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# Two Shope Papillomavirus-Associated VX2 Carcinoma Cell Lines with Different Levels of Keratinocyte Differentiation and Transplantability

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**Two cell lines, named VX2T and VX2R, were isolated from the transplantable VX2 carcinoma, a wholly anaplastic tumor established from a carcinoma induced by the Shope cottontail rabbit papillomavirus (CRPV) (J. G. Kidd and P. Rous, J. Exp. Med. 71:813–838, 1940). The CRPV genome was found to be maintained and transcribed in both cell lines, as in the VX2 carcinoma. The VX2T cells retained the tumor-producing capacity in the rabbit and the low expression of epidermal keratinocyte differentiation of the VX2 tumor cells. The VX2R cells, although tumorigenic for nude mice, were no longer serially transplantable in the rabbit and expressed differentiated functions of keratinocytes. These data indicate that the anaplastic characteristic and the transplantability of VX2 carcinoma cells to immune competent allogeneic hosts may be lost without any detectable modification of the physical state and transcription of the CRPV genome.**

Papillomaviruses induce benign and malignant tumors of keratinocytes in animals and in human beings (11, 21). The transformation of keratinocytes by papillomaviruses has not yet been obtained in vitro (31). Cultures of papillomavirus-associated tumor cells should provide models to define the phenotypes of papillomavirus-transformed keratinocytes and to analyze the expression of the viral genome and its regulation in tumor cells. The VX2 carcinoma of the domestic rabbit represents a suitable system for such studies. This serially transplantable tumor, established in 1940 by Kidd and Rous (9), was isolated from a carcinoma derived from a domestic rabbit skin wart induced by the Shope cottontail rabbit papillomavirus (CRPV) (26, 30). It can be considered to express to the utmost the properties of CRPV-associated carcinomas (28). The VX2 tumor is a wholly anaplastic carcinoma, that is, a tumor whose keratinocytes never keratinize (9, 27). VX2 cells grow rapidly in allogenic adult recipients, frequently metastasizing in the lungs (9). The tumor cells still contain multiple copies of the CRPV genome integrated into the cellular DNA as head-to-tail tandem repeats (5, 15, 29, 33) and express two major spliced and colinear CRPV-specific transcripts with sizes of 1.25 and 2 kilobases, similar to those found in non-virus-producing domestic rabbit warts (8, 17). These mRNAs correspond to the E6 and E7 genes, which are assumed to be involved in the production of CRPV-induced warts and to play a role in the establishment and the maintenance of the malignant characteristics of the carcinoma cells (3, 8). No evidence for the production of viral particles or viral structural proteins has been found in VX2 cells since tumor transplant 22 (27).

In this paper we report the isolation of two cell lines from the VX2 carcinoma which have been named VX2T and VX2R. The CRPV genome is maintained and transcribed in both lines, as in the VX2 tumor. While the VX2T cells are most probably the in vitro counterpart of the VX2 tumor cells, the VX2R cells have lost the capacity to be serially transplanted in rabbits and have reacquired some of the differentiated features of the epidermal keratinocytes.

