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Physical State and Transcription of the Cottontail Rabbit Papillomavirus Genome in Warts and Transplantable VX2 and VX7 Carcinomas of Domestic Rabbits

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The physical state and the transcription of the genome of cottontail rabbit papillomavirus (CRPV) in non-virus-producing warts and in the VX2 and VX7 transplantable carcinomas of domestic rabbits were compared. The CRPV DNA present in VX2 and VX7 carcinomas (10 to 20 and 100 to 200 genome equivalents per diploid cell, respectively) was found to be entirely integrated into the cellular DNA, most probably as head-to-tail tandem repeats, in contrast to warts, in which viral DNA (10 to 100 copies per diploid cell) was found only as free, mainly monomeric, molecules. In the VX7 tumor, ca. 50% of the viral DNA molecules were found to be longer than one genome length, indicating that viral DNA rearrangements had occurred. A major viral transcript of 1,250 bases was detected in warts and in VX2 and VX7 carcinomas. Complementary sequences were localized within the E region, the putative transforming region inferred from the nucleotide sequence of the CRPV genome (I. Giri, O. Danos, and M. Yaniv, manuscript in preparation). Analysis of heteroduplexes formed between single-stranded CRPV DNA and polyadenylated RNAs from the VX2 tumor showed that the 1,250-base RNA resulted from the splicing of the sequences corresponding to the open reading frame E6 to those corresponding to the 3′ third of E2. A second viral transcript, measuring 2,000 bases, was detected in warts and, in lesser amounts than the 1,250-base species, in VX2 carcinoma, and a 2,100-base RNA was found in VX7 carcinoma. Complementary sequences to these messengers were localized to the same part of the genome as the 1,250-base species and to a contiguous fragment situated upstream. Heteroduplex analysis showed that the 2,000-base species from VX2 carcinoma resulted from the splicing of the sequences corresponding to E6 and E7 to those corresponding to the 3′ third of E2. The sequences spliced out upon the maturation of the two messengers of VX2 carcinoma correspond to E1, the two-thirds of E2, and most of E4. Additional transcripts were found in VX7 carcinoma, a major 3,100-base species transcribed from the E region, and several minor species, measuring from 2,400 to 5,400 bases, which all hybridize with a subgenomic fragment contained in the L region encoding the viral capsid polypeptides. This could account for the antiviral antibodies found in animals bearing the VX7 carcinoma.

The cottontail rabbit papillomavirus (CRPV) induces cutaneous papillomas (warts) in its natural host and, under experimental conditions, in domestic rabbits (37). The transformation of warts into metastasizing squamous cell carcinomas is observed in up to 25% of the infected cottontail rabbits and up to 75% of the infected domestic rabbits (33, 42). Under experimental conditions, cancerogenic polycyclic hydrocarbons play a synergic role in this conversion (34, 35). The CRPV-induced tumors can, therefore, be used as a model for the analysis of the role of a virus in a multistage carcinogenesis process (14, 27).

CRPV replication is usually high in cottontail rabbit warts (3, 25, 28, 37). Viral replication is low or absent in domestic rabbit warts (3, 25, 28, 37), although recoverable CRPV strains, transmissible serially in the domestic rabbit, have been isolated (38). No viral replication is detected in carcinomas (3, 42), which led to the concept of virus masking in tumors (3). Recently, evidence has been obtained for the persistence and expression of the CRPV genome in non-virus-producing tumors (24, 40, 41, 44–47). The presence of multiple monomeric or oligomeric CRPV DNA molecules has been demonstrated in domestic rabbit papillomas, as well as in primary carcinomas and in metastases (40, 41, 44, 46). The viral genomes are highly methylated (41, 47) and usually maintained in an episomal state (44, 46); the integration of the viral DNA into the cell genome has, however, been observed in certain cases (46). Two major CRPV-specific RNAs of 1.3 and 2 kilobases (kb) have been found in warts and primary carcinomas of domestic rabbits, with differences in the relative abundance of the two transcripts in the two types of tumors (24). Thus, the continuous expression of viral genomes is most probably involved in the transformation of a wart into a carcinoma and in the maintenance of the malignant characteristics.

A suitable experimental model to analyze this problem is provided by the transplantable VX2 and VX7 carcinomas of the domestic rabbit (13, 29, 30, 36). These tumors were initially isolated to demonstrate the presence of a CRPV variant, or cancervirus, responsible for the transformation of papillomas into carcinomas (13, 36). Anti-CRPV antibodies were found, up to passage 22, in animals with VX2 carcinoma, but no sign of viral replication was found afterwards in this tumor (36). On the other hand, the isolation of an infectious CRPV identical to the original recoverable strain from the first transplants of VX7 carcinoma (29, 30), the infectivity for rabbits of nucleic acid extracted from tumor

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transplant 83 (10), and the detection of antiviral antibodies in animals with grafts up to at least tumor transplant 165 (22, 26) suggest a persistent, low viral replication in the VX7 carcinoma. The tumor cells contain multiple copies of the viral genome detected in the DNA extracted from the tumors as complexes of low electrophoretic mobility (7, 21, 41) and interpreted, contradictorily, as being integrated head-to-tail tandem repeats (21, 41) or free oligomers (7) of the CRPV genome. Two major viral transcripts similar to those present in the papillomas and carcinomas of the domestic rabbit were found in a transplantable carcinoma named VX7 tumor (21) but probably deriving from the VX2 tumor (47) as well as in a cell line originating from the latter tumor (24). Mapping of the viral RNAs detected in this cell line by hybridization of blotted RNAs with subgenomic probes has shown that they were transcribed from the same region of the genome and were probably spliced and colinear (24).

In the present article, the physical state of the CRPV genome in warts and transplantable VX2 and VX7 carcinomas of the domestic rabbit has been reexamined before transcription studies. CRPV-specific transcripts isolated from the transplantable carcinomas have been compared with those detected in warts as to the number and size of the different species, as well as to their localization on the genome, by blot hybridization with genomic or subgenomic probes. The messengers expressed in VX2 carcinoma have been mapped and oriented on the CRPV physical map by electron microscope analysis of RNA-to-single-stranded DNA heteroduplex molecules. The transcription map of the CRPV genome in VX2 carcinoma has been compared with the map of the open reading frames recently available (I. Giri, O. Danos, and M. Yaniv, manuscript in preparation).

MATERIALS AND METHODS

Tumors. The CRPV particles were purified from cottontail rabbit warts (Vearl Johnson, Rago, Kans.) (26). Domestic rabbit papillomas were induced by application of a suspension of virus particles in glycerin-saline to the scarified skin of the back and flanks of New Zealand white rabbits (28). The transplantable VX2 carcinoma (13), kindly provided in 1970 by C. Trench (Newcastle-upon-Tyne, United Kingdom) after an unknown number of passages, has since been serially propagated in Fauve de Bourgogne domestic rabbits (7, 28). The transplantable VX7 carcinoma (29, 30), kindly provided in 1971 by S. Rogers at passage 116, has since been serially propagated in adult New Zealand white rabbits (7, 26).

DNA preparations. Supercoiled circular CRPV DNA molecules were purified from DNA extracted from cottontail rabbit warts as previously described (7). The linear CRPV DNA was obtained from CRPV-pBR322 recombinant plasmids (a kind gift of I. Giri, Institut Pasteur, Paris). The recombinant plasmids were treated with endonuclease *Eco*RI, and the linear CRPV DNA was purified by sedimentation in a 5 to 21% sucrose gradient in the presence of ethidium bromide (7). The CRPV DNA, purified from either cottontail rabbit warts or recombinant plasmids, shows the same sensitivity to various restriction endonucleases (7). Total cellular DNA was prepared from domestic rabbit warts and from VX2 and VX7 carcinomas as previously described (7).

