Differences Exist Between Viral Transcripts in Cottontail Rabbit Papillomavirus-Induced Benign and Malignant Tumors as Well as Non-Virus-Producing and Virus-Producing Tumors

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Five major cottontail rabbit papillomavirus-specific polyadenylated RNA species with sizes of 4.8, 2.6, 2.0, 1.3, and 0.9 kilobases (kb) were found in virus-producing tumors of cottontail rabbits (the natural host for the virus). Two of the RNA species (sizes, 2.0 and 1.3 kb) are indistinguishable with respect to size and map position from the RNA species detected previously in non-virus-producing benign and malignant tumors (Nasseri et al., *J. Virol.* 44:263-268, 1982). The 2.0-kb RNA in virus-producing benign tumors is more abundant than the 1.3-kb RNA. This, together with similar observations of benign non-virus-producing tumors, suggests that the predominance of the 2.0-kb RNA is a general feature of benign tumors. The change to a preferential synthesis of the 1.3-kb RNA appears to be a phenomenon of tumor progression from papillomas to carcinomas. Three transcripts of 4.8, 2.6, and 0.9 kb are unique to virus-producing tumors. The RNA molecules were mapped in two steps. First, hybridization of Northern blots with subgenomic probes revealed the approximate map position of the transcripts. Second, with nuclease S1 and exonuclease VII mapping procedures and end-labeled probes, the major exons of the 4.8-, 2.6-, 2.0-, and 1.3-kb RNAs were mapped precisely, and it is shown that all RNAs are transcribed from the same DNA strand. Both 1.3- and 2.0-kb RNAs consist of two exons which are separated by an identical 2.45-kb intron. The 5' ends of the 5'-proximal exons of the 2.0- and 1.3-kb RNAs map to positions 0.07 and 0.16, respectively. Some of the 2.0-kb RNA molecules, especially in the carcinoma, have an alternative 5' end at position 0.06. The 3' ends of both exons map to position 0.22, where two ends were found about seven nucleotides apart. The sizes of the 5'-proximal exons of the 2.0- and 1.3-kb RNAs are 1.23 and 0.48 kb, respectively. The 1.3- and 2.0-kb RNAs share a common 3'-proximal exon of 0.66 (0.61) kb. This exon has two 5' ends 50 nucleotides apart at map position 0.53 and a 3' end at map position 0.61. Only the 3'-proximal part of the 4.8- and 2.6-kb RNAs have been mapped precisely. Both RNAs share a common 3' end at position 0.99. The 2.6-kb RNA part consists of a single 1.59-kb exon which extends to map position 0.79. The 4.8-kb RNA is heterogeneous. Some molecules have one or two small introns at map position 0.79 or 0.61 or both, whereas in others this part consists of a single 3.7-kb exon extending to position 0.53. The positions of the leader sequences for the 2.6- and 4.8-kb RNAs as well as that of the 0.9-kb RNA have not been mapped in detail.

Cottontail rabbit (Shope) papillomavirus (CRPV) induces tumors in both cottontail and domestic rabbits. Tumors, at first, are benign (papillomas), but carcinomas usually develop at the same site several months later. However, among the tumors induced by CRPV, virus production is only observed in papillomas of the cottontail rabbit, the natural host for the virus. Although domestic rabbit tumors are virus negative, cells of both types of tumors contain from 10 to more than 100 viral gene copies (21), and the viral DNA in most tumors is exclusively extrachromosomal (23, 25). So far, nothing is known about any viral gene product present in these tumors. Antisera which stain virus-producing tumors do not stain non-virus-producing tumors when immunofluorescent techniques are employed (21); however, virus-specific RNA of low abundance can be detected (24).

Previously, we showed that in non-virus-producing rabbit tumors two major spliced colinear transcripts of 1.3 and 2.0 kilobases (kb) are present. To determine whether synthesis of viral structural proteins was associated with the synthesis of different polyadenylated [poly(A)⁺] RNA species, RNA isolated from virus-producing cottontail rabbit papillomas (CPs) was analyzed. Here we show that in virus-producing

tumors, three unique transcripts are present in addition to those found in non-virus-producing tumors. Further, since evidence from previous experiments suggested a quantitative difference between carcinomas or a carcinoma-derived cell line and a papilloma, RNAs isolated from additional domestic rabbit tumors were included in these analyses.

Finally, using hybridization of RNA blots with different subgenomic probes as well as S1 nuclease (S1) and exonuclease VII (Exo VII) mapping procedures with 5'- and 3'-end-labeled probes, we have mapped the transcripts present in cottontail rabbit tumors.