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The VX2 tumor was propagated in the thigh muscles of New Zealand rabbits (8). Living tumor tissue was obtained from the epithelial lining of the tumor cyst, minced, and washed thoroughly in Hanks balanced salt solution containing penicillin (100 U/ml), streptomycin (100 μg/ml), erythromycin (10 μg/ml), colimycin (100 U/ml), and amphotericin B (Fungizone, 5 μg/ml). Fragments were incubated with moderate stirring at 37°C for 1 h in a collagenase solution (3.5 mg/ml) in Eagle minimum essential medium containing Earle salts and nonessential amino acids (MEM) and supplemented with 2% fetal calf serum, antibiotics, and amphotericin B. DNase I (Sigma Chemical Co., St. Louis, Mo.), at a final concentration of 20,000 U/ml, was added to the suspension of cell clumps thus obtained (14). After a 5-min incubation at room temperature, the cell aggregates were centrifuged (350 × *g*) and washed in serum-free MEM and then incubated for 20 min at 37°C in a trypsin solution (3 mg/ml) with mild stirring. The cell suspensions were seeded in plastic or collagen-coated dishes (12), in the presence or absence of lethally irradiated 3T3 mouse cells (22, 23), and in a variety of media (MEM, Dulbecco-Vogt modified Eagle medium, Ham F12 medium, and medium 199) or medium combinations, supplemented with 20% fetal calf serum, 10 μg of hydrocortisone per ml, antibiotics, and amphotericin B. In vitro, the VX2 cells assumed the uniform rounded morphology described for cells grown from VX2 tumor explants (19). Serial subcultures were successful only when the cells were plated at high density in collagen-coated dishes seeded with feeder cells with a 1:1 mixture of Ham F12 medium and medium 199 containing 20% fetal calf serum, 10 μg of hydrocortisone per ml, antibiotics, and amphotericin B at the same concentrations as above. The cultures were split in half when they reached confluency, first after 5 weeks and then after 3 weeks, and maintained in 10% fetal calf serum after subculture 2 and then in the absence of feeder cells after subculture 4. After 10 passages, the cultures were routinely split in half every week and changed to MEM containing 10% fetal calf serum and 0.5 μg of hydrocortisone per ml after 25 subcultures. Having reached subculture 67, the cells retained their rounded or

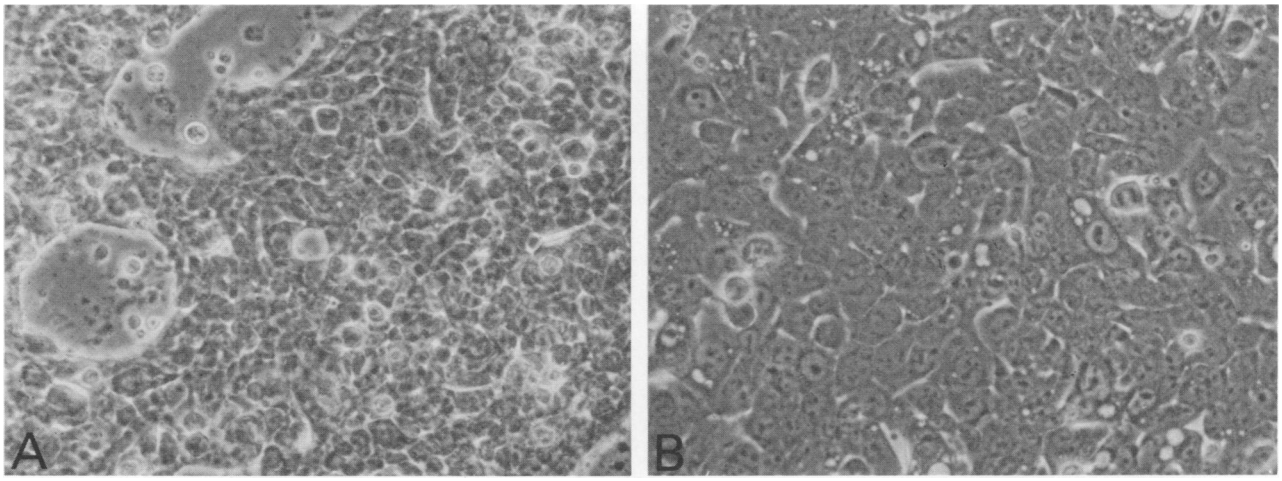


FIG. 1. Morphology of cultured VX2T (A) and VX2R (B) cells. Note the smaller size and the rounded morphology of VX2T cells (subculture 46) and the larger size of VX2R cells and their pavement-like arrangement (subculture 54). Phase-contrast microscopy; magnification, ×200.

cuboidal morphology. They grew as compact sheets and tended to pile up and slough into the medium (Fig. 1A). When about $5 \times 10^7$ cells from subcultures 12 and 38 were inoculated into the thigh muscles of seven New Zealand rabbits, they gave rise to highly necrotizing, metastasizing tumors in all animals, showing the histological features of the anaplastic VX2 carcinoma (9; data not shown). Since these cells retained the tumorigenic properties of VX2 cells, they were designated VX2T cells.