DNA analysis by blot hybridization. Total cellular DNA was digested with the restriction endonucleases *Bgl*I, *Kpn*I, *Eco*RI, *Sac*I, and *Bst*EII used in the conditions described by the suppliers (Boehringer Mannheim or Amersham International). After electrophoresis in 0.5% horizontal agarose slab gels, DNA was depurinated, denatured, and transferred to nitrocellulose filters (Schleicher and Schüll; type BA85), using 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (19, 39). The blots were hybridized with cloned ^{32}P-labeled CRPV DNA (19) in the presence of 10% dextran-sulphate as described by Thomas (43). The specific activity of the [α-^{32}P]dCTP or TTP, used for nick translation, was 800 Ci/mmol (Amersham International). The filters were exposed to X-ray films (Kodak XAR-5) at −70°C between two intensifying screens (Dupont Cronex Lightning Plus).

RNA extraction. After elimination of the superficial keratinized parts, the papillomas were minced, washed with Hanks balanced salt solution, and incubated in the presence of a trypsin solution (3 mg/ml) for 1 h at 37°C. The total RNAs were extracted from papilloma cells as described by Amtmann and Sauer (1) and from minced VX2 and VX7 tumor tissues by the method of Auffray and Rougeon (2). The contaminating DNA was eliminated by incubation with DNase I (DPFF Worthington) (50 μg/ml) in 10 mM Tris-hydrochloride (pH 7.5)–10 mM $MgCl_2$ for 2 h at 37°C. The polyadenylated [poly(A)$^+$] RNAs were purified from the total RNAs by chromatography on an oligodeoxythymidylic acid [oligo (dT)]-cellulose column (Collaborative Research, Inc.) (31). The RNAs were kept in 70% ethanol–0.2 M sodium acetate (pH 5.0) at −20°C.

RNA analysis by blot hybridization. Samples of RNA were incubated for 10 min at 60°C in MOPS buffer (20 mM morpholinepropanesulfonic acid, 5 mM sodium acetate, 1 mM EDTA [pH 7.0]) containing 6% formaldehyde and 50% deionized formamide (8, 17). The fragments obtained by digestion of the CRPV DNA with *Bam*HI (5,548 and 2,320 base pairs [bp]), *Sal*I + *Eco*RI (4,360 and 3,508 bp), and *Hin*dII (2,420, 1,885, 1,613, 1,520, and 430 bp) (7, 24, 44) were processed similarly and used as size markers. The sizes of the fragments were deduced from the complete nucleotidic sequence of the CRPV DNA (Giri et al., in preparation). The RNAs and DNA markers were fractionated by electrophoresis in denaturing (6% formaldehyde) 1% agarose gels in MOPS buffer for 14 to 18 h at 1 to 2 V/cm (8, 17) and transferred to nitrocellulose filters, using 10× SSC. The RNA blots were hybridized with cloned ^{32}P-labeled CRPV DNA or ^{32}P-labeled subgenomic probes (19) in conditions described by Thomas (43). The filters were exposed to X-ray films as described above.

Electron microscope analysis of RNA-to-single-stranded DNA heteroduplexes. RNA-to-single-stranded DNA heteroduplexes were formed in the conditions described by Chow and Broker (4), using poly(A)$^+$ RNAs isolated from VX2 carcinoma and either *Eco*RI-cleaved CRPV DNA purified from recombinant plasmids or form I DNA treated with *Sal*I or *Bam*HI. A mixture of poly(A)$^+$ RNA (20 μg) and CRPV DNA (50 ng) was heated in 10 μl of 0.1 M HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)–0.4 M NaCl–0.01 M EDTA (pH 7.9) in the presence of 80% deionized formamide for 10 min at 59°C; i.e., 6°C higher than the strand separation temperature determined by electron microscopy (4). Hybridization was allowed for 90 min at 54°C and stopped by a 10-fold dilution in ice-cold distilled water. Samples of 2 μl were spread in the presence of 40% formamide on hypophases containing 10% formamide (4). The preparations were observed in a Siemens Elmiskop 101 electron microscope, and the heteroduplex molecules were photographed at a magnification of ×16,000. Measurements were carried out on fourfold enlarged micrographs with the aid of a Numonics graphic calculator connected to a Nova 3D computer (Data General).

RESULTS

Physical state of the CRPV genome in domestic rabbit tumors. The VX2 and VX7 carcinomas used in this study have characteristics very similar to those described for early passages of these tumors (11, 29, 30, 36). The VX2 carcinoma has the histological characteristics of an anaplastic carcinoma and metastasizes frequently in the lungs (11); furthermore, its growth is not accompanied by the production of antiviral antibodies (26, 36). The VX7 carcinoma has kept some of the characteristics of squamous cell carcinomas (29, 30) and metastasizes in the regional lymph nodes but only exceptionally in the lungs (11); antiviral antibodies are regularly detected in the serum of the animals with grafts (22, 26).

The physical state of the CRPV genome in warts and transplantable carcinomas was analyzed by blot hybridization, using low-concentration (0.5%) agarose gels and no-cut enzymes for CRPV DNA which are insensitive to DNA methylation (*Bgl*I, *Kpn*I). In papillomas from four domestic rabbits, the viral DNA was found mostly as free monomers (Fig. 1a), the intensity of the signal corresponding to 10 to 100 copies of the viral genome per diploid cell in agreement with the results of others (44, 46). The mobility of slowly migrating molecules present in low amounts was not modified after treatment with a no-cut enzyme for CRPV DNA (Fig. 1a, b), indicating that they correspond to free oligomeric molecules. Digestion with one-cut enzymes for CRPV DNA (*Eco*RI, *Sac*I) yielded only one-genome-length linear molecules (Fig. 1c, d), rendering unlikely the integration of some viral sequences into the cell genome. In unrestricted VX2 and VX7 tumor DNAs, the viral DNA was exclusively associated with the bulk of cellular DNA (Fig. 1e, i) in agreement with previous results (7) and corresponded to 10 to 20 and 100 to 200 viral genome equivalents per diploid cell for VX2 and VX7 tumors, respectively. A clear-cut effect of the no-cut enzymes *Bgl*I (Fig. 1e, f, i, j) and *Kpn*I (data not shown) was observed on the migration of high-molecular-weight viral DNA for some DNA preparations from both tumors. This indicates that viral DNA is integrated into the cell genome in contrast with previous conclusions (7). The phenomenon was masked in some instances by the degradation of the DNA obtained from the necrotic VX2 and VX7 carcinomas. Cleavage of VX2 tumor DNA with two different one-cut enzymes (*Eco*RI, *Sac*I) gave rise to several faint bands, with distinct mobilities for each enzyme, in addition to the one-genome-length viral DNA molecules (Fig. 1g, h). These patterns indicate that the viral DNA is integrated into the cellular DNA, as head-to-tail tandem repeats, in agreement with the data reported by McVay et al. (21). Cleavage of VX7 tumor DNA with the one-cut enzymes *Eco*RI, *Sac*I (Fig. 1k, l), and *Bst*EII (data not shown) gave rise to two intense bands with mobilities corresponding to 8,000 bp (one-genome-length viral DNA molecules) and 9,000 bp and to one less intense band with a mobility of ca. 9,400 bp (Fig. 1k, l), confirming that an important fraction of the viral sequences is constituted of rearranged molecules (7). Minor bands were observed with mobilities between 16,000 and 45,000 bp, the mobility of the latter being close to that of viral sequences after cleavage of VX7 DNA with no-cut enzymes (Fig. 1j, k, l). These bands correspond most probably to incomplete digestion products, since they decreased in intensity or disappeared upon increasing the amount of enzyme or the time of incubation (data not shown). In addition, at least two faint bands were observed with distinct mobilities for each enzyme (Fig. 1k, l). Similar results were obtained with the DNA extracted from transplants 211 to 215 of the VX7 tumor. Data obtained after cleavage of VX7 DNA with no-cut or one-cut enyzmes for CRPV DNA indicate that in this tumor, as in VX2 tumor, viral sequences are integrated into the cellular genome most probably as head-to-tail tandem repeats.

