and cofactors interact have been handicapped by the lack of an animal model system which allows high yields of malignant transformation in relatively brief periods of time. Transgenic animals offer a potential solution to this problem. Some attempts have been made to produce mice transgenic for bovine papillomavirus (11) or HPV-16 (9), but the lesions that developed were stromal in origin and not squamous cell carcinomas, similar to those of the uterine cervix. In the

present report, we describe preliminary studies with transgenic rabbits carrying cottontail rabbit papillomavirus (CRPV) DNA, which develop lesions remarkably similar in etiology and mechanism to some naturally occurring lesions associated with the oncogenic HPVs (10). Animals were produced in which the entire integument developed either benign papillomas or squamous cell carcinomas which expressed CRPV DNA.

Transgenic rabbits were produced by the procedure described previously (1, 8) with some modifications. Male and female rabbits (New Zealand White) were purchased from Hazelton Research Products, Inc., Denver, Pa. The donor and recipient female rabbits were mated at the same time with fertile or sterile males, respectively. Eggs were flushed out of the oviducts of donor rabbits 19.5 to 20 h after mating. The flushing, washing, and microinjection of eggs were carried out in medium consisting of phosphate-buffered saline (PBS) supplemented with 20% fetal bovine serum at a pH of 7.3 to 7.4. The DNA solution containing 1 μ g of CRPV DNA linearized by *SalI* and 1 μ g of EJ-*ras* linearized by

FIG. 1. Transgenic rabbit TRI (left) and its normal littermate (right) at the age of 2.5 months. The dark foci on TRI are cutaneous papillomas which rise above the fur; the entire integument was covered with papillomas.

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FIG. 2. Transgenic rabbit TRII (bottom) and its normal littermate (top) at the age of 3 days. Note the folding and thickening of the skin of TRII, which was very hard to the touch.

*Bam*HI per ml was microinjected into the pronucleus of fertilized eggs at the single-cell stage by the standard protocol for mice (6). The microinjected eggs, at the two-cell stage, were then transferred via an abdominal incision to the oviducts of the recipient rabbits. Three of 39 rabbits obtained from 248 injected eggs were identified as CRPV transgenic by Southern blot analysis under stringent conditions by the protocol in the Zeta-Probe Blotting Membrane System (Bio-Rad, Richmond, Calif.). Two of them (TRI and TRIII) carried only CRPV DNA; the other one (TRII) carried both CRPV DNA and *EJ-ras*. TRI and TRIII were phenotypically normal for the first 1 to 2 weeks after birth. They were then smaller than their littermates and had hyperkeratotic skin. Small papillomas appeared on the body

skin at about 20 to 30 days of age. Papillomas later developed spontaneously over almost the entire skin (Fig. 1). These two rabbits died from pneumonia and septicemia at 40 and 75 days of age, respectively. Complete necropsy and histologic examination did not show tumors or neoplastic changes in any other organs except the skin in either rabbit. Sections of skin tumors stained with hematoxylin and eosin showed obvious epidermal papillomatosis and koilocytosis. However, epidermal differentiation and maturation were normal, and there was no malignant change.

Rabbit TRII (Fig. 2) was found to carry both CRPV DNA and *EJ-ras* by Southern blot and to have extensive thickening of the entire skin on the first day after birth. The animal died at 3 days of age. Sections of skin stained with hematoxylin and eosin showed epidermal papillomatosis and highly malignant progression, with many regions of invasion of papillary dermis (Fig. 3). The entire skin was an extended squamous carcinoma. No neoplastic changes were found in other tissues.

Cellular DNA isolated from a variety of nonneoplastic tissues and tumors was digested with appropriate restriction enzymes and analyzed by Southern blot analysis. CRPV DNA was found in tumors and in nonneoplastic tissues, including brain, heart, intestine, stomach, kidney, liver, and lung, from all three transgenic rabbits. DNA from the lungs was representative of DNA from other normal tissues (Fig. 4). DNA samples from tumors always showed much higher copy numbers of CRPV DNA than samples from nonneoplastic tissues. There was no obvious difference in CRPV DNA copy numbers among nonneoplastic tissues. These results indicate that CRPV DNA was amplified only in tumors. The papillomas of rabbits TRI and TRIII contained higher copy numbers of CRPV DNA than the carcinomas of rabbit TRII, but *EJ-ras* was only detectable in TRII. There was no difference in *EJ-ras* copy numbers between tumors and nonneoplastic tissues. Figure 4 also shows the results when tumor DNA and lung DNA from all three rabbits were

FIG. 3. Microscopic section of the skin of transgenic rabbit TRII. Nests of epidermal carcinoma composed of sheets of polygonal cells are trapped in the dermal fibroblasts. Magnification, $\times 170$.