During a culture carried out in collagen-coated dishes in MEM without feeder cells, an island of cells tightly adherent to each other and to the substrate was observed in subculture 2, while the rounded VX2 cells became detached, and the contaminating fibroblasts gradually invaded the dish. The island cells were larger than the VX2T cells and showed a typical epithelial morphology and a growth pattern recalling those of keratinocytes isolated from human squamous cell carcinomas (22). After the area covered by the epithelial cells had reached a reasonable size, the cells were selectively trypsinized (22) and transferred to a collagen-coated dish in the presence of feeder cells in a 1:1 mixture of Hams F12 medium and medium 199 containing 10% fetal calf serum, 10 μg of hydrocortisone per ml, antibiotics, and amphotericin B. The cultures were split in half every 10 days. After 7 subcultures the cells grew in the absence of feeder cells, and after 36 subcultures the medium was changed to MEM supplemented with 0.5 μg of hydrocortisone per ml and 10% fetal calf serum. After subculture 10, the cells could be split in half every 3 days; they were then at subculture 65. They retained their initial morphology and growth pattern (Fig. 1B). They tended to form pluristratified areas in dense cultures. When about $5 \times 10^7$ cells from subcultures 26 and 37 were inoculated into the thigh muscles of New Zealand rabbits, they gave rise 7 to 10 days later to perceptible nodules in only four of the seven grafted rabbits. One rabbit was killed, and the histological examination showed that the nodule consisted of strands of carcinomatous tissue. In the three remaining rabbits, the nodules regressed 15 to 21 days after inoculation. The histological examination of a regressing tumor showed an abundant infiltrate of inflammatory cells (data not shown). Because of the regression of the induced tumors, these cells were designated VX2R cells.

The capacity of VX2R cells to induce tumors in immune deficient hosts was compared with that of VX2T cells. When about $10^7$ cells from subcultures 10 and 56 (VX2R) and 60 (VX2T) were inoculated subcutaneously into 6- to 7-week-old nude mice (Swiss Nu/Nu; Iffa Credo, L'Arbresle France), large cystic tumors developed at the site of injection, reaching a 2- to 3-cm diameter after 2 to 3 weeks. VX2T cell tumors showed the characteristic histological features of the VX2 carcinoma (9), while VX2R cell tumors showed more differentiated features with evidence for abnormal keratinization of many areas (data not shown). VX2R and VX2T cells were also compared for anchorage-independent growth. Cells at various concentrations were seeded in a 0.4% agarose-containing medium onto a 0.6% agarose gel (13). When plated at $5 \times 10^5$ cells per 60-mm dish, VX2R cells formed colonies that grew progressively over a 2- to 3-week period to a maximum size of about 50 to 100 cells, with a colony-forming efficiency ranging from 0.4 to 0.8%. In contrast, VX2T cells were repeatedly found to be unable to grow in the same conditions.

VX2T and VX2R cells were analyzed for the presence of CRPV DNA sequences. Total cellular DNA was extracted and analyzed by the blot hybridization technique with a $^{32}$P-labeled cloned CRPV DNA probe under the conditions previously described (8). The presence of the CRPV genome was demonstrated in both cell lines, and the patterns observed were similar to those previously described for the VX2 tumor (8, 29). In an undigested DNA preparation, the CRPV DNA sequences migrated with the bulk of the cellular DNA (Fig. 2A, lanes 1 and 5). After digestion with *Eco*RI, a single-cut restriction endonuclease for CRPV DNA, the viral sequences migrated as linear CRPV DNA molecules (Fig. 2A, lanes 2 and 6). The intensity of the signal indicated the presence of about 10 copies of CRPV genome per diploid amount of cell DNA. Digestion with *Bam*HI (Fig. 2A, lanes 3 and 7) and *Hin*dII (Fig. 2A, lanes 4 and 8) endonucleases gave rise to two and five fragments, respectively, with the sizes expected for the CRPV DNA cleavage products (5, 8). Fainter bands, corresponding most probably to virus-cell DNA junctions, were also detected, suggesting that the CRPV genomes were integrated as head-to-tail tandem repeats as reported for the VX2 carcinoma (8, 29) and for a rabbit carcinoma cell line (15) probably derived from the