MATERIALS AND METHODS

Animals and virus. The source of animals and virus and the mode of infection were as described earlier (21).

Isolation of RNA. RNA was isolated from tumors by extraction with guanidium hydrochloride, and poly(A)⁺ RNA was selected by oligodeoxythymidylate-cellulose column chromatography (3) as previously described (17).

RNA analysis by RNA transfer (Northern) blot hybridization. Poly(A)⁺ RNA was glyoxalated (14) and electrophoresed as described previously (17). RNA was transferred to Gene Screen (New England Nuclear Corp.) and hybridized with nick-translated (18), full-genomic or subgenomic probes characterized previously (17, 26). The [³²P]dCTP used in

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nick translations had a specific activity of 3,000 Ci/mmol and was purchased from Amersham Corp. or New England Nuclear Corp. Filters were hybridized with 10^6 cpm of full-genomic probe per ml and washed, and autoradiographs were prepared as described previously (17). With subgenomic probes, the amount of label was reduced in relation to the reduced complexity of the probes.

RNA analysis by S1 and Exo VII mapping. S1 and Exo VII mapping was by the method of Berk and Sharp (4, 5), employing end-labeled probes (22). The end-labeled probes were prepared by the digestion of the whole recombinant CRPV pBR322 plasmid or by the digestion of preisolated subgenomic fragments with appropriate restriction endonucleases. For 5'-end labeling, the cut DNA was digested with alkaline phosphatase and reisolated by phenol extraction and ethanol precipitation. The ends were labeled with ^{32}P , using T4 polynucleotide kinase and $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ with a specific activity of 5,000 Ci/mmol purchased from Amersham Corp. 3' ends were labeled with the Klenow fragment of DNA polymerase I with $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$ or TTP with a specific activity of 2,000 or 3,000 Ci/mmol, respectively, purchased from Amersham Corp. or New England Nuclear Corp. After labeling, the probes were recut with restriction endonucleases to provide probes labeled only on one end or separated directly from other labeled DNA fragments by neutral (Tris-acetate [20]) or Tris-borate (0.089 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA) or by alkaline (0.03 M NaOH, 0.001 M EDTA) agarose gel electrophoresis. Probe fragments were isolated by electroelution in neutral agarose buffer containing 10 μg of tRNA per ml, phenol extraction, and ethanol precipitation. The restriction enzymes were purchased from New England Biolabs or Bethesda Research Laboratories and were used under the conditions recommended by the manufacturers.

Hybridization mixtures contained (per 10 μl): ca. 0.02 to 0.05 pmol of probe DNA, 5 or 10 μg of poly(A)⁺ CP RNA or domestic rabbit tumor RNA, respectively, and tRNA to bring the total amount of RNA to 25 μg . DNA and RNA were dissolved in 80% deionized formamide–0.4 M NaCl–40 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid; pH 6.4)]–1 mM EDTA. Samples (5 or 10 μl) were sealed in 10- μl glass capillaries, denatured for 10 min at 68°C, and hybridized for 3 h at 50°C. Standard conditions for S1 digestion were 4,000 U/ml (Miles Laboratories) for 30 min at 37°C. Exo VII (Bethesda Research Laboratories) digestion was for 30 min at 37°C with various concentrations of enzyme. Digestion products were ethanol precipitated with sonicated denatured calf thymus DNA serving as a carrier. Analysis of the digestion products, equivalent to 2.5 μl of hybridization mixture containing 1 μg of carrier DNA, was on neutral (Tris-acetate) or alkaline agarose gels or on polyacrylamide-urea gels (13). Polyacrylamide gels were autoradiographed directly with or without an amplifying screen. Alkaline agarose gels were neutralized, fixed in 70% ethanol–0.1 M NaCl, dried between filter paper, and autoradiographed with amplifying screens. Neutral agarose gels were fixed, dried, and autoradiographed as alkaline gels.