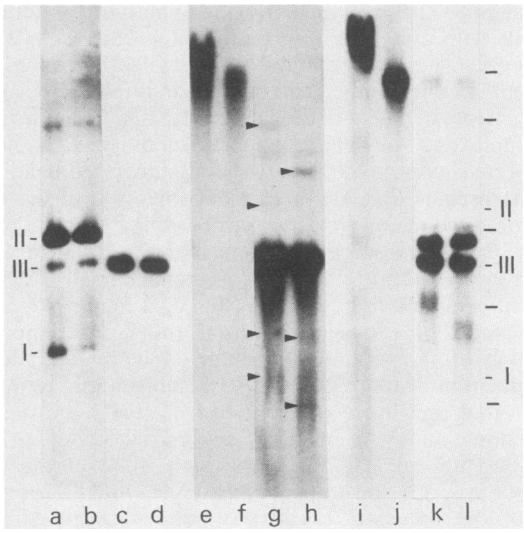

FIG. 1. Blot hybridization analysis of the CRPV DNA sequences in domestic rabbit warts and in the transplantable VX2 and VX7 carcinomas. Total cellular DNA (10 μg) extracted from domestic rabbit warts (lanes a to d) and from the VX2 (lanes e to h) and VX7 tumors (transplant 214, lanes i and j; transplant 215, lanes k and l) were submitted to electrophoresis in horizontal 0.5% agarose slab gels, either untreated (lanes a, e, i) or treated with *Bgl*I (lanes b, f, j), *Eco*RI (lanes c, g, k), or *Sac*I (lanes d, h, l). The DNA was depurinated, denatured, and transferred to nitrocellulose filters. The blots were hybridized with ^{32}P-labeled cloned CRPV DNA in the presence of 10% dextran-sulfate (43) and exposed to Kodak XAR-5 films at −70°C for 10 h (lanes a to d), 36 h (lanes e to h), 4 h (lanes i and j), and 8 h (lanes k and l). The analyses of the DNAs extracted from the warts and from the VX2 and VX7 tumors were performed in separate experiments. Bars indicate the positions of phage λ DNA (48,507 bp) and of four λ *Hin*dIII fragments (23,130, 9,419, 6,557, and 4,371 bp) (New England Biolabs, Inc.) used as size markers in the VX7 DNA experiments (lanes i to l). The migration of CRPV DNA forms I, II, and III are indicated. Arrowheads indicate the faint bands unique to *Eco*RI (lane g) and *Sac*I (lane h) cleavage patterns.

Comparative study of the CRPV-specific transcripts from domestic rabbit tumors. Since RNAs are difficult to isolate from highly keratinized warts, the total RNAs were extracted from wart cell suspensions. The RNAs were used without further fractionation due to the low yield of the extraction (2 to 10 μg of RNA per 10^6 cells). The total RNAs isolated from papillomas and the poly(A)$^+$ RNAs isolated from VX2 and VX7 carcinomas were fractionated by electrophoresis in formaldehyde agarose gels, transferred to nitrocellulose filters, and hybridized with ^{32}P-labeled CRPV DNA. Two major transcripts, whose migration corresponded to 1.25 and 2 kb, were detected in similar quantities in papillomas and with a predominance of the 1.25-kb species in the VX2 tumor (Fig. 2a, b) in agreement with the results of Nasseri et al. (24). A 1.25-kb RNA and an RNA with a migration slightly lower than that of the 2-kb RNA, corresponding to 2.1 kb, were detected in the poly(A)$^+$ RNAs from VX7 carcinoma, with a predominance of the 2.1-kb species (Fig. 2c). Addi-

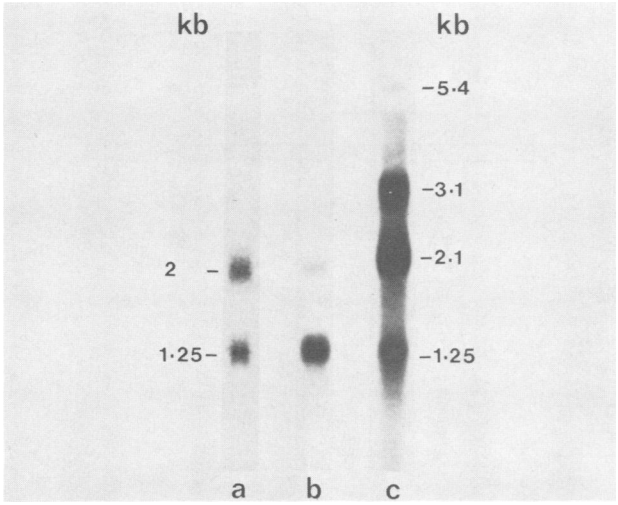

FIG. 2. Blot hybridization analysis of CRPV-specific RNAs from domestic rabbit tumors. Total RNAs (40 μg) extracted from (a) papillomas and poly(A)$^+$ RNAs (2 μg) purified from (b) VX2 and (c) VX7 carcinomas were denatured, separated by electrophoresis in 1% formaldehyde agarose gels (17), and transferred to nitrocellulose filters (43). The RNAs obtained from papillomas and transplantable carcinomas were analyzed in two separate experiments. The blots were hybridized with cloned ^{32}P-labeled CRPV DNA (19, 43) and exposed to X-ray films (Kodak XAR-5) as described in the text. The fragments obtained after cleavage of CRPV DNA with *Hin*dII, *Bam*HI and a mixture of *Sal*I + *Eco*RI were used as molecular weight standards.

tional transcripts, undetectable after RNase treatment, were also observed: a major 3.1-kb species and minor species with sizes varying from 4 to 5.4 kb (Fig. 2c). The same results were obtained with three RNA preparations extracted from papillomas of different rabbits, preparations isolated from VX2 tumors at two different passages and from lung metastases, and RNA preparations obtained from transplants 214 and 223 of the VX7 tumor.

