Transcriptional Organization of Bovine Papillomavirus Type 4

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SUMMARY

Seven virus-specific RNA transcripts have been identified in tumours induced by bovine papillomavirus type 4 (BPV-4). The RNAs measured 4.2, 3.6, 3.0, 2.8, 1.9, 1.6 and 1.0 kilobases (kb). They were mapped on the viral genome by Northern blot hybridization to subgenomic probes, by cDNA hybridization to viral DNA fragments and by S1 analysis of unlabelled and 3' and 5' end-labelled DNA fragments. All the RNA species are transcribed from the same DNA strand, are polyadenylated and with the exception of the 1.0 kb RNA internally spliced. The 3.0, 1.9, 1.6 and 1.0 kb RNAs share the same 3' polyadenylation site at nucleotide 4009 whereas the 4.2 kb RNA and the 2.8 kb RNA terminate near a polyadenylation site at nucleotide 7187. The 4.2 kb and the 2.8 kb RNAs are transcribed from the late open reading frames and encode the structural polypeptides; the 3.0, 1.9, 1.6 and 1.0 kb RNAs are transcribed from the early open reading frames and are the transcripts involved in viral replication and cellular transformation.

INTRODUCTION

Papillomaviruses are implicated in the aetiology of squamous cell carcinomas both in humans and in animals. Although a multiplicity of papillomaviruses exists, only a few virus types are associated with naturally occurring cancers.

These include several human papillomaviruses (HPV) (Gissmann, 1984), the cottontail rabbit papillomavirus (CRPV) (Rous & Beard, 1938) and the bovine papillomavirus (BPV) type 4 (Campo et al., 1980). BPV-4 induces epithelial papillomas of the upper alimentary canal which become the focus for malignant transformation into squamous carcinomas in cattle feeding on bracken fern (Jarrett et al., 1978, 1980). BPV-4 is one of the members of subgroup B of bovine papillomaviruses, which cause proliferation of epithelial cells only, have a small genome, and are evolutionarily distant from the viruses of subgroup A, all of which induce proliferation of both fibroblasts and keratinocytes (Jarrett et al., 1984). Subgroup A includes BPV-1, probably the best known of all papillomaviruses. As no papillomavirus replicates productivity in cell culture, virus functions have to be analysed in papillomas and in cells transformed in vitro. The association of BPV-4 with malignancies prompted us to investigate transcription of its genome in productive alimentary canal papillomas, and to identify the viral products responsible for keratinocyte transformation. By Northern blot hybridization and S1 nuclease analysis, we have identified seven viral mRNAs and have constructed their physical map. They have been assigned to the late (L) and early (E) open reading frames (ORFs), identified by nucleotide sequencing and by comparison of the amino acid sequences of BPV-4 with those of other papillomaviruses (Danos & Yaniv, 1984; Danos et al., 1984; Schwarz et al., 1983). Expression of one of the early mRNAs is confined to keratinocytes.

METHODS

RNA purification. Total cellular RNA was prepared from papillomas of the upper alimentary canal by the method of Chirgwin et al. (1979). Frozen tumours were ground in 4 M-guanidinium thiocyanate solution and the RNA was purified from cellular debris and DNA by centrifugation through a 5-20% CsCl cushion. The pellet was...
resuspended in 7 M-guanidine hydrochloride and the RNA was re-precipitated by adding 0.025 vol. 1 M-acetic acid and 0.5 vol. absolute ethanol. The solution was kept at −20 °C for at least 4 h before centrifugation. The final pellet was resuspended in sterile water and stored at −20 °C. The polyadenylated RNA [poly(A) RNA] was purified by chromatography on oligo(dT)-cellulose columns twice (Chirgwin et al., 1979).

