Identification and Mapping of Human Papillomavirus Type 1 RNA Transcripts Recovered from Plantar Warts and Infected Epithelial Cell Cultures

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Multiple spliced transcripts of human papillomavirus type 1 were detected by electron microscopic analysis of R-loops formed with total RNA extracted from plantar warts and with poly(A)⁺ RNA isolated from cultured keratinocytes infected with human papillomavirus type 1. The 5' ends of the RNAs were mapped to sites in the E7 open reading frame (ORF), just upstream of the E6 ORF and in the upstream regulatory region. Species with 5' ends in E7 accounted for over 95% of all transcripts seen. Two polyadenylation sites were used, one at the end of the early (E) region of the viral DNA, the other at the end of the late (L) region. The most abundant species had a short 5' exon of approximately 100 nucleotides spanning the junction of the E7 and E1 ORFs spliced to a 3' exon of 800 nucleotides in the region with overlapping E2 and E4 ORFs; it was polyadenylated at the end of the E region. This species probably encodes the abundant E4 protein found in plantar warts (F. Breitburd, O. Croissant, and G. Orth, *Cancer Cells*, vol. 5, in press; J. Doorbar, D. Campbell, R. J. A. Grand, and P. H. Gallimore, *EMBO J.* 5:355-362, 1986). Other transcripts had exons spanning the E6-E7 ORFs, the E4-E5-L2-L1 ORFs, or the L1 ORF. The infrequent L1 transcript, probably the mRNA coding for the major capsid protein, had the same 5' exon in E7 as the abundant mRNA spliced from E1 and E4 ORFs, suggesting genetic regulation via the choice of the alternative polyadenylation sites or mRNA processing.

Papillomaviruses are small DNA viruses containing circular chromosomes of approximately 7,900 base pairs (bp). They infect humans and animals with strict host and tissue specificity. Over 40 types of human papillomaviruses (HPV) have been molecularly cloned, characterized, and linked to specific infectious diseases involving benign or malignant hyperproliferation of either mucosal or cutaneous epithelia at different body sites (for reviews, see references 2, 22, 38, 46; L. B. Taichman and R. F. LaPorta, in P. M. Howley and N. P. Salzman, ed., *The Papovaviridae: the Papillomaviruses*, in press). Despite their ubiquitous distribution and acute medical impact, molecular and genetic studies have, until recently, been largely precluded since the viruses have never been successfully propagated in cell culture. HPV type 1 (HPV-1) is associated with plantar warts and is not known to have malignant potential. Unlike lesions caused by other papillomaviruses, plantar warts produce a large number of virus particles that can be purified by banding in CsCl gradients. Infection of cultured keratinocytes with HPV-1 virions results in transient HPV-1 DNA replication which is eventually lost from the culture (24, 30). Apparently virus development is closely linked to unknown aspects of epithelial cell differentiation which currently cannot be reproduced in vitro. The DNA sequences of many human and animal papillomavirus genomes have been determined (4, 10, 13, 15, 18, 20, 21, 33, 35). They possess similar genetic organization of regulatory regions and open reading frames (ORFs) that are distributed along a single transcribed strand of their genomes (Fig. 1). With few exceptions, HPVs do not transform cells in culture (3, 9, 40-42, 45). In contrast, bovine papillomavirus type 1 (BPV-1) readily induces cellular transformation in certain mouse cell lines, C127 and NIH 3T3,

and the transfecting DNA remains as extrachromosomal plasmids, as found in vivo (17, 25). Therefore, BPV-1 has been used as a model system for the genetic analysis of papillomaviruses.