The quantity of poly(A)$^+$ CRPV-specific RNAs present in VX2 and VX7 carcinoma cells was evaluated by dot blot hybridization. The poly(A)$^+$ RNAs were denatured by heating at 60°C for 15 min in 12× SSC containing 15% formaldehyde (48), and twofold serial dilutions in 15× SSC were applied to nitrocellulose filters pretreated with 20× SSC. The blots were heated and hybridized with ^{32}P-labeled CRPV DNA. The intensity of the autoradiograms revealed an approximately fourfold higher amount of CRPV-specific RNA in VX7 carcinoma than in VX2 carcinoma (Fig. 3). The DNA reconstruction experiment showed that the intensity of the dots containing 200 ng of poly(A)$^+$ RNA isolated from VX2 and 50 ng of poly(A)$^+$ RNA isolated from VX7 corresponded to ca. 10 pg of DNA. This indicated that the viral transcripts represented ca. 0.005 to 0.02% of the poly(A)$^+$ RNAs of the tumoral cells, an amount close to that reported for bovine papillomavirus type 1 (BPV-1)-specific transcripts in mouse-transformed cells (9).

The regions of the viral genome transcribed in papillomas and transplantable carcinomas were identified by hybridization of blotted RNA with subgenomic probes corresponding to four fragments generated by cleaving the CRPV DNA with *Eco*RI, *Bam*HI, and *Sal*I endonucleases. The linear CRPV, excised from the recombinant plasmid with *Eco*RI, was purified and treated with a mixture of *Bam*HI and *Sal*I. The fragments were separated by electrophoresis and purified by electroelution and then by sedimentation in a 5 to 21% sucrose gradient containing ethidium bromide (12). Each of the fragments labeled by nick translation revealed only one fragment after hybridization of Southern blots of CRPV DNA digested with a mixture of *Eco*RI, *Bam*HI, and *Sal*I endonucleases (data not shown). Figure 4 (top) shows both the localization of these fragments (named A to D according to a decreasing size order) on the physical map of the CRPV DNA (7, 24, 44) and the mapping of the CRPV-specific transcripts reported for a carcinoma cell line by Nasseri et al. (24). The map was oriented in the 5′ → 3′ direction of the open reading frames of the CRPV genome (Giri et al., in preparation). The 1.25-kb transcripts of papillomas and VX2 and VX7 carcinomas were detected only by probes corresponding to fragments A and D (Fig. 4a to l), in agreement with the data obtained by Nasseri et al., using other subgenomic fragments (Fig. 4, top) (24). The 2-kb RNAs of papillomas and VX2 carcinoma as well as the 2.1-kb RNA of VX7 carcinoma were detected not only by the A- and D-labeled fragments but also by fragment B (Fig. 4g to i), in contrast with the results of Nasseri et al. (Fig. 4, top) (24). The 3.1-kb transcript, specific for VX7 carcinoma, hybridized strongly with fragments A and D (Fig. 4c, l) and weakly with fragments B and C (Fig. 4f, i). Fragment C did not detect any viral RNA from papillomas and VX2 carcinoma but revealed all of the minor transcripts of VX7 carcinoma. In addition, this probe detected a 2.4-kb RNA (Fig. 4f) masked by the high labeling of the 2.1-kb messenger after hybridization with a full genomic probe (Fig. 2c).

Heteroduplex mapping of CRPV-specific RNAs in the VX2 carcinoma. To map the sequences represented in the RNAs

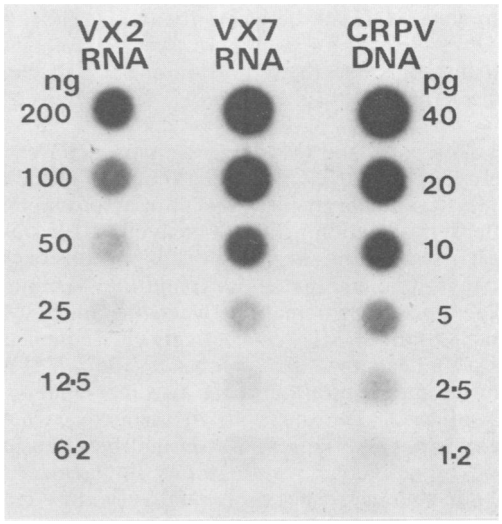

FIG. 3. Quantitation of CRPV-specific RNAs in VX2 and VX7 carcinoma cells by dot blot analysis. Poly(A)$^+$ RNAs, isolated from VX2 and VX7 carcinomas were heat denatured in the presence of 15% formaldehyde (48). Twofold serial dilutions in 15× SSC (from 200 to 6.2 ng) were dot blotted (30-μl portions) on a nitrocellulose filter pretreated with 20× SSC with the aid of a Minifold apparatus (Schleicher and Schüll). Twofold dilutions of denatured CRPV DNA (from 40 to 1.2 pg) were used as standards. The blots were hybridized with ^{32}P-labeled CRPV DNA (1.2×10^8 cpm/μg; 1.5×10^4 cpm/cm^2) as described in the text. The blots were exposed to a Kodak XAR-5 film for 3 days at −70°C.

and to determine the direction of the transcription, RNA-to-single-stranded DNA heteroduplexes (4) were formed between poly(A)$^+$ RNAs isolated from VX2 tumor and either CRPV DNA purified after excision from the plasmid by *Eco*RI or form I CRPV DNA cleaved by *Bam*HI or *Sal*I endonucleases. After hybridization of the RNAs with CRPV cleaved by *Sal*I, two types of heteroduplexes corresponding to the 1.25- and 2-kb messengers were observed at low frequency (Fig. 5a, b). These heteroduplexes show two double-stranded segments separated by a single-stranded loop of similar size, which demonstrates directly the occurrence of a splicing. One of the double-stranded segments terminates near the extremity of the DNA strand and has a similar size in both types of heteroduplexes. This segment is often prolonged by a short fork, which is indicative of the poly(A)$^+$ end of the RNAs. This localizes the 3′ end in the neighborhood of the *Sal*I site situated 44.6 map units (MU) from the *Eco*RI site taken as the origin of the CRPV map. The double-stranded segment which corresponds to the 5′ portion of the messengers has a different size in the two types of heteroduplexes (Fig. 5a, b). Since the 1.25- and 2-kb messengers do not hybridize with the *Sal*I-*Bam*HI fragment, which maps between 44.6 and 67.3 MU (Fig. 4e), the two messengers are colinear. After hybridization of the RNAs with the CRPV DNA cleaved by *Bam*HI, the heteroduplex molecules formed by the 1.25-kb RNA have the size of the *Bam*HI A fragment (70.5% of the genome length) (Fig. 5c). In these heteroduplexes, as well as in the heteroduplexes formed between the 1.25-kb RNA and the CRPV DNA cleaved by *Eco*RI (Fig. 5e), the duplex segment corresponding to the 5′ portion starts in the neighborhood of one of the DNA extremities. This means that the 5′ end of the 1.25-kb messenger is located between 96.8 and 100 MU (Fig. 4, top). The heteroduplex molecules formed between the 2-kb RNA and the CRPV DNA cleaved by *Bam*HI have the size of a complete DNA strand (Fig. 5d), whereas the heteroduplexes formed between the 2-kb RNA and the CRPV DNA cleaved by *Eco*RI are circular molecules (Fig. 5f). This indicates that the 5′ portion of the 2-kb messenger spans the *Eco*RI site and the *Bam*HI site localized at 96.8 MU.

The positions of the messengers on the CRPV physical map were determined by measuring the sizes of the double- and single-stranded segments present in heteroduplex molecules after hybridization with *Sal*I-cleaved CRPV DNA (Fig. 6; Table 1). The results show that the 5′ portions of the 1.25-kb messenger (ca. 400 nucleotides) and the 2-kb messenger (ca. 1,200 nucleotides) map between 99 and 4 MU and between 88.8 and 4 MU, respectively. A sequence of ca. 2,500 nucleotides situated between 4 and 35.5 MU is eliminated during the maturation of the two messengers. The 3′ portions of the 1.25- and 2-kb messengers, which were mapped between 35.3 and 42.5 MU and between 35.6 and 42.3 MU, respectively, have, therefore, similar sizes (ca. 570 and 530 nucleotides, respectively) and very close map positions.