RESULTS

RNA (Northern) blot hybridizations. The Northern blots of oligodeoxythymidylate-cellulose-selected RNA isolated from several types of CRPV-induced tumors are shown in Fig. 1. Lanes 1 and 2 represent the RNA analysis of a primary domestic rabbit carcinoma (DC), lanes 3 to 8 represent duplicate analyses of three domestic rabbit papillomas (DPs), and, finally, lanes 8 and 9 show the analysis of

FIG. 1. RNA transfer (Northern) blot analysis of virus-specific transcripts from CRPV-induced cottontail and domestic rabbit tumors. Poly(A)⁺ RNA was isolated from a primary DC (lanes 1 and 2; 5 μg per track), three different non-virus-producing DPs (lanes 3 to 8; 5 μg per track), and two virus-producing CPs (lanes 9 and 10; 0.5 and 1 μg per track, respectively). Exposure of the hybridized filters to X-ray films in the presence of amplifying screens as described previously (17) was for 5 days, except for CPs, for which exposure was 3 days. The position of 28S and 18S RNA is indicated by the arrowheads.

two different CPs. Two RNA bands, 1.3 and 2.0 kb in size, are present in all domestic rabbit tumors. In the DC the 1.3-kb RNA is more abundant than the 2.0-kb band; this is in agreement with previous data from malignant tumors (17). In contrast, the three different DPs show a predominance of the 2.0-kb RNA. The RNA of the two different CPs (lanes 8 and 9) is resolved into five bands representing sizes of 4.8, 2.6, 2.0, 1.3, and 0.9 kb. Here again, the 2.0-kb bands are more prominent than the 1.3-kb bands. Indeed, the higher relative intensity of the 2.0-kb RNA band compared with that of the 1.3-kb RNA band seems to be even more pronounced in virus-producing papillomas than in non-virus-producing ones. Thus these results show that virus-producing tumors contain three unique transcripts in addition to two which are identical in size to those found in non-virus-producing tumors, and further, the higher abundance of the 2.0-kb RNA compared with the 1.3-kb RNA appears to be a general feature of benign tumors.

The results of hybridizations with six subgenomic probes (*Msp*I fragments 5, 8, 4, 1, and 6 and *Bgl*II fragment II) are shown in Fig. 2B (lanes 1, 3, 4, 6, 7, and 2, respectively). The hybridization with whole genomic probe (Fig. 2B, lane 5) serves as a reference. The map locations of the probes are indicated in Fig. 2A. The data show that *Bgl*II fragment II and *Msp*I fragment 1 hybridized strongly with the 4.8- and 2.6-kb RNAs (lanes 2 and 6). These probes did not hybridize with the major RNAs of non-virus-producing tumors (17). Probes 4 and 6 (lanes 6 and 7, respectively) hybridized to all except the 2.6-kb RNA. Two probes, 5 and 8 (tracks 1 and 3, respectively) hybridized only to one band each, the 2.0- and 4.8-kb RNA, respectively. Hybridization of the *Msp*I fragment 5 to the 2.0-kb RNA was weak, and hybridization of

FIG. 2. Mapping of CRPV-specific transcripts in virus-producing CPs. (A) Restriction map of CRPV DNA. The map positions of *Bgl*II, *Eco*RI, and *Sal*I restriction endonuclease sites as well as the fragments generated by *Bgl*II digestion (I, II, and III) are indicated above the map line, and those generated by *Msp*I are shown below the line. At the bottom of A is a schematic presentation of the subgenomic probe hybridization shown in B, and it includes the results of some previous experiments with the 1.3- and 2.0-kb RNAs (17). (B) RNA transfer (Northern) blot analysis with full genomic or subgenomic probes. Lanes: 1, *Msp*I fragment 5; 2, *Bgl*II fragment II; 3, *Msp*I fragment 8; 4, *Msp*I fragment 4; 5, whole genomic probe; 6, *Msp*I fragment 1; 7, *Msp*I fragment 6. Exposure of the hybridized filters was for 5 days for fragments 5 and 8, 3 days for *Msp*I fragments 4, 6, and 1 and *Bgl*II fragment II, and overnight for the whole genomic probe. The positions of 28S and 18S rRNA are marked on both sides of the figure. Not all agarose gels were electrophoresed to the same extent, and bars connect positions of major RNA bands.

this probe with the 2.0-kb RNA of the Vx-7 cell line was not detected previously (17). However, when hybridizations with *Msp*I fragment 5 were repeated with poly(A)⁺ RNA isolated from the parent Vx-7 tumor, which contains a higher concentration of viral RNA than does the derived cell line, *Msp*I fragment 5 did indeed hybridize to the 2.0-kb RNA (data not shown).