**RNA blot hybridization.** Total or polyadenylated RNAs were fractioned by electrophoresis in 1.4% agarose gels containing 2-2 M-formaldehyde (Lehrach et al., 1977). After electrophoresis, rRNA markers were stained with 1 µg/ml ethidium bromide. The RNA was transferred directly to Pall Biodyne A nylon membranes in 20 × SSC and hybridized to 32P-labelled nick-translated total BPV-4 DNA or subgenomic clones (Rigby et al., 1977; Thomas, 1980).

**Nomenclature and numbering system.** The circular 7261 bp genome of BPV-4 has been opened at the end of the late open reading frames; thus, putative promoter sequences are positioned 5' to the early genes. Coordinates of restriction endonuclease sites are given as the first nucleotide of the recognition sequence.

**Splice donor and acceptor sites** are numbered from the nucleotide preceding or following the cut respectively. A full description of the genomic sequence and organization of BPV-4 will be published elsewhere by K. R. Patel & M. S. Campo.

**Molecular cloning of viral fragments.** Subgenomic fragments of BPV-4 were prepared by the cleavage of the whole recombinant pBV4 plasmid (Campo & Coggins, 1982) with appropriate restriction endonucleases. The digested DNA was electrophoresed in low melting point (LMP) agarose gels and the required fragment was eluted from the gel and purified (Weislander, 1979). The DNA fragments were ligated to the vector pAT153 (Twigg & Sherratt, 1980) cleaved with the appropriate restriction endonuclease, and recombinant plasmids were identified as described previously (Campo & Coggins, 1982). The subclones are referred to by the first letter of the restriction endonuclease sites bordering the fragments and by the size in kilobases (kb) with nucleotide positions in brackets.

**DNA blot hybridization.** Molecularly cloned BPV-4 DNA (Campo & Coggins, 1982) or derived subclones were digested with the appropriate restriction endonucleases, subjected to electrophoresis and transferred to Pall Biodyne A nylon membranes (Southern, 1975). Hybridization was done following the manufacturers' recommendations.

**Synthesis of cDNA probes.** Oligo(dT)-primed cDNA was prepared according to Amtmann & Sauer (1982) except that 2 µg poly(A) RNA was used and the concentration of deoxynucleotides was limited to ensure that the cDNA represented only the 3' end of mRNA.

**5' end-labeling of mRNA.** Polyadenylated RNA from papillomas was decapped and dephosphorylated with calf intestinal alkaline phosphatase (Boehringer), phenol-extracted and re-precipitated with ethanol. The 5' ends were labelled using T4 polynucleotide kinase and [γ-32p]ATP (sp. act. 5000 Ci/mmol, Amersham). The RNA was partially degraded to an average size of 250 bases by treatment with 0.2 M-KOH at 0 °C for 70 s, neutralized with 0.1 M-KH2PO4, and hybridized to subgenomic restriction fragments of BPV-4 DNA immobilized in a nylon membrane.

**S1 nuclease analysis.** S1 nuclease analysis of viral RNA was performed by the method of Berk & Sharp (1977) as described by Favaloro et al. (1980). Mapping of the 3' and 5' ends of the RNAs was achieved using end-labelled probes (Weaver & Weissman, 1979). Required viral DNA fragments were obtained by cleaving the recombinant plasmids with appropriate restriction endonucleases, and by fractionation in LMP agarose gels (Weislander, 1979). For 5' end-labeling, the purified DNA was treated with calf intestinal alkaline phosphatase (Boehringer), phenol-extracted twice and re-precipitated with ethanol. The 5' ends were labelled by the use of T4 polynucleotide kinase and [γ-32P]ATP as above. 3' ends were labelled using the Klenow fragment of DNA polymerase I and the appropriate radioactive dNTP.