The BPV-1 genome has been functionally divided into an early (E) region, a late (L) region, and an upstream regulatory region (URR, synonymous with NCR for noncoding region or LCR for long control region) (for a review, see reference 2). The E region functions are necessary for transformation, plasmid replication, and transcriptional regulation. The L region encodes virion structural proteins. The URR contains an origin of DNA replication, enhancers, several promoters, and the 5' exons for some messages. The L-region ORFs are expressed in virus-producing papillomas, but not in transformed cells in culture. Recent analyses of transcripts and cDNAs of BPV-1 have demonstrated that differential RNA splicing enables some bovine papillomavirus ORFs to be used individually or in combinations to increase the variety of potential viral gene products (1, 37, 44). Analyses of the transcripts of cottontail rabbit papillomavirus isolated from transplantable tumors, papillomas, or carcinomas have also revealed spliced species (12, 19, 26, 27, 29). We report here the characterization by electron microscopic analysis of R-loops formed with HPV-1 RNAs isolated from plantar warts and from keratinocyte cultures infected by HPV-1.

MATERIALS AND METHODS

Symbols for representing ORFs in transcripts. The ^ indicates an RNA splice; the / indicates that one or the other ORF is used in the message; and the - means that the transcript spans the ORFs indicated.

Virus isolation and keratinocyte infection. Virus extraction was carried out as described by LaPorta and Taichman (24).

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FIG. 1. Schematic representation of HPV-1 transcripts mapped by the R-loop method. The HPV-1a DNA restriction map is shown permuted from the conventional *HpaI* site by linearization at the *SacII* site in the URR to reflect the recombinant DNA used for the R-loop analysis. The ORFs were inferred from the DNA sequence. The structures of RNA species detected in two plantar warts and in HPV-1 virion-infected epithelial cell cultures are shown below and are grouped into the E and L region species. Their relative abundances are described in the text. Beads mark the positions of 5' ends, arrowheads are the poly(A)⁺ sites, and gaps in the arrows correspond to intervening sequences deleted from the transcripts.

Keratinocyte cultures were initiated from newborn foreskin explants as described by Rheinwald and Green (31) with some modifications described previously (39). Cells were maintained in the medium of Wu et al. (43) and were infected at about 60% confluency in passage 2 at an estimated multiplicity of 4×10^4 viral particles per cell.

Typing of viruses isolated from plantar warts. DNA purified from virus suspensions by proteinase K digestion (100 μ g/ml) in the presence of 1% sodium dodecyl sulfate was treated with various restriction enzymes, electrophoresed in a 1% agarose gel, blotted onto a nitrocellulose filter, and hybridized to nick-translated, ³²P-labeled HPV-1 DNA in a pBR322 vector. Only those viruses yielding restriction patterns identical to cloned HPV-1 were used for infection.

Extraction of RNA from plantar warts and cultured keratinocytes. Plantar warts were frozen in liquid nitrogen and stored at -70°C until processing. Total RNA from warts was prepared according to Chirgwin et al. (5) by precipitation directly from the guanidine isothiocyanate buffer. Total RNA was isolated from cell cultures 48 h postinfection according to Chirgwin et al. (5) by pelleting it through a CsCl cushion. The RNA pellet recovered was extracted with phenol-chloroform-isoamyl alcohol and precipitated with ethanol. Poly(A)⁺ RNA was obtained by chromatography on an oligo(dT)-cellulose column.

HPV-1 DNA for R-loop analysis. The HPV-1 DNA used was cloned at its *SacII* site (at nucleotide [nt] 7634) (converted to a *SalI* site by linker ligation) into the vector PF1 (28) modified as described previously (9). The total length was approximately 13,600 bp. The DNA, purified by CsCl-ethidium bromide equilibrium density gradient centrifugation, was linearized with the single-cut enzyme *XhoI*, ex-

tracted with phenol and ether, precipitated from ethanol, and dissolved in water. The *XhoI* site was located in the vector, 180 bp from the cloning site, or about 340 bp from nt 1 of HPV-1. This asymmetrical placement of the HPV-1 sequence in the linearized recombinant permitted the unambiguous correlation of R-loop coordinates with the genetic map of the HPV-1 DNA.

R-loop formation and analyses. One microgram of linearized HPV-1 recombinant DNA and 10 μ g of either total RNA extracted from plantar warts or poly(A)⁺ RNA purified from infected keratinocytes were used for R-loop formation, as described before (7, 9). R-loops were stabilized with glyoxal (23). Electron microscopic grids prepared by the formamide technique were examined in a Zeiss 10 CA transmission electron microscope and photographed at a magnification of 10,000. The negatives were projected from a photographic enlarger, and the R-loop structures were measured with a Numonics electronic planimeter. The positions of R-loops were aligned with the HPV-1 DNA sequence and its ORFs (13, 33).