The results of the hybridizations shown in Fig. 2B and additional ones (data not shown) are summarized in Fig. 2A. Interestingly, two areas of the viral genome are only represented by a major poly(A)⁺ RNA isolated from virus-producing CPs. The first one maps to *Msp*I fragment 1, and the second one maps to *Bgl*II fragment II. Further, the 0.9-kb RNA also unique to virus-producing papillomas is colinear with the 3'-proximal exon of the 2.0- and 1.3-kb RNAs. The data presented also show that the 2.6-kb RNA is spliced.

S1 and Exo VII mapping of transcripts. In the second step, the major exons of the 4.8-, 2.6-, 2.0-, and 1.3-kb RNAs were more precisely examined by S1 mapping procedures (4, 5), in which 5'- and 3'-end-labeled probes were used (22). Labeling sites were chosen within transcribed regions identified by subgenomic probe hybridization of Northern blots shown in Fig. 2. The autoradiograms of S1 and Exo VII mapping experiments are shown in Fig. 3, panels A through J. The map location of the restriction sites used and the results of the mapping data are summarized in the center section of Fig. 3. Finally, the location of open reading frames of CRPV DNA determined by Giri, I. O. Danos, and M.

Yaniv (submitted for publication) (O. Danos, I. Giri, F. Thierry, and M. Yaniv, *J. Invest. Dermatol.*, in press) are shown at the bottom of the figure.

The position of 5' ends of the left-hand exons of the 1.3- and 2.0-kb RNAs were determined by probes 5'-end labeled at the *Bam*HI (map position, 0.15), *Eco*RI (map position, 0.18) and *Hind*III (map position, 0.19) sites. Shown in Fig. 3, panel A, are the results of probes labeled at the *Hind*III site as an example. The S1 digests reveal only one major band of 0.95 kb and several minor bands in all tumors. The minor bands were also observed when hybridizations were carried out at 45°C or when S1 digestion was with 1,000 or 8,000 Miles units of S1 per ml. However, few minor bands were detected when hybridization was carried out at 55°C. With the *Eco*RI and *Bam*HI 5'-labeled probes, the major bands were 1.0 and 0.65 kb, respectively (data not shown). Thus the three different probes mapped the 5' end to position 0/1.0.

Exo VII digestion of the *Hind*III probe hybridizations (Fig. 3, panel A) showed the same major band as S1 digestion; however, the CP contained a second weak band of about 1.4 kb, and the end of the parent RNA maps to position 0/1.0.

Based on the subgenomic probe hybridizations of the Northern blots (Fig. 2), we would have expected to see two bands in S1 digestions, at least with the *Hind*III and *Eco*RI labeled probes. This is so since both the 2.0- and 1.3-kb RNAs hybridized to probes representing segments to the left and right of the *Eco*RI site (Fig. 3; 17). When probes labeled at *Hin*fI sites were used, the 5' ends of both RNAs could be

FIG. 3. Mapping of CRPV transcripts with end-labeled probes. Poly(A)⁺ RNA isolated from CPs, DPs, or DCs or tRNA was hybridized to different 3'- or 5'-end-labeled probes, digested with nuclease S1 or Exo VII, and analyzed on acrylamide-urea gels (panels A, B, C, F, G, H, and J) or on neutral (panels D and E) or alkaline agarose (panel J) gels as described in the text. The top section shows autoradiograms which were obtained after exposure with amplifying screens for 18 h (panels A, B, D, E, F, G, H, and J) or 3 days (panel I) or without an amplifying screen for 3 days (panel C). The map positions of the labeled ends of the hybridizing probe strands are indicated below the autoradiograms. The probes used in the autoradiograms shown were: A, *Hind*III 0.19 to *Hind*III 0.77 (the same results were obtained with the probe recut at *Bam*HI 0.15); B (left), *Hinf*I 0.21 to *Hinf*I 0.12 (the same results were obtained with the probe recut at *Bam*HI); B (right), *Hinf*I 0.12 to *Hinf*I 0.04; C, *Hind*III 0.19 to *Hind*III 0.26; D, *Bst*EII 0.54 to *Sal*I 0.63; E, *Bst*EII (full-sized recombinant DNA with pBR322 inserted at the *Eco*RI site); F, same as D; G (left), same as E; G (right), *Bam*HI 0.86 to *Bgl*II 0.02; H, *Bam*HI 0.86 to *Eco*RI 0.18; I and J, *Sal*I 0.63 to *Eco*RI 0.18 and extending 650 nucleotides to the *Sal*I site in pBR322. The molecular weight markers in the tracks labeled M were end-labeled fragments of *Hind*III-digested λ DNA with sizes of 23.1, 9.41, 6.55, 4.37, 2.32, 2.02, and 0.564 kb; *Hae*III-digested replicative form of ϕ X174 with sizes of 1.353, 1.078, 0.872, 0.603, and 0.310 kb and weak bands at 0.281 and 0.271 kb; *Msp*I-digested pBR322 DNA with sizes of 622, 527, 403, 309 (not resolved from the 310-base-pair band of *Hae*III-digested replicative form of ϕ X174), 243, 238, 217, 201, 190, 180, 160 (double band), 147 (double band), 122, 100, 90, and 76 bases. In panels A, B, C, G, and I, both ϕ X174 and pBR322 markers are present; the smallest markers shown are 122, 147, 122, 147, and 76 bases, respectively. In panels F and H, only pBR322 markers are present, and the smallest ones are 76 and 110 bases, respectively. In panel D, the markers are λ DNA *Hind*III digested. In E and J, the λ DNA *Hind*III and the four largest ϕ X174 replicative form *Hae*III marker positions are indicated to the right of the panels. The center portion shows a restriction map of CRPV and a map of CRPV transcripts. The results of Northern blot hybridizations (Fig. 2) were combined with those of nuclease S1 and Exo VII mapping (top of figure). Exons are marked by heavy lines, and introns are marked by light brackets. The mapped ends of exons are marked by vertical bars, and the ends of exons not mapped are indicated by extending the exon line as a dashed line. Dashed intron brackets in the 4.8-kb RNA indicate that some RNA molecules do not have introns. The arrowheads point from the end of the labeled probe in the direction of probe extension. The bottom portion depicts open reading frames of CRPV DNA. The reading direction is from left to right; the vertical arrowheads indicate the positions of potential polyadenylation sites (AATAAA sequences) (Giri et al., submitted for publication).