Ten to 20 ng of probe DNA, 10 or 20 µg of total RNA, or 5, 10 or 20 µg of poly(A) RNA, and λRNA to total 25 µg were ethanol-precipitated and redissolved in 10 µl of hybridization buffer consisting of 80% denaturated formamide, 0.4 M-NaCl, 40 mM-PIPES pH 6.5, and 1 mM-EDTA. Samples, in screw-cap Eppendorf tubes, were denatured at 85 °C for 15 min and hybridized at 57 °C for 3 h. Hybridization was stopped by adding 0.3 ml ice-cold S1 buffer (280 mM-NaCl, 30 mM-sodium acetate pH 4.4, 4.5 mM-zinc acetate), 20 µg/ml denatured calf thymus DNA, and 400 units/ml S1 nuclease (Boehringer) and incubated at either 20 °C or 37 °C for 1 h or 30 min respectively. Digestion products were precipitated with ethanol and trRNA as carrier. Analysis of the digestion products was on neutral gels or denaturing urea/polyacrylamide gels. In experiments where no labelled probe was used, the digestion products were transferred to Pall Biodyne A nylon membranes and hybridized with appropriate nick-translated probes. Where radioactive probes were used the gels were dried and autoradiographed.

**RESULTS**

**Identification and mapping of viral mRNA**

The number and sizes of the BPV-4-specific RNAs in productive papillomas were determined by Northern blot analysis. Total RNA from papillomas was fractionated on a denaturing
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agarose gel, transferred to nylon membranes and hybridized to either the complete BPV-4 genome or subgenomic fragments labelled with $^{32}$P.

When probing with the whole BPV-4 genome, seven transcripts were observed with sizes of 4.2, 3.6, 3.0, 2.8, 1.9, 1.6 and 1.0 kb (Fig. 1a). In some RNA preparations the 3.6 and 1.9 kb transcripts were absent or present at very low levels (data not shown). Each of these virus-specific RNAs was present in the polyadenylated fraction of the papilloma RNA as shown by their retention on an oligo(dT)-cellulose column (data not shown). The hybridization pattern of the mRNAs to subgenomic viral DNA probes (Fig. 1a, lanes 2 to 7) indicated that many of these transcripts were spliced since DNA from widely separate regions of the viral genome hybridized to the same RNAs. For example the 1.9 kb RNA appeared to contain sequences complementary to many of the subgenomic fragments. However, two major mRNAs, 2.8 kb and 1.0 kb long, appeared to be transcribed from two discrete regions of the genome: from subclones H-H2.2 (bases 3705 to 5954) and H-E2.0 (6106 to 904), and from B-H1.0 (2601 to 3705) and H-H2.2 respectively (Fig. 1b).

Nuclease S1 analysis of DNA/RNA hybrids

The transcribed regions of the viral genome were mapped more precisely using S1 nuclease digests of DNA/RNA hybrids. The several subgenomic viral DNA fragments were denatured and hybridized to either total or poly(A) RNA. The hybrids were digested with S1 nuclease and the protected fragments were separated on agarose gels. After transfer to nylon membranes the DNA was probed with $^{32}$P-labelled BPV-4 DNA. A 1.0 kb fragment deriving from E-B1.6 (1142 to 2601) (Fig. 2a, lane 2) and a 0.75 kb fragment from H-H3.6 (6106 to 2360) (Fig. 2a, lane 10) were detected, consistent with the length difference of 241 bp at the 3' end of the subclones. In addition, a strong band of 1.2 kb was seen with both H-E2.0 (6106 to 904) and H-H3.6 (Fig. 2a, lanes 8 and 10), and therefore RNA must be transcribed from the overlapping region of the two subclones. Fragment P-P1.6 (5403 to 7126) consistently produced a band of 1.5 kb, nearly the full length of the probe (Fig. 2a, lanes 8 and 10), and therefore RNA extends approximately 600 bases 5' to the H-E2.0 fragment.

The 1.0 kb transcript would be expected to protect approximately 1.0 kb of a DNA fragment spanning subclones B-H1.0 (2601 to 3705) and H-H2.2 (3705 to 5954), and indeed a strong band of 700 bp was seen when such a fragment was used (Fig. 2a, lane 4). The difference of approximately 300 bases between the size of the transcript and the size of the protected fragment may indicate that the RNA is spliced, e.g. has a discontinuous leader sequence, or may be due to the presence of a poly(A) tail on the RNA.