VX2 tumor (33). CRPV-specific transcripts were analyzed by blot hybridization of polyadenylated RNAs purified from the VX2T and VX2R cells as previously described (8). Two major transcripts of 1.25 and 2 kilobases were identified in both cell lines (Fig. 2B, lanes 1 and 2), in the same relative proportions as in vivo (8), or in the above-mentioned rabbit carcinoma cell line (17). Both species, as well as a minor 4.6-kilobase species present in both cell lines (Fig. 2B) and in the VX2 tumor (3), hybridized only with subgenomic probes corresponding to the region of the CRPV genome analogous to the transforming region of the bovine papillomavirus type 1 genome (8a). While these data demonstrate that both the VX2T and VX2R cell lines derive from the VX2 tumor, they do not disclose any detectable difference in the copy number, the physical state, or the transcription of the CRPV genome which could account for the difference in morphology and biological properties of the cell lines.

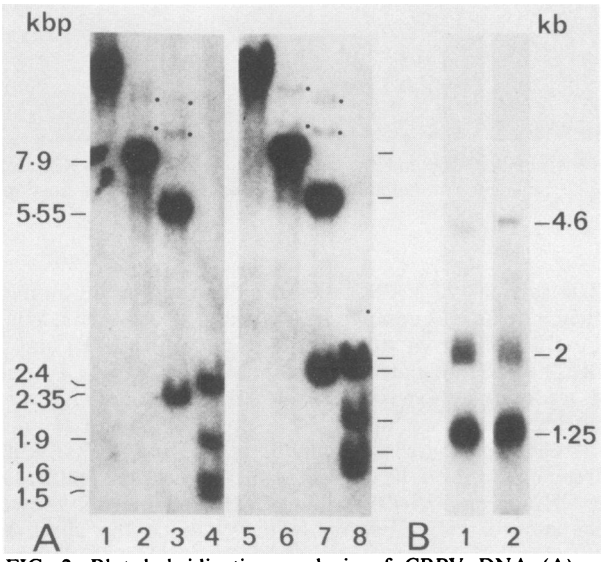


FIG. 2. Blot hybridization analysis of CRPV DNA (A) and CRPV-specific transcripts (B) from VX2T and VX2R cells. All the procedures have been previously described (8). (A) The total cellular DNA was extracted from VX2T cells (subculture 33, lanes 1 through 4) and VX2R cells (subculture 38, lanes 5 through 8). Samples (10 μg) were electrophoresed in 1.2% agarose slab gels without prior treatment (lanes 1 and 5) or after digestion with *Eco*RI (lanes 2 and 6), *Bam*HI (lanes 3 and 7), or *Hin*dII (lanes 4 and 8) restriction endonucleases (Amersham International). The DNA was depurinated, denatured, and transferred to GeneScreen membranes (New England Nuclear Corp.). The membranes were hybridized with $^{32}$P-labeled cloned CRPV DNA ($10^8$ cpm/μg) and processed as recommended by the membrane supplier. The membranes were exposed to Kodak XAR-5 films for 10 days with Du Pont Cronex Lightning-Plus intensifying screens. The migration of the linear CRPV DNA (7.9 kilobase pairs), the *Bam*HI fragments (5.55 and 2.35 kilobase pairs), and the *Hin*dII fragments (2.42, 1.89, 1.61, and 1.52 kilobase pairs) (8) is indicated for the two separate experiments illustrated (lanes 1 through 4 and 5 through 8). The smallest *Hin*dII fragment (0.43 kilobase pairs) ran out of the gels. The presumed virus-cell DNA junctions are indicated by •. (B) After lysis of the cells in 33% phenol–150 mM sodium acetate buffer (pH 7.5)–0.25% SDS–2.5 mM EDTA, the total RNAs were extracted with chloroform-isoamyl alcohol (24:1) and selectively precipitated with 2 M LiCl. After treatment with RNase-free DNase I (50 μg/ml, 2 h at 37°C), the polyadenylated RNAs were purified on oligodeoxythymidilic acid-cellulose columns (Collaborative Research). A 2-μg portion of polyadenylated RNAs from VX2T cells (subculture 32, lane 1) or VX2R cells (subculture 38, lane 2) were denatured, fractionated in formaldehyde-agarose gels, and transferred to nitrocellulose filters (BA85; Schleicher & Schuell). The RNAs on the filters were hybridized with $^{32}$P-labeled cloned CRPV DNA ($10^8$ cpm/μg). The sizes of the RNAs, indicated in kilobases, were evaluated with *Hin*dII CRPV DNA fragments as the standard (8). The filters were exposed to Kodak XAR-5 films for 20 h with intensifying screens.