mapped. Hybridization with *Hinf*I fragment 0.04 to 0.12 (Fig. 3, panel B, right side) again identified the 5' end of the 2.0-kb RNA at map position 0.07. Further, in DC particularly, a second minor 2.0-kb RNA species exists with a 5' end 70 bases upstream. With *Hinf*I fragment 0.12 to 0.21 (Fig. 3, panel B, left side), a major 0.4-kb band is present, mapping the 5' end of the 1.3-kb RNA to position 0.16. In Fig. 3, panel B, left side, the bands visible in panel B, right side, are detectable as weak bands due to a minor contamination with the other *Hinf*I probe. The detection of the 5' end of the 1.3-kb RNA with the *Hinf*I probe, but not with the *Hind*III and *Eco*RI probes, suggests that unusual sequences near the two sites prevent stable hybrid formation.

The 3' ends of the 5'-proximal exons were mapped with probes labeled 3' at the *Hind*III (map position, 0.19) and *Eco*RI (map position, 0.18) sites. A double band of 0.28 kb is present in all tumors (Fig. 3, panel C). The two bands are less than 10 nucleotides different in size. In the CP and DP tumors, the smaller band is more intense, whereas in the DC tumor, both are of about equal intensity. Since the hybridization mixtures appeared to contain about equal amounts of virus-specific RNA, the difference in intensity may be significant, and a tentative conclusion could be that the smaller band represents the 2.0-kb RNA, which is more prominent in papillomas, and the larger band represents the 1.3-kb RNA. Alternately, the exons of both RNAs could have two 3' ends. The hybridizations with *Eco*RI labeled probes mapped the ends of the exons to the same position as did the *Hind*III probes (data not shown).