Mapping of the 3' ends

To identify the polyadenylated 3' ends of the viral mRNA, short $^{32}$P-labelled cDNA (Amtmann & Sauer, 1982) representing the 3' ends of the papillomavirus RNA was used to probe Southern blots of BPV-4 fragments. Two major signals were obtained, one in fragment P-P1.0 (4334 to 5403) and one in fragment H-P0.63 (3705 to 4334) (Fig. 3a, lanes 3 and 2). In confirmation, a virus-specific oligo(dT)-primed cDNA from papilloma RNA (4A6) mapped to H-P0.63 and was shown by sequence analysis to contain the polyadenylation signal at nucleotide 4009. However, the hybridization of labelled cDNA to P-P1.0 may have been spurious binding to the several A-T regions present in this subclone which therefore may not contain a true 3' terminus.

The positions of the 3' ends and the splice sites of the viral mRNA were established by S1 nuclease analysis of 3' labelled DNA fragments (Weaver & Weissman, 1979). The protected labelled fragments were analysed by acrylamide gel electrophoresis and visualized by autoradiography.

Subclone E-B1.6 (1142 to 2601) hybridized to a fragment of 1.1 kb, placing the 3' end at position 2242 (Fig. 4a, lane 4) near a splice donor site (AAG/GTGTAG) at nucleotide 2188. Similarly, a fragment of 335 bases was protected in subclone B-H1.0 (2601 to 3705) (Fig. 4a, lane 8) mapping to nucleotide 2936 with a splice donor site identified at nucleotide 2932 (AAG/GCTAGT). Therefore, these 3' ends are likely to represent splicing rather than termini,
Fig. 1. Mapping of BPV-4-specific transcripts in oesophageal papillomas. (a) Northern blots were prepared by electrophoresing 250 μg of denatured total papilloma RNA across a 12 cm well. After transfer the membrane was cut into 0.5 cm strips and hybridized to probes shown diagrammatically in (b): lane 1, pBV4; lane 2, E-B1.6; lane 3, B-H1-0; lane 4, H-H2-2; lane 5, P-P1-0; lane 6, H-E2-0; lane 7, 4A6. (b) In this and subsequent figures, a schematic representation of the BPV-4 genome and subgenomic probes is shown. The map positions of BamHI, EcoRI, HindIII, and the relevant PstI restriction enzyme sites are shown as B, E, H and P respectively and sizes are given in bp. Established ORFs are shown above the restriction map with the positions of the first and last nucleotide (K. R. Patel & M. S. Campo, unpublished). Clone 4A6 is a cDNA derived from papilloma RNA containing a polyadenylation site at nucleotide 4009 within subclone H-H2-2.
and this was confirmed by the lack of hybridization of cDNA to these regions of the viral genome (Fig. 3).

A fragment of 325 bases mapping to nucleotide 4030 was protected in subclone H-H2·2 (3705 to 5954) (Fig. 4a, lane 6) and in subclone H-P0·63 (3705 to 4334) (Fig. 4a, lane 14) but not in

Fig. 2. Nuclease S1 analysis of DNA/RNA hybrids. (a) Subgenomic probes were denatured and hybridized to papilloma RNA; after digestion with S1 nuclease the fragments were separated in agarose gels, transferred to nylon membranes and probed with 32P-labelled BPV-4 DNA. Lanes 1 and 2, E-B1·6; lanes 3 and 4, fragment 18; lanes 5 and 6, P-P1·6; lanes 7 and 8, H-E2·0; lanes 9 and 10, H-H3·6; odd-numbered lanes contained no RNA; lane 2, 5 μg poly(A) RNA; lanes 4, 6, 8 and 10, 20 μg total RNA. The numbers represent the sizes in bp of pAT153 digested with HinfI. (b) Described in Fig. 1.
Fig. 3. Hybridization of cDNA to BPV-4 DNA. (a) Short oligo(dT)-primed \(^{32}\)P-labelled cDNA was hybridized to BPV-4 fragments immobilized on a nylon membrane. Lane 1, H-E2-0, restricted with \(PstI\); lane 2, H-H2-2 restricted with \(PstI\); lane 3, P-P1-0; lane 4, fragment 18 restricted with \(HindIII\); lane 5, E-H0-5; lane 6, B-E0-5. Fragments showing significant hybridization are shown below the restriction map in (b).