The distinct morphology and growth pattern of VX2T and VX2R cells suggest some differences in their cytoskeleton and cell junctions. The cytoskeleton of keratinocytes is mainly composed of abundant 8-nm filaments constituted of keratin polypeptides and anchored in the numerous desmosomes joining adjacent keratinocytes (16). When examined by transmission electron microscopy, VX2T and VX2R cells displayed a striking difference in the abundance of keratin filaments and desmosomes. In VX2T cells, the desmosomes were rare and the keratin filaments scarce or absent. In contrast, VX2R cells contained abundant filament bundles and numerous desmosome-filament complexes (Fig. 3). The keratin content of VX2T and VX2R cells was further analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and immune blotting experiments. Cytoskeletal proteins were extracted from a rabbit epidermal keratinocyte suspension prepared as previously described (18) and from VX2 tumor cells and VX2T and VX2R cells. The cells were broken in a Dounce homogenizer and extracted in a 10 mM Tris-1 mM EDTA buffer (pH 7.6) containing 2% Nonidet P-40, as described by Fuchs and Green (6). The cytoskeletal proteins, present in the pellet, were solubilized in 3% SDS–5% mercaptoethanol–4 M urea by sonication and incubation for 5 min at 100°C. The polypeptides were analyzed by electrophoresis in a 7.5 to 15% polyacrylamide gradient slab gel in the presence of SDS (24). After electrophoretic transfer from the gels to nitrocellulose membranes (BA85; Schleicher & Schuell, Dassel, West Germany), the keratins were further characterized by immune blotting experiments (24, 32) with pooled antisera against total keratin polypeptides obtained from a human plantar wart (F. Breitburd and G. Orth, unpublished results) and peroxidase-labeled protein A (Amersham International). The keratin polypeptides of rabbit epidermal cells migrated as 65,000-molecular-weight (65K), 59K, 55K, and 52K species, values close to those reported by other authors (4, 6), and represented the bulk of cytoskeletal proteins when compared with the amount of detectable actin (Fig. 4A and B, lane 1). In contrast, the keratin polypeptides of the VX2 tumor cells were not found in amounts larger than that of actin, and the 65K keratin specific for differentiating keratinocytes (6, 7) was not detected (Fig. 4A and B, lane 2). This is in agreement with the anaplastic features of the VX2 carcinoma (9, 27). The keratin pattern of VX2T cells was similar to that of VX2 tumor cells (Fig. 4A and B, lane 3). In contrast, the VX2R cells showed abundant 52K, 55K, and 59K keratin polypeptides when compared with the amount of actin but no 65K keratin, recalling the pattern of rabbit epidermal keratinocytes in primary cultures (4). A 40K keratin, expressed in some human squamous cell carcinoma lines and typically prominent in nonkeratinizing secretory epithelia (7, 10, 34), was also detected (Fig. 4A and B, lane 6).