The ends of the 3'-proximal exons of the 1.3- and 2.0-kb RNAs were mapped with *Bst*E-II labeled probes. The mapping experiments with 5'-labeled probes (Fig. 3, panel F) show two bands of 0.14 and 0.09 kb in all tumors. Since the relative intensity of the bands is very similar in papillomas and carcinomas, the two different ends cannot be assigned uniquely to one or the other RNA species. To show that both the 1.3- and 2.0-kb RNAs were mapped, we analyzed S1 digests on neutral agarose gels (Fig. 3, panel D). In this analysis, the domestic carcinoma revealed two well-defined bands of 0.7 and 1.4 kb, which are the sizes expected for the segments of the 1.3- and 2.0-kb RNAs, respectively. The CP in addition shows a major band (0.5 kb) and two minor bands (0.9 and 1.1 kb). These probably derived from the 4.8- or 0.9-kb RNA or both since the two species are unique to CPs and hybridize strongly to the genomic segment (*Msp*I fragment 4) containing the *Bst*E-II site (Fig. 2B). The 3' ends of the exons were mapped with *Bst*E-II 3'-labeled probes, and the S1 digests revealed one major band of 0.52 kb (Fig. 3, panel G, left side). A weak band with the mobility (but not necessarily the size) of the probe can be seen in all tracks including the control (tRNA); however, this band is distinctly more pronounced in the CP, suggesting that in this tumor, some RNA molecules do not terminate at map position 0.61. The neutral agarose gel analysis (Fig. 3, panel E) of the same S1 digests revealed one strong band of about 0.6 kb in all tumors; in addition, the CP contained a band of 3.6 kb. The relative intensity of the 3.6-kb band to the 0.6-kb band in the neutral agarose gel is higher than the relative intensity of the "probe-sized" band to the 0.52-kb band in the acrylamide gel. This suggests that some of the parent RNA molecules giving rise to the 3.6-kb band have an intron 0.52 kb downstream from the *Bst*E-II site. Finally, a probe labeled 3' at the *Bst*E-II site and extending leftward to the *Eco*RI site did not give rise to any bands (data not shown), and this further confirms that all RNA species which contain the *Bst*E-II site in their exons (4.8-, 2.0-, 1.3-, and 0.9-kb

RNA) have the polarity 5' to 3' from left to right on the map (Fig. 3).

The 3'-proximal exons of the 2.6- and 4.8-kb RNAs were mapped with probes labeled 3' and 5' at the *Bam*HI site (map position, 0.86). The 3'-labeled probe gave rise to a single band of about 1.05 kb (Fig. 3, panel G, right side), mapping the ends of both RNAs to position 0.99. The 5'-end-labeled probe revealed one major band of 0.54 kb (Fig. 3, panel H); in addition, in some analyses (data not shown) a minor probe-sized band could be detected. The results suggest that the majority of the RNA molecules mapping to this area of the genome contain an exon extending 5' from map position 0.79 to map position 0.99 at the 3' end and that a small minority of molecules representing probably a fraction of the 4.8-kb RNA have an exon extending further upstream.

Our Northern blots (Fig. 2) indicated that *Msp*I fragment 8 hybridized only to the 4.8-kb RNA, and therefore, a probe labeled within this segment could be used to map the 4.8-kb RNA. A *Sal*I (map position, 0.63) 5'-labeled probe extending upstream to the *Eco*RI site (map position, 0.18) and 650 base pairs into pBR322 sequences was used. The S1 digest (Fig. 3, panel I, left side) revealed a major band of 0.85 kb, which maps the 5' end of the major exon to position 0.53. A faint minor band has a size of 0.23 kb, locating the end of a minor exon at position 0.61. The analysis of Exo VII digests on acrylamide gels showed the same 0.85-kb band, but the 0.23-kb band could not be detected (Fig. 3, panel I, right side). The Exo VII digests were also analyzed on agarose gels (Fig. 3, panel J). As can be seen, a second band of about 3.4 kb is detectable. The 5' end of the larger protected fragment maps close to the *Eco*RI site, thus linking the major 3'-proximal portion of the 4.8-kb RNA to sequences also transcribed in the 5'-proximal exons of the 1.3- and 2.0-kb RNAs. The relative intensities of the 0.85- and 3.4-kb RNAs have no true significance, since the Exo VII commercially available also has endonuclease activity. With increasing levels of enzyme, Exo VII digests increasingly resemble S1 digests. The presence of this endonuclease activity made it impossible to map across large introns. This difficulty and the apparent colinearity of leader sequences of the 2.6- and 4.8-kb RNAs with the 5' exons of the 1.3- and 2.0-kb RNA have prevented the definitive mapping of the leaders of the 2.6- and 4.8-kb RNA.

DISCUSSION

We have analyzed the viral transcripts in CRPV-induced non-virus-producing benign and malignant domestic rabbit tumors and in virus-producing CPs. The non-virus-producing tumors contain two transcripts of 1.3 and 2.0 kb, and virus-producing CPs contain five transcripts of 4.8, 2.6, 2.0, 1.3, and 0.9 kb. In virus- and non-virus-producing benign tumors, the 2.0-kb RNA is more prominent than the 1.3-kb RNA, whereas the opposite is found in malignant tumors. The transcripts were mapped by Northern blot hybridizations with subgenomic probes and by S1 and Exo VII mapping with 3'- and 5'-end-labeled probes, and the results are summarized in Fig. 3.