Subclone P-H0-55 (5403 to 5954) (Fig. 4a, lane 12), thus showing that it derived from the 5' end of H-H2-2. A polyadenylation site has been found at position 4009 (ATTAAA), and this, in conjunction with the strong hybridization of cDNA to this fragment confirmed that this site was a 3' terminus.
Subclone P-P1-0 (4334 to 5403), which hybridized only to the 4.2 kb transcript (Fig. 1a, lane 5), produced a fragment of 870 bases (Fig. 4a, lane 10), terminating at position 5204 corresponding to a splice donor site at 5225 (AAG/GTCAGA).

In agreement with the results obtained with the same unlabelled fragments (Fig. 2a, lanes 8 and 10), subclones H-E2-0 (6106 to 904) and H-H3-6 (6106 to 2360), which have a common 5' end, produced a fragment of 1.2 kb (Fig. 4a, lane 2; H-H3-6 not shown) terminating at nucleotide 45, near to positions 7151 and 7187 where two more polyadenylation sites (AATAAAA) were located. We conclude that this site is a 3' terminus. The other protected fragment of 700 bases was not consistently observed and may represent alternative splicing.

A 128 base fragment was protected in E-E0-23 (903 to 1142) which mapped at nucleotide 1031 near to a splice donor site at 1016 (AAG/GTACTA) (Fig. 4a, lane 17).

5' ends of the viral RNA

To identify the 5' ends of the major viral transcripts, the RNA was labelled at the 5' end, partially degraded and hybridized to subgenomic restriction fragments of BPV-4 DNA. The partial degradation ensured monitoring of the hybridization of the labelled terminus only. The RNA hybridized most strongly to H-H2-2 (3705 to 5954) and H-P0-63 (3705 to 4334) and E-H0-55 (3151 to 3705) (Fig. 5a, lanes 4, 6 and 7) indicating that the 5' termini of the most abundant viral transcripts mapped between nucleotides 3151 and 4334.

S1 nuclease analysis of 5' end-labelled subgenomic DNA probes established the 5' termini and identified the splice sites. In agreement with the results obtained with the same unlabelled subclone (Fig. 2a, lane 10), labelled H-H3-6 (6106 to 2360) hybridized to a fragment of 750 bases (Fig. 6a, lane 2) the 5' end of which mapped to nucleotide 1610 near a possible splice acceptor site at 1553 (CTTTGACAAG/A). Subclone B-H1-0 (2601 to 3705) produced a 350 bp fragment (Fig. 6a, lane 4) ending at nucleotide 3355, subclone H-H2-2 (3705 to 5954) a 400 bp fragment mapping to nucleotide 5554 (Fig. 6a, lane 6) and H-E2-0 (6106 to 904) three fragments of 700, 550 and 250 bases (Fig. 6a, lane 8) mapping to nucleotides 204, 354 and 654 respectively. Splice acceptor sites have been found at positions 3376 (CTGTTACCAG/T) and 5595 (TGTTTTTCAAG/A), but not around positions 204, 354 or 654, indicating that the 5' ends at 3355 and 5554 may have arisen from splicing, whereas those at 204, 354 and 654 may be 5' termini.

Another splice acceptor site has been found at nucleotide 4028 (GTTCGTGCAG/C), at the start of the L2 ORF. Although not mapped by S1 analysis, this site is probably used, as indicated by the protection of 870 bases at the 3' end of the P-P1-0 subclone: transcription must span the PstI site at 4334 for this fragment to be protected.