The removal of vitamin A from the culture media of normal human keratinocytes or squamous cell carcinoma lines leads to an extensive pluristratification of the cultures and to the synthesis of a high-molecular-weight keratin (67K for human keratinocytes), which is one of the earliest in vivo indications that keratinocytes are committed to terminal

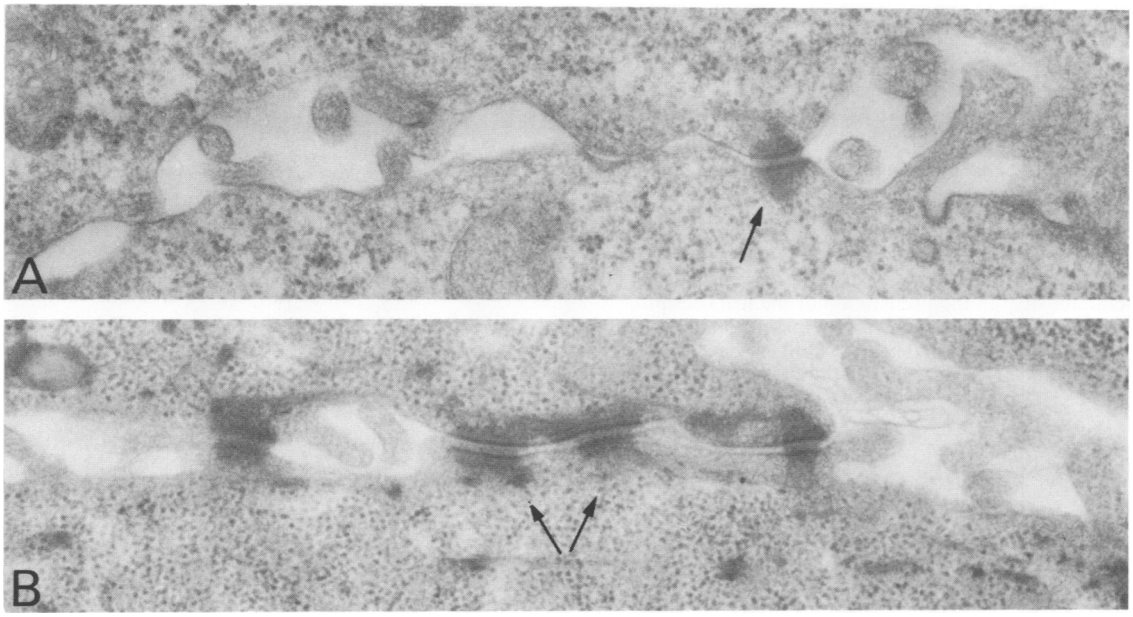


FIG. 3. Ultrastructural features of VX2T and VX2R cells. VX2T cells (subculture 29)(A) and VX2R cells (subculture 37)(B) grown on collagen-coated dishes were fixed in 1% glutaraldehyde and 1% osmic acid and embedded in situ in Epon-araldite (17). Ultrathin sections were performed perpendicularly to the surface of the cultures. The figure illustrates a region of the borders of adjacent cells. Desmosome-filament complexes (arrows) were only rarely encountered in VX2T cells but were observed in great number in VX2R cells. Magnification, ×36,000.

differentiation (7, 10). Simultaneously, the synthesis of the 40K keratin is greatly reduced (7, 10). The capacity of the VX2T and VX2R cells to respond to vitamin A deprivation was analyzed by growing the cells as described by Fuchs and Green (7). The cells were transferred in a medium supplemented with delipidized serum (25). After a second transfer in the same medium, the VX2R cells were collected after 3 weeks, while the progressive detachment of VX2T cells precluded studies beyond 6 days. No effect was observed on the keratin synthesis in VX2T cells (Fig. 4A and B, lanes 4 and 5). The VX2R cells underwent a marked pluristratification and produced a 65K keratin, while the synthesis of the 40K keratin was suppressed (Fig. 4A and B, lanes 6 and 7).