When the maps of the transcripts present in non-virus-producing tumors are compared with the map of open reading frames, several facts can be established. The major 5' end of the 2.0-kb RNA maps just within E6, whereas the minor 5' end maps to the end or outside E6. Since the transcript continues uninterrupted by introns past the end of E6, no sequences in addition to E6 could be translated from this RNA. The significance of the increased presence of a slightly larger transcript in the carcinoma cannot be assessed

at present. The 1.3-kb transcript starts toward the 3' end of E6. In both human papillomavirus 1a (7) and bovine papillomavirus type 1 (BPV-1) (6) there are no ATG codons in this segment of E6; the next ATG is in E7, and if the situation in CRPV is equivalent, then translation could start here. Since the 3' end of the exon maps to the end of E7, the amino-terminal portion of the proteins translated from the 1.3-kb RNA could be coded for by E7 and the carboxy-terminal portion could be coded for by E4 or E2 or both.

The 1.3- and 2.0-kb RNA could be generated by two mechanisms: differential splicing or transcription from two different sites. In BPV-1 there is only one TATA-like sequence in this segment of the genome, and it is close to the beginning of E6 (6); in contrast, in human papillomavirus 1a and human papillomavirus 6b there are two such sequences: one again at the beginning of E6 and the other at the beginning of E7 (7, 19). Our experimental evidence suggests that the two transcripts are initiated at different sites. First, in the DP (Fig. 3, panel A) there is no difference between the S1 and the Exo VII digests, indicating that the transcription for the 2.0-kb RNA starts close to the beginning of E6. Second, preliminary primer extension experiments mapped the 5' ends to the same location as S1 experiments. The notion that the 1.3- and 2.0-kb RNAs may be transcribed from different sites, together with the fact that the 2.0-kb RNA is more prominent in papillomas, is particularly intriguing since we have previously found that CRPV DNA in carcinomas is methylated to a higher degree compared with that in papillomas (26). It is possible then that changes in methylation could affect the efficiency of transcription from different sites differentially and thus be responsible for the observed differences in transcription between papillomas and carcinomas.

There are differences between transcripts in non-virus-producing tumors induced by CRPV and transcripts in BPV-1-transformed cells (1, 2, 8, 9, 11). A most striking difference is that there is no major RNA species in CRPV-induced tumors which could code for the large open reading frame E1, a segment of the genome which appears to be required in BPV-1-transformed cells for the maintenance of BPV-1 DNA as a plasmid (12, 16). Further, CRPV tumors also do not contain RNA species which could code for the entire E2 and E4 open reading frames. Finally, it has been proposed that the transcripts in BPV-1-transformed cells have a common 5'-leader sequence of at least 150 base pairs and that the 5' end maps close to the beginning of the E6 open reading frame (1), whereas our data suggest that there is no common leader for the 1.3- and 2.0-kb RNA.

The definitively mapped segments of the 2.6- and 4.8-kb RNAs correspond to 1.59 and 3.7 kb, leaving segments of 0.9 and 1.0 kb, respectively, unmapped. The Northern blots indicate that these segments are present in a part of the genome which is represented in the 1.3- and 2.0-kb RNAs (Fig. 2B). It was further shown by alkaline agarose gel electrophoresis of Exo VII digests that the *Eco*RI site is contained in 5'-proximal sequences of the 4.8-kb RNA (Fig. 3, panel J).

There are some similarities between the RNAs unique to virus-producing tumors induced by CRPV and BPV-1 (9). The 3' exon of the 2.6-kb RNA is equivalent to an RNA mapped in BPV-1-induced tumors: in both cases it starts close to the 5' end, but within the L1 reading frame, and extends beyond the reading frame to a polyadenylation site. The main body of the 4.8-kb RNA is similar to a BPV-1 RNA in that it starts 5' within E2 and E4 and coterminates with the other virus-producing tumor-specific RNA. However, a dif-

ference is that this segment here has one or two small introns, at least in some of the molecules. Particularly significant may be the intron located at map position 0.79, since this intron could link reading frames L1 and L2. Such an RNA could code for proteins larger than L1 or L2, and such larger proteins have been described in BPV-1 and CRPV (10, 15).

Although the mapping of papilloma transcripts is incomplete, at this stage it appears that human papillomavirus-1 (L. T. Chow and T. R. Broker, in M. L. Pearson and N. L. Steinberg, ed., *Gene Transfer and Cancer*, in press) and CRPV resemble each other more closely than they resemble BPV-1.