FIG. 4. Southern blot of DNAs isolated from tumors (lanes 1, 3, 5, 7, 9, and 11) and lungs (lanes 2, 4, 6, 8, 10, and 12) of transgenic rabbits TRI (lanes 1 to 4), TRIII (lanes 5 to 8), and TRII (lanes 9 to 12). Lanes 13 to 14 are *SalI*-linearized CRPV DNA (50 copies each lane) reconstructed with normal rabbit cellular DNA as controls. The DNAs (10 μ g in each track) were digested with the restriction enzyme *XhoI* (lanes 1, 2, 5, 6, 9, 10, and 13) or *BamHI* (lanes 3, 4, 7, 8, 11, 12, and 14). All digestions were done with 10 U of enzyme per μ g of DNA at 37°C for 3 h. Hybridization was done with a 32 P-labeled full-length CRPV DNA probe. Sizes are shown in kilobases.

digested with the restriction enzymes *XhoI* and *BamHI*, respectively, and hybridized with the CRPV DNA probe. *XhoI* does not cleave CRPV DNA; *BamHI* cleaves CRPV DNA at two sites. This Southern analysis demonstrates that all these rabbits contain integrated CRPV DNA with head-to-tail tandem repeats. In addition, tumor samples from all the rabbits show additional extrachromosomal CRPV DNA, which might represent the amplified CRPV DNA. The

Southern analysis also indicates that the sites of integration of CRPV DNA were different among the three rabbits because of the different patterns of such integration bands after digestion with *BamHI*. These results suggest that the copy numbers and integration sites might not be directly related to the cancer development.

For detecting the transcription of CRPV and EJ-*ras* DNA, cellular RNA was isolated by a single-step protocol (2) and analyzed by Northern (RNA) blot with the Zeta-Probe Blotting Membrane System (Bio-Rad). CRPV mRNA was detected only in ear skin and/or tumors from all three rabbits (Fig. 5A, B, and C). The two major transcripts of 1.3 and 2.0 kb are the same as in the controls from CRPV-induced rabbit papilloma and carcinoma (controls not shown) and previous reports (15, 16). No transcripts were detectable in any other nonneoplastic tissues. One 1.2-kb transcript of EJ-*ras* was demonstrated only in the skin carcinoma from rabbit TRII and not in nonneoplastic tissues (Fig. 5D).

The tissue-specific expression of CRPV genes may be related to less methylation of CRPV DNA in skin tumors than in other normal tissues (data not shown). However, the specific expression of EJ-*ras* may be initiated or transactivated by the expression of some other gene(s), such as those of the CRPV genome. Cell transfection studies demonstrate that *ras* oncogenes alone could not immortalize primary cells (13, 17), but the *ras* oncogene linked to more than one transcriptional enhancer could immortalize normal rat and hamster fibroblasts (23). However, our study suggests a more direct interaction between CRPV DNA and EJ-*ras* in carcinomatous progression. The activation and expression of *ras* genes may be initiated by the presence and expression of oncogenic papillomavirus genes in the carcinomas.

Thus, this study demonstrates that transgenic rabbits provide a new model for the investigation of tissue-specific expression of CRPV DNA, the mechanism of CRPV onco-

FIG. 5. Northern blots of total cellular RNAs (15 μ g in each track) isolated from nonneoplastic tissues and tumors of transgenic rabbits TRI (A), TRIII (B), and TRII (C and D). Blots were hybridized with a 32 P-labeled full-length CRPV DNA probe (A, B, and C) or a 32 P-labeled full-length EJ-*ras* probe (D). Skin 1 and skin 2 in blot A indicate RNAs isolated from ear skin at age 28 days and 51 days, respectively. The RNA in skin 1 seems to be degraded. Sizes are shown in kilobases.

genesis, and the interaction between CRPV and oncogenes in neoplastic progression. This transgenic model should provide an opportunity to explore papillomavirus tumor initiation and progression.

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