Since the complete nucleotide sequence of the CRPV DNA has been recently determined (Giri et al., in preparation), the transcription map of the CRPV genome in VX2 carcinoma could be compared with the map of the open reading frames (Fig. 7). The mean values of the coordinates of the 5′ portions correspond to the nucleotides 984 and 1378 and to the nucleotides 182 and 1378 for the 1.25- and 2-kb RNAs, respectively. These values are very close to the coordinates of E7 and of E6 and E7, respectively (Fig. 7). Furthermore, the 5′ end of the 2-kb RNA is mapped ca. 50

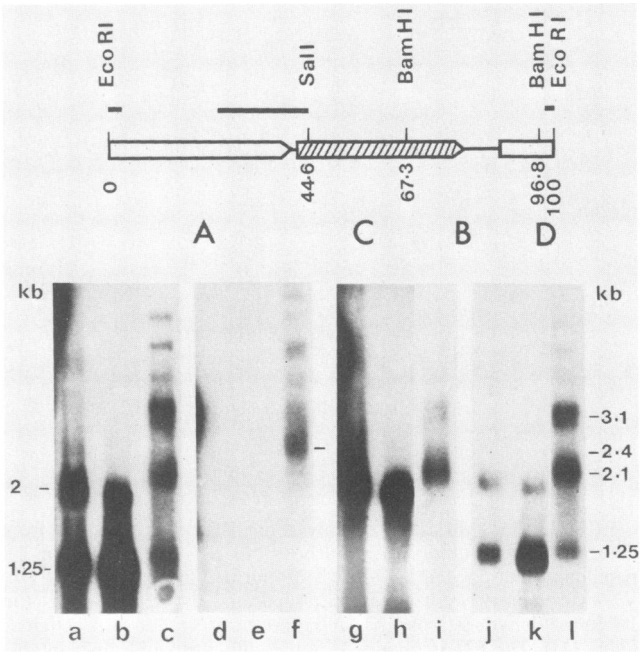

FIG. 4. Mapping of CRPV-specific RNAs by hybridization of RNA blots with subgenomic probes. The four fragments generated by *Sal*I and *Bam*HI digestion of the CRPV DNA excised from the recombinant plasmid by *Eco*RI were purified and ^{32}P labeled by nick translation (specific activities, 1×10^7 to 8×10^7 cpm/μg). A simplified map (top of the figure) gives the positions of the fragments, named A to D according to their decreasing sizes, as well as the localization of the CRPV-specific transcripts reported for a carcinoma cell line (24) (upper discontinuous solid line). The positions of the restriction sites (7, 24, 44) have been adjusted to fit the nucleotide sequence of the CRPV DNA (Giri et al., in preparation). Open and hatched arrows represent the regions containing the E and L open reading frames, respectively (see Fig. 7). Total RNAs from papillomas (20 μg) (lanes a, d, g, j) and poly(A)$^+$ RNAs from VX2 (5 μg) (lanes b, e, h, k) and VX7 carcinoma (2 μg) (lanes c, f, i, l) were fractionated in 1% formaldehyde agarose gels and transferred to nitrocellulose filters. Blots were hybridized with the ^{32}P-labeled fragments A (lanes a to c), B (lanes g to i), C (lanes d to f), and D (lanes j to l) and exposed to Kodak XAR-5 films for 15 h (lanes c, i), 24 h (lanes a, b, g, h), 30 h (lanes d, e, j, k), and 4 days (lanes f, l). The VX7 RNA was not analyzed in the same experiment.

nucleotides downstream from a potential promoter element (TATA sequence). The mean values of the coordinates of the 3′ portions correspond to the nucleotides 3840 and 4407 and to the nucleotides 3864 and 4391 for the 1.25- and 2-kb RNAs, respectively. These values correspond to the 3′ third of E2. The 3′ ends of the two RNAs are localized between two polyadenylation sites, which are distant from each other by 120 nucleotides. The sequence spliced out upon maturation of the two messengers correspond to E1, the 5′ two-thirds of E2, and most of E4.

DISCUSSION

In this paper, we report the results of the analysis of the physical state and the transcription of the CRPV genome in domestic rabbit warts and in the transplantable VX2 and VX7 carcinomas, maintained for more than 10 years in our laboratory and still presenting characteristics similar to those described for early passages (11, 29, 30, 36). The VX7 cells contain ca. 10 times more copies of the viral genome than the VX2 cells. Unlike the warts studied, the viral DNA is integrated into the cellular DNA in both carcinomas, most