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LITERATURE CITED

- Ahola, H., A. Stenlund, J. Moreno-Lopez, and U. Pettersson. 1983. Sequences of bovine papillomavirus type 1 DNA—functional and evolutionary implications. *Nucleic Acids Res.* **11**:2639–2650.
- 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.
- Bantle, J. A., I. H. Maxwell, and W. E. Hahn. 1976. Specificity of oligo (dt) cellulose chromatography in isolation of polyadenylated RNA. *Anal. Biochem.* **72**:413–427.
- Berk, J. A., and P. A. Sharp. 1977. Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 nuclease-digested hybrids. *Cell* **12**:721–723.
- Berk, J. A., and P. A. Sharp. 1978. Spliced early mRNAs of simian virus 40. *Proc. Natl. Acad. Sci. U.S.A.* **75**:1274–1278.
- Chen, E. Y., P. M. Howley, A. D. Levinson, and P. H. Seeburg. 1982. Primary structure and genetic organization of the bovine papillomavirus type 1 genome. *Nature (London)* **299**:529–534.
- Danos, O., M. Katinka, and M. Yaniv. 1982. Human papillomavirus 1a complete DNA sequence: a novel type of genome organization among papovaviridae. *EMBO J.* **1**:231–246.
- Engel, L. W., C. A. Heilman, and P. M. Howley. 1982. Virus-specific transcription in bovine papilloma-virus-transformed mouse cells. *Virology* **119**:22–35.
- Engel, L. W., C. A. Heilman, and P. M. Howley. 1983. Transcriptional organization of bovine papillomavirus type 1. *J. Virol.* **47**:516–528.
- Favre, M., F. Breitburd, O. Croissant, and G. Orth. 1975. Structural polypeptides of rabbit, bovine, and human papillomaviruses. *J. Virol.* **15**:1239–1247.
- Freese, U. K., P. Schulte, and F. Pfister. 1982. Papilloma virus-induced tumors contain a virus-specific transcript. *Virology* **117**:251–257.
- Lusky, M., and M. R. Botchan. 1984. Characterization of the bovine papillomavirus plasmid maintenance sequences. *Cell* **36**:391–401.
- Maxam, A., and W. Gilbert. 1980. Sequencing end-labeled DNA with base specific chemical cleavages. *Methods Enzymol.* **65**:499–560.
- McMaster, G. K., and G. G. Carmichael. 1977. Analysis of single and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridin orange. *Proc. Natl. Acad. Sci. U.S.A.* **74**:4835–4838.

15. **Meinke, W., and G. Meinke.** 1981. Isolation and characterization of the major capsid protein of bovine papillomavirus type 1. *J. Gen. Virol.* **52**:15–24.
16. **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.
17. **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.
18. **Rigby, P. W., M. Dieckmann, C. Rhodes, and P. Berg.** 1977. Labeling deoxyribonucleic acid to high specific activity in vitro by nicktranslation with DNA polymerase I. *J. Mol. Biol.* **113**:237–251.
19. **Schwarz, E., M. Dürst, C. Demankowski, O. Lattermann, R. Zech, E. Wolfsperger, S. Suhai, and H. zur Hausen.** 1983. DNA sequence and genome organization of genital human papillomavirus type 6b. *EMBO J.* **2**:2341–2348.
20. **Sharp, P. A., B. Sugden, and J. Sambrook.** 1973. Detection of two restriction endonuclease activities in *Haemophilus parainfluenzae* using analytical agarose-ethidium bromide electrophoresis. *Biochemistry* **12**:3055–3063.
21. **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.
22. **Weaver, R. F., and C. Weissmann.** 1979. Mapping of RNA by a modification of the Berk-Sharp Procedure: the 5' termini of 15s β -globin mRNA precursor and mature 10s β -globin mRNA have identical map coordinates. *Nucleic Acids Res.* **7**:1175–1193.
23. **Wettstein, F. O., and J. G. Stevens.** 1980. Distribution and state of viral nucleic acid in tumors induced by Shope papilloma virus, p. 301–307. *In* M. Essex, G. Todaro, and H. zur Hausen (ed.), *Viruses in naturally occurring cancers*, vol. 7. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **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.
25. **Wettstein, F. O., and J. G. Stevens.** 1982. Variable size 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.
26. **Wettstein, F. O., and J. G. Stevens.** 1983. Shope papilloma virus DNA is extensively methylated in nonvirus-producing neoplasms. *Virology* **126**:493–504.