Potential CAAT and TATA promoter sequences were found at nucleotides 86 (CAAAAT) and 115 (TATAAA), supporting the conclusion that transcription initiates in this region.

DISCUSSION

We have identified seven BPV-4-specific transcripts in virus-producing epithelial papillomas of the upper alimentary canal of cattle. The three major RNAs of 4.2, 2.8 and 1.0 kb and the two minor species of 3.0 and 1.6 kb are invariably present in the RNA population of all papillomas examined, whereas the 3.6 and 1.9 kb RNAs are present in low amounts and are occasionally absent in some papillomas. As the production of viral progeny is strictly dependent on cell differentiation (Orth et al., 1971), the different relative amounts of viral transcripts may reflect the age of the tumour and the degree of keratinization. However, we have not attempted to follow the fate of the viral RNA as the papillomas age or to establish their location in the several cell layers within the tumour.

In agreement with findings in other papillomaviruses only one strand of BPV-4 DNA is transcribed; all the viral RNAs are polyadenylated and, with the exception of the 1.0 kb RNA, internally spliced. The pattern of splicing is complex and there is considerable overlap between the bodies of the several RNA species (Fig. 7).

The late and the early RNAs have been identified by comparing the sequence of amino acids encoded by the transcribed regions of the BPV-4 genome with that of other papillomaviruses.
Thus, the 2.8 kb and the 4.2 kb RNAs have been identified as structural, whereas the 3.0, 1.9, 1.6 and 1.0 kb RNAs are involved in viral replication and transformation.

As the map coordinates defined by the nucleotide sequence are more accurate than those calculated from the size of the S1-protected fragments, we shall use the former in the description of the structure of the RNAs.

The 2.8 kb transcript encodes the L1 ORF (5550 to 7112); it is continuously transcribed from nucleotide 5595 to nucleotide 45, 119 bases downstream from the polyadenylation site at 7187 (Fig. 2a, lane 6; 4a, lane 2; 6a, lane 6). The 5' end of the 2.8 kb RNA most likely consists of 812 bases from position 204 to 1016 (Fig. 6a, lane 8), since no other region of the BPV-4 genome hybridized with the transcript (Fig. 1).

The 4.2 kb transcript starts at nucleotide 654 and is spliced three times: from nucleotide 1016 to nucleotide 1553, from 2932 to 4028 and from 5225 to 5595. The first 1.5 kb are transcribed from the early region; after the second splice the RNA encodes the L2 ORF (3991 to 5085) (Fig. 1a, lane 5; 2a, lane 2; 4a, lane 2; 6a, lane 6); after the third splice it encodes a 1.8 kb sequence from L1 which is shared with the 2.8 kb transcript, and it ends at the same polyadenylation site.

Despite differences in their molecular weight, the structural RNAs of BPV-4, BPV-1 and CRPV show a similar organization: transcripts of 2.8, 1.7 and 2.6 kb respectively span the L1 ORF, whereas a larger transcript, of 4.2, 3.8 and 4.8 kb respectively, covers both the L2 and L1 ORFs. None of the viruses produces a transcript encoding the L2 ORF alone (Engel et al., 1983; Nasseri & Wettstein, 1984; Phelps et al., 1985).

The 4.2 kb RNA of BPV-4 is the only transcript that contains information from the L2 ORF. It may code for an L1/L2 fusion peptide; capsid proteins larger than both the L1 and the L2 proteins have been observed in papillomaviruses (Favre et al., 1975) and it has been suggested that the removal of small introns from the 4.8 kb RNA of CRPV leads to the production of an L1/L2 fusion protein (Nasseri & Wettstein, 1984).
Fig. 5. Hybridization of 5'-end labelled RNA to BPV-4 DNA. (a) Partially degraded 5'-end-labelled RNA was hybridized with subgenomic BPV-4 restriction fragments immobilized in a nylon membrane. Lane 1, E-B1-6; lane 2, H-E2-0 digested with EcoRI and PstI; lane 3, P-P1-6 digested with HindIII; lane 4, H-H2-2; lane 5, P-P1-0; lane 6, H-H2-2 digested with PstI; lane 7, B-H1-0 digested with HindIII and EcoRI. Fragments showing significant hybridization are shown below the restriction map. (b) Described in Fig. 1.