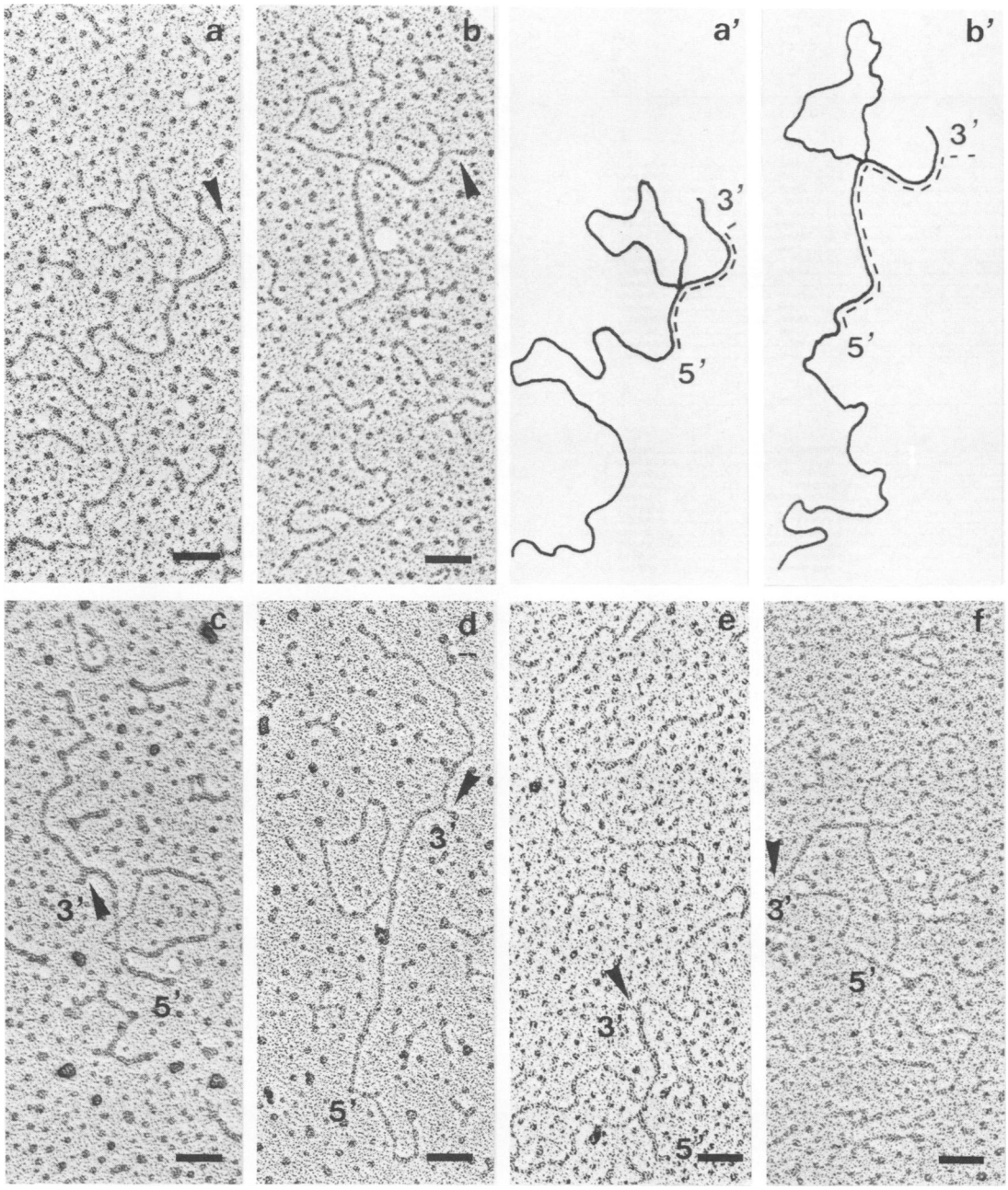

FIG. 5. Electron micrographs of RNA-to-single-stranded DNA heteroduplexes formed between poly(A)$^+$ RNA isolated from VX2 carcinoma and CRPV DNA. Mixtures of poly(A)$^+$ RNAs, prepared from VX2 carcinoma and CRPV DNA cleaved with either *Sal*I, *Bam*HI, or *Eco*RI, were heated in the presence of 80% formamide at 59°C for 10 min and then incubated at 54°C for 90 min (4). The samples were spread and mounted for electron microscopy as described in the text. Heteroduplexes formed between 1.25-kb RNA (a, c, e) or 2-kb RNA (b, d, f) and CRPV DNA cleaved by *Sal*I (a, b), *Bam*HI (c, d), and *Eco*RI (e, f) are shown. The heteroduplexes formed between the two RNAs and CRPV DNA cleaved by *Sal*I have been drawn (a', b'). The 5' → 3' orientations of the mRNAs have been deduced from the positions of the poly(A)$^+$ tails, indicated by arrows. Bar = 0.1 μm.

likely as head-to-tail tandem repeats, in contrast with previously reported results (7). The integration of papillomavirus sequences into the cellular DNA has already been reported for some domestic rabbit papillomas and primary carcinomas (46) as well as for transplantable carcinomas (21, 41). It has also been observed for a highly tumorigenic transplantable hamster sarcoma induced by BPV-1 (12) in contrast with the episomal state of the BPV-1 genome in mouse cells transformed in vitro (15). This points to the possible role played by the integration of viral genomes into cellular DNA in the conversion of a papilloma to a carcinoma or in the transplantability of the carcinoma cells. About 50% of the viral genomes present in VX7 carcinoma show a greater size (9,000 bp) after cleavage with one-cut enzymes and yield additional fragments after cleavage with *Bam*HI, *Hin*dII, and *Hin*dIII endonucleases (data not shown) (7), confirming

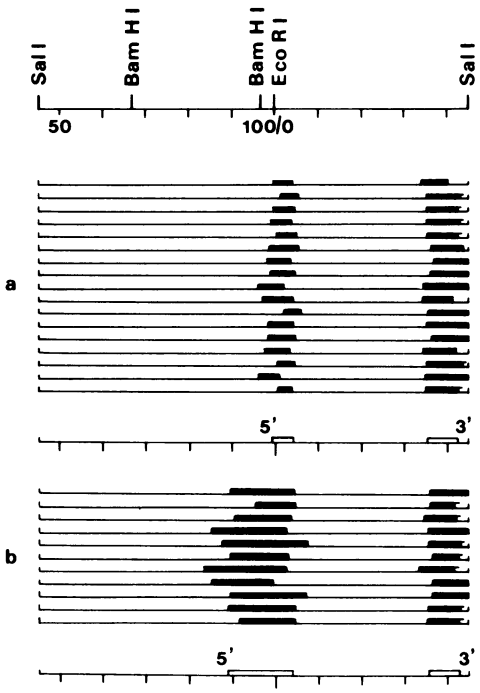

FIG. 6. Diagram of heteroduplex molecules formed between poly(A)$^+$ RNA isolated from VX2 carcinoma and CRPV DNA cleaved by *Sal*I. The solid boxes designate the duplex regions of the heteroduplexes corresponding to (a) 1.25-kb RNA and (b) 2-kb RNA. The open boxes indicate the position of the transcripts on the map of the CRPV genome shown at the top of the figure, as deduced from the mean values of the coordinates expressed as percent units of the genome length (Table 1).

that rearrangements of the viral genomes have occurred (7). Whether this is related to the recoverability of the CRPV strain, used to induce the domestic rabbit tumor from which the VX7 carcinoma was derived (29, 30), remains to be determined.

Two viral transcripts with mobilities of 1.25 and 2 kb have been detected in the warts and the VX2 carcinoma. They correspond in size to those described by others (24) for CRPV-induced tumors. Three major RNA species (1.25, 2.1, and 3.1 kb), together with several minor transcripts, have been found in the VX7 carcinoma. Hybridization of RNA blots with subgenomic probes has shown that the complementary sequences to the 1.25-, 2-, 2.1-, and 3.1-kb RNAs are localized in the E region and that the minor species found in the VX7 carcinoma hybridize with a fragment contained in the L region. Some of these minor RNAs are likely to code for the structural proteins of the virus, a major 54,000-molecular-weight species and a minor 76,000-molecular-weight species (26), whose synthesis is responsible for the production of antiviral antibodies in animals carrying the VX7 carcinoma (22, 26, 29, 30). Differences in the transcription patterns may be related to the rearranged genomes present in VX7 carcinoma. Additional species and the abundance of the 2.1- and 3.1-kb mRNAs may explain the 4-fold higher amount of CRPV-specific RNA detected in the VX7 tumor, as compared with the VX2 tumor, rather than the 10-fold higher copy number of the viral genome.

The analysis of RNA-to-single-stranded DNA heteroduplexes directly demonstrates that the 1.25- and 2-kb messengers found in VX2 carcinoma are colinear and spliced, as assumed by Nasseri et al. (24). Comparison of the transcription map with the nucleotidic sequence (Giri et al., in preparation) indicates clearly that the messengers result from the splicing of the E7 (1.25 kb) or the E6 and E7 (2 kb) sequences to the 3′ third of the E2 sequence. This splicing leads to the elimination of sequences, including the E1 open reading frame, a region responsible for the episomal state of the viral DNA in BPV-1-transformed mouse cells (23). The potential promoter nearest to the 5′ end of the 1.25-kb RNA is that localized upstream, next to the 5′ end of the 2-kb RNA. This suggests that the 1.25-kb RNA comprises a short leader sequence undetected by heteroduplex analysis. Two termination codons are present between the E6 and E7 open reading frames (Giri et al., in preparation). However, E6 and E7 could be placed in the same translation frame by a splicing event undetected by heteroduplex analysis. Another possibility would be that the E6 or E7 reading frame or both are translated separately from the 2-kb messenger. Finally, it remains to be determined whether the spliced E7 and E2 sequences can be translated into a fusion polypeptide. It is worth stressing that the 3′ part of E2 is expressed in cells transformed by BPV-1 (1, 6, 9, 12) and is required for the transformation of mouse cells (23). Furthermore, a human papillomavirus type 1-specific spliced messenger containing the 3′ portion of E2 has been detected in COS cells (monkey CV1 cells transformed by simian virus 40) transfected with a human papillomavirus type 1-vector recombinant plasmid (L. T. Chow and T. R. Broker, *Gene Transfer and Cancer*, in press).