Described since its isolation as a completely anaplastic carcinoma (9), the VX2 tumor has retained this characteristic throughout numerous passages in domestic rabbits (8, 27). The VX2T cell line is highly tumorigenic, produces keratins at a very low level, and most likely represents the in vitro counterpart of the VX2 tumor. The inability of VX2T cells to grow in semisolid medium is a property shared by some other established tumorigenic cell lines derived from human squamous cell carcinomas (22). It was thus unexpected to isolate a cell line unable to be successfully transplanted into rabbits and expressing some phenotypic traits of epidermal keratinocytes. Since VX2R cells grow as an established cell line, show anchorage-independent growth, and induce tumors in nude mice, they are still transformed. Furthermore, the expression of epidermal keratins and the 40K keratin has been observed for other tumorigenic squamous cell carcinoma lines (10, 34). Cytogenetic studies have shown structural and numerical chromosomal changes in VX2 carcinoma cells (2, 20). The culture conditions may have selected a cell with a chromosomal distribution resulting in the reexpression of differentiated characteristics, different requirements for growth, and an antigenic makeup which renders the cells susceptible to

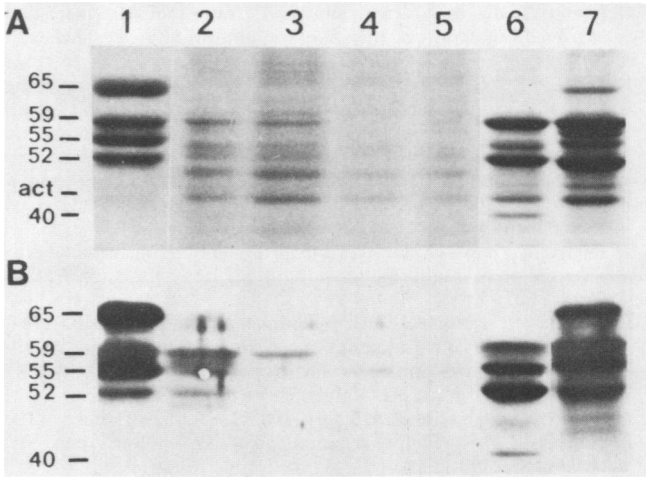


FIG. 4. Electrophoretic (A) and immune blotting (B) characterization of the cytoskeletal proteins of VX2 tumor cells and VX2T and VX2R cells, compared with normal rabbit epidermal keratinocytes. The lanes show the cytoskeletal proteins extracted from cell suspensions obtained from: 1, rabbit ear skin epidermis; 2, VX2 tumor; 3, VX2T cells (subculture 49); 4 and 5, VX2T cells (subculture 22) grown in normal and vitamin A-depleted media, respectively; 6 and 7, VX2R cells (subculture 38) grown in normal and vitamin A-depleted media, respectively. After heat dissociation in the presence of SDS, urea, and mercaptoethanol, portions corresponding to about $5 \times 10^5$ cells were electrophoresed in 7.5 to 15% polyacrylamide gradient slab gels in the presence of SDS (24). The polypeptides were either stained in the gels with Coomassie blue (A) or transferred to nitrocellulose membranes (24, 32)(B). The membranes were incubated with a pool of guinea pig antisera against human keratins, and the immune complexes were revealed by an immunoperoxidase test (24). The molecular weights of the major rabbit epidermal keratin polypeptides (65,000, 59,000, 55,000, and 52,000) and of the 40K keratin were evaluated by comparison with the migration of standard proteins (Pharmacia) run in the same gel. The migration of actin is indicated (act).

the immune reactions of the host (1). This would preclude the development of such cell variants in vivo.

In conclusion, our results show that the anaplastic properties of the VX2 epidermal carcinoma are reversible, that this reversion is associated with a loss of transplantability of the tumoral cells to allogenic hosts, and that these events do not depend on detectable modifications of the physical state or transcription of the CRPV genome.

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