The transcriptional organization of the region of the BPV-4 genome encoding the replication and transformation functions is similar to that of BPV-1 and CRPV (Danos et al., 1985; Nasseri & Wettstein, 1984; Phelps et al., 1985; Yang et al., 1985). The early RNAs show extensive sequence overlap and use the common poly(A) site at position 4009.

The 1-0 kb RNA is not spliced (Fig. 1, 2), it starts at nucleotide 3376 and terminates at 4030. The 3-0, 1-9 and 1-6 kb RNAs have different 5' ends and are internally spliced. The 3-0 kb RNA starts at nucleotide 354 and is spliced twice, from position 1016 to 1553 and from 2932 to 3376. Its main body is transcribed from the E1 ORF (1549 to 2778) and the 680 bases at its 3' end overlap the 1-0 kb RNA. The 1-9 and 1-6 kb RNAs start at 1153 and near nucleotide 1142 respectively; they are both spliced once and utilize the same acceptor site but different donor sites. The splice in the 1-9 kb RNA is the same as the second splice in the 3-0 kb RNA. They
Fig. 6. Mapping of the 5' ends and splice sites of viral RNA. (a) Subgenomic DNA fragments labelled at the 5' end were denatured and hybridized to papilloma RNA and analysed as described in Fig. 4. Lanes 1 and 2, H-H3.6; lanes 3 and 4, B-E1.0; lanes 5 and 6, H-H2.2; lanes 7 and 8, H-E2.0. Odd-numbered lanes, no RNA; even-numbered lanes, 5 µg poly(A) RNA. Molecular weight markers: M1 and M3, φX174 DNA digested with HaeIII; M2 and M4 pBR322 digested with HaeIII. The sizes are given in nucleotides (M2, M4) and kb (M1 and M3). The protected DNA segments are shown below the subgenomic fragments and their labelled 5' end is marked (×). (b) Described in Fig. 1; splice acceptor sites are indicated (△); C and T, CAT box and TATA box respectively.

resemble the 1.8 and 1.1 kb transcripts of CRPV (Danos et al., 1985; Nasseri & Wettstein, 1984), and similar RNAs have been reported for BPV-1 and HPVs (Chow & Broker, 1984; Lehn et al., 1985; Yang et al., 1985).
Non-spliced precursor RNAs may exist but we have not detected them. The 3.6 kb RNA has only been observed sporadically and has not been mapped. Our data cannot eliminate alternative splice patterns. For instance, an additional 1.6 kb RNA may exist, which would be transcribed from nucleotide 204 to nucleotide 1016 (812 bp), spliced to nucleotide 3376 and further transcribed to nucleotide 4030 (654 bp).

The 3.0 kb and 1.6 kb transcripts are the major viral RNAs present in NIH 3T3 mouse fibroblasts transformed in vitro by BPV-4 DNA, whereas the 1.0 kb RNA, which is the most abundant species in papillomas, is not present (K. T. Smith & M. S. Campo, unpublished results). Interestingly, a similar RNA of 0.9 kb transcribed from the same region of the genome is found in productive CRPV papillomas only (Nasseri & Wettstein, 1984), suggesting that transcription of these RNAs is keratinocyte-specific. It is possible that the 1.0 kb RNA of BPV-4 encodes a product necessary for the expression of papilloma-specific viral functions, similar to the E4 product recently described in HPV-1-induced warts (Doorbar et al., 1986).

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