The mechanisms involved in the transformation of a wart into a carcinoma are far from being understood. Discussing the possible mechanisms, Rous proposed "a variation of the virus which makes it express itself in somewhat different terms, making the papilloma cells, which are already vigorously neoplastic, become cancer cells (32)." This seemed unlikely, since the biological properties of the virus recovered from several transplantable carcinomas, among which was the VX7 carcinoma, were not different from those of the original CRPV strain (3, 29, 30). Two processes, acting synergistically, are probably necessary to transform a wart into a carcinoma and maintain its malignant characteristics. The first one may be the continuous expression of the

TABLE 1. Coordinates of the CRPV-specific transcripts found in VX2 carcinoma

RNAs (kb)	Coordinates (MU)[a]				Lengths[b]	
	5′	3′	5′	3′	RNAs	Intervening sequences
1.25	99 ± 1.7 (17)	4 ± 1.2 (17)	35.3 ± 0.8 (17)	42.5 ± 1.1 (10)	12.2	31.3
2	88.8 ± 3.4 (11)	4 ± 2.2 (11)	35.6 ± 0.9 (11)	42.3 ± 0.7 (7)	21.9	31.6

[a] The values are given as mean value ± standard deviation, taking the *Eco*RI site as the origin of the map. The number of measurements is indicated in parentheses.

[b] The lengths are expressed as percentage of genome length (7,868 bp [Giri et al., in preparation]). The RNA lengths do not include the contribution of the poly(A)$^+$ sequence at the 3′ ends.

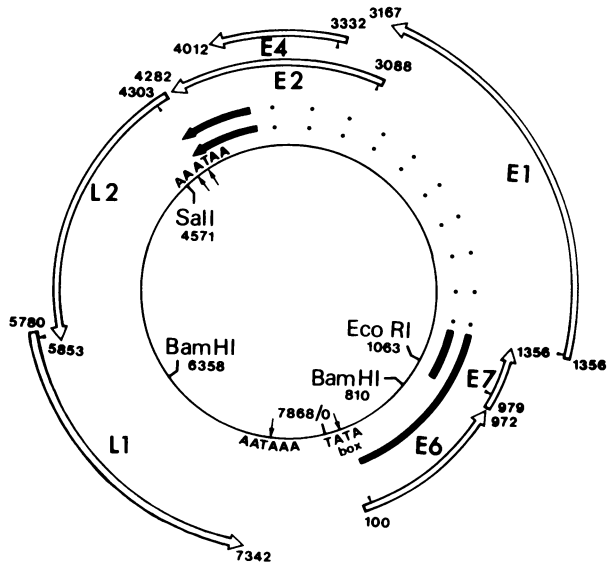

FIG. 7. Transcription map of the CRPV genome in VX2 carcinoma. The inner circle represents a simplified restriction endonuclease map of CRPV, based on the DNA nucleotide sequence (Giri et al., in preparation). Spliced segments of the CRPV-specific, 1.25- and 2-kb transcripts and sequences spliced out are indicated by solid lines and dots, respectively. The 5′ → 3′ orientation is given by the arrowheads. The organization of the open reading frames (Giri et al., manuscript in preparation) is indicated by the outer open arrows. Numbers refer to the start and end of the frames, and arrowheads refer to the first methionine. Open reading frames within the part of the genome homologous to the transforming region of BPV-1 genome have been designated E1 to E7, and those within the part of the genome homologous to the region only expressed in productive infection have been designated L1 and L2 (5, 6). The positions of a potential promoter element for the transcription of the E region (TATA sequence) starting at nucleotide 129 and the polyadenylation recognition sites (AATAAA) starting at nucleotides 4348, 4468, and 7397 are indicated by arrows on the inner circle.

products, not yet identified, of the 1.25- and 2-kb RNAs, still detectable in the VX2 carcinoma isolated 45 years ago (13). That a virus-coded protein is involved is suggested by the transforming effect of CRPV on papillomas induced by tarring the skin of the domestic rabbit (34) and on carcinomas induced in a similar way in the cottontail rabbit (35). The second process involved could be the activation of one or more cellular protooncogenes. Such activation could result from the activity of a viral promoter or a viral enhancer (18), after the integration of CRPV genomes into the cellular DNA, or be due to cellular DNA rearrangements. Abnormalities in the structure and number of chromosomes are indeed observed with increasing frequency and extent during the different phases of the progression of a papilloma into a carcinoma (20). It has recently, been shown that the cooperation between a gene of an oncogenic DNA virus and an activated cellular oncogene permits the tumorigenic conversion of primary cells in culture (16). A similar synergy could lead to the in vivo production of malignant CRPV-infected epidermal cells.

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LITERATURE CITED

1. **Amtmann, E., and G. Sauer.** 1982. Bovine papilloma virus transcription: polyadenylated RNA species and assessment of the direction of transcription. J. Virol. **43**:59–66.
2. **Auffray, C., and F. Rougeon.** 1980. Purification of mouse immunoglobulin heavy-chain messenger RNAs from total myeloma tumor RNA. Eur. J. Biochem. **107**:303–314.
3. **Beard, J. W.** 1956. The fallacy of the concept of virus "masking": a review. Cancer Res. **16**:279–291.
4. **Chow, L. T., and T. R. Broker.** 1981. Mapping RNA:DNA heteroduplexes by electron microscopy, p. 139–188. *In* J. D. Griffith (ed.), Electron microscopy in biology. John Wiley & Sons, New York.
5. **Danos, O., L. W. Engel, E. Y. Chen, M. Yaniv, and P. M. Howley.** 1983. Comparative analysis of the human type 1a and bovine type 1 papillomavirus genome. J. Virol. **46**:557–566.
6. **Engel, L. W., C. A. Heilman, and P. M. Howley.** 1983. Transcriptional organization of bovine papillomavirus type 1. J. Virol. **47**:516–528.
7. **Favre, M., N. Jibard, and G. Orth.** 1982. Restriction mapping and physical characterization of the cottontail rabbit papillomavirus genome in transplantable VX2 and VX7 domestic rabbit carcinomas. Virology **119**:298–309.
8. **Goldberg, D. A.** 1980. Isolation and partial characterization of the drosophila alcohol dehydrogenase gene. Proc. Natl. Acad. Sci. U.S.A. **77**:5794–5798.
9. **Heilman, C. A., L. Engel, D. R. Lowy, and P. M. Howley.** 1982. Virus-specific transcription in bovine papilloma virus-transformed mouse cells. Virology **119**:22–34.
10. **Ito, Y.** 1970. Induction of papillomas in rabbits with nucleic acid extracts from VX7 carcinomas. Brit. J. Cancer **24**:535–541.
11. **Ito, Y., I. Kimura, and T. Miyake.** 1968. Comparative studies on pathogenesis of two strains of transplantable carcinomas of rabbit, VX2 and VX7. Proc. Soc. Exp. Biol. Med. **127**:1106–1111.
12. **Jaureguiberry, G., M. Favre, and G. Orth.** 1983. Bovine papillomavirus type 1 genome in hamster sarcoma cells in vivo and in vitro: variation in the level of transcription. J. Gen. Virol. **64**:1199–1204.
13. **Kidd, J. G., and P. Rous.** 1940. A transplantable rabbit carcinoma originating in a virus-induced papilloma and containing the virus in masked or altered form. J. Exp. Med. **71**:813–838.
14. **Kreider, J. W., and G. L. Bartlett.** 1981. The Shope papilloma-carcinoma complex of rabbits: a model system of neoplastic progression and spontaneous regression. Adv. Cancer Res. **35**:81–110.
15. **Lancaster, W. D., and C. Olson.** 1982. Animal papillomaviruses. Microbiol. Rev. **46**:191–207.
16. **Land, H., L. F. Parada, and R. A. Weinberg.** 1983. Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature (London) **304**:596–602.
17. **Lehrach, H., D. Diamond, J. M. Wozney, and H. Boedtker.** 1977. RNA molecular weight determinations by gel electrophoresis under denaturing conditions, a critical reexamination. Biochemistry **16**:4743–4751.
18. **Lusky, M., L. Berg, H. Weiher, and M. Botchan.** 1983. Bovine papilloma virus contains an activator of gene expression at the distal end of the early transcription unit. Mol. Cell. Biol. **3**:1108–1122.
19. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
20. **McMichael, H., J. E. Wagner, P. C. Nowell, and D. A. Hungerford.** 1963. Chromosome studies of virus-induced rabbit papillo-

mas and derived primary carcinomas. J. Natl. Cancer Inst. **31**:1197–1215.
21. **McVay, P., M. Fretz, F. O. Wettstein, J. G. Stevens, and Y. Ito.** 1982. Integrated Shope virus DNA is present and transcribed in the transplantable rabbit tumor VX7. J. Gen. Virol. **60**:271–278.
22. **Mellors, R. C.** 1960. Tumor cell localization of the antigens of the Shope papilloma virus and the Rous sarcoma virus. Cancer Res. **20**:744–746.
23. **Nakabayashi, Y., S. K. Chattopadhyay, and D. R. Lowy.** 1983. The transforming function of bovine papillomavirus DNA. Proc. Natl. Acad. Sci. U.S.A. **80**:5832–5836.
24. **Nasseri, M., F. O. Wettstein, and J. G. Stevens.** 1982. Two colinear and spliced viral transcripts are present in non-virus-producing benign and malignant neoplasms induced by the Shope (rabbit) papilloma virus. J. Virol. **44**:263–268.
25. **Noyes, W. F., and R. C. Mellors.** 1957. Fluorescent antibody detection of the antigens of the Shope papilloma virus in papillomas of the wild and domestic rabbit. J. Exp. Med. **106**:555–562.
26. **Orth, G., F. Breitburd, and M. Favre.** 1978. Evidence for antigenic determinants shared by the structural polypeptides of (Shope) rabbit papillomavirus and human papillomavirus type 1. Virology **91**:243–255.
27. **Orth, G., F. Breitburd, M. Favre, and O. Croissant.** 1977. Papillomaviruses: possible role in human cancer, p. 1043–1068. *In* H. H. Hiatt, J. D. Watson, and J. A. Winsten (ed.), Origins of human cancer. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
28. **Orth, G., P. Jeanteur, and O. Croissant.** 1971. Evidence for and localization of vegetative viral DNA replication by autoradiographic detection of RNA-DNA hybrids in sections of tumors induced by Shope papilloma virus. Proc. Natl. Acad. Sci. U.S.A. **68**:1876–1880.
29. **Rogers, S., J. G. Kidd, and P. Rous.** 1950. An etiological study of the cancers arising from the virus-induced papillomas of domestic rabbits. Cancer Res. **10**:237.
30. **Rogers, S., J. G. Kidd, and P. Rous.** 1960. Relationships of the Shope papilloma virus to the cancers it determines in domestic rabbits. Acta Unio Int. Contra Cancrum **16**:129–130.
31. **Rosenthal, L. J.** 1976. Isolation and characterization of poly(A)-containing polyoma "early" and "late" messenger RNAs. Nucleic Acids Res. **3**:661–676.
32. **Rous, P.** 1943. The nearer causes of cancer. J. Am. Med. Assoc. **122**:575–581.
33. **Rous, P., and J. W. Beard.** 1935. The progression to carcinoma of virus-induced rabbit papillomas (Shope). J. Exp. Med. **62**:523–548.
34. **Rous, P., and J. G. Kidd.** 1938. The carcinogenic effect of a papilloma virus on the tarred skin of rabbits. I. Description of the phenomenon. J. Exp. Med. **67**:399–428.
35. **Rous, P., and J. G. Kidd.** 1940. The activating transforming and carcinogenic effects of the rabbit papilloma virus (Shope) upon implanted tar tumors. J. Exp. Med. **71**:787–812.
36. **Rous, P., J. G. Kidd, and W. E. Smith.** 1952. Experiments on the cause of the rabbit carcinomas derived from virus-induced papillomas. II. Loss by the VX2 carcinoma of the power to immunize hosts against the papilloma virus. J. Exp. Med. **96**:159–174.
37. **Shope, R. E.** 1933. Infectious papillomatosis of rabbits (with a note on the histopathology by E. W. Hurst). J. Exp. Med. **58**:607–624.
38. **Shope, R. E.** 1935. Serial transmission of virus of infectious papillomatosis in domestic rabbits. Proc. Soc. Exp. Biol. Med. **32**:830–832.
39. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
40. **Stevens, J. G., and F. O. Wettstein.** 1979. Multiple copies of Shope virus DNA are present in cells of benign and malignant non-virus-producing neoplasms. J. Virol. **30**:891–898.
41. **Sugawara, K., K. Fujinaga, T. Yamashita, and Y. Ito.** 1983. Integration and methylation of Shope papillomavirus DNA in the transplantable VX2 and VX7 rabbit carcinomas. Virology **131**:88–99.
42. **Syverton, J. T.** 1952. The pathogenesis of the rabbit papilloma-to-carcinoma sequence. Ann. N.Y. Acad. Sci. **54**:1126–1140.
43. **Thomas, P. S.** 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. U.S.A. **77**:5201–5205.
44. **Watts, S. L., R. S. Ostrow, W. C. Phelps, J. T. Prince, and A. T. Faras.** 1983. Free cottontail rabbit papilloma virus DNA persists in warts and carcinomas of infected rabbits and in cells in culture transformed with virus or viral DNA. Virology **125**:127–138.
45. **Wettstein, F. O., and J. G. Stevens.** 1981.Transcription of the viral genome in papillomas and carcinomas induced by the Shope virus. Virology **109**:448–451.
46. **Wettstein, F. O., and J. G. Stevens.** 1982. Variable-sized free episomes of Shope papilloma virus DNA are present in all non-virus-producing neoplasms and integrated episomes are detected in some. Proc. Natl. Acad. Sci. U.S.A. **79**:790–794.
47. **Wettstein, F. O., and J. G. Stevens.** 1983. Shope papilloma virus DNA is extensively methylated in non-virus-producing neoplasms. Virology **126**:493–504.
48. **White, B. A., and F. C. Bancroft.** 1982. Cytoplasmic dot hybridization. Simple analysis of relative mRNA levels in multiple small cell or tissue samples. J. Biol. Chem. **257**:8569–8572.