RESULTS

Analysis of HPV-1 RNAs from plantar warts. To analyze the HPV-1 transcripts isolated from plantar warts, total RNA preparations were processed individually and were examined by electron microscopy of R-loops formed with linearized, cloned HPV-1 DNA. Only two samples contained HPV-1 RNA abundant enough to be studied. From one of the preparations, a number of transcripts were identified, and virtually all were spliced. Their 5' ends were derived from three locations: in E7, at the beginning of E6, and in the

FIG. 2. R loops of RNAs from plantar warts. The HPV-1 DNA was linearized at an *Xho*I site in the vector 180 bp upstream from the *Sac*II cloning site (nt 7634) or 340 bp from nt 1 of HPV-1. The rest of the vector sequences are at the distal end from the R-loop. Small and medium arrowheads point to the 5' and 3' ends, respectively; large arrowheads point to the intervening sequences evident as DNA deletion loops. Panels A, B, C, and D correspond to RNA species a, b, f, and d of Fig. 1.

URR. Of the transcripts, 95% had their 5' ends in E7. There were two polyadenylation sites, one near nt 4000 at the 3' end of the E region, the other near nt 7400 at the 3' end of the L region. The major species accounted for 80 to 90% of all R-loops; 30 examples were measured. It had a short 5' exon about 100 nt long spanning the junction of the E7 and E1 ORFs (from nt 700 to 800) spliced to an 800-nt-long 3' exon extending from the region where the E2 and E4 ORFs overlap to the polyadenylation site at the end of the E region (from nt 3,200 to 4,000) (Fig. 1, species a; Fig. 2A). The next two more frequently seen species (over 12 molecules measured for each) had the same 5' exon and RNA splice donor and acceptor sites as the most abundant species just described. In one, the 3' exon extended continuously from nt 3200 to 7400 at the end of the L region, where it was polyadenylated (Fig. 1, species g). In the other, the second exon measured about 360 nt long (nt 3200 to 3560) and was spliced again to a third exon from nt 5400 through the L1 ORF to nt 7400, where it was polyadenylated (Fig. 1, species d; Fig. 2D). Two other minor species had 3' exons very similar to the once- or twice-spliced species described above except that they had a different, short 5' exon derived from the URR near the cloning site (nt 7634) (Fig. 1, species f and h). The URR⁺E2/E4-L1 species h was seen once, while 10 examples of the URR⁺E2/E4-L1 species f were measured (Fig. 2C). One infrequent species had a long 5' exon of about 700 nt (from nt 100 to 800) spliced to the same 3' E2/E4 exon

of 800 nt as present in the major species (Fig. 1, species b; Fig. 2B). Additional rare species are shown in Fig. 1 (species c, e, i, and j).

HPV-1 RNAs from the second preparation were similarly analyzed. A total of 31 and 35 molecules corresponding to species a through c and d through j, respectively, of Fig. 1 were measured. The structures were practically identical except that many mRNAs were truncated at the 3' end because of degradation. Species b (Fig. 1) with the 5' exon spanning the E6 and E7 ORFs was seen more frequently in this preparation.

Analysis of HPV-1 RNAs from infected keratinocytes. Poly(A)⁺ RNAs from infected keratinocyte cultures were also studied by the R-loop method. Thirty-three molecules that were polyadenylated at the end of the E region (Fig. 1, species a and b) and 10 molecules polyadenylated at the end of the L region (Fig. 1, species d and g) were measured. They were a subset of those seen in the wart RNA preparations and had identical splice patterns. The major difference was the absence of transcripts with exons derived from URR. The relative abundance of the various species was approximately the same as that observed with the wart RNA preparations.

DISCUSSION

A number of spliced species of HPV-1 RNAs from plantar warts and from cultured foreskin epithelial cells infected

with HPV-1 particles were mapped by the electron microscopic R-loop method. The more abundant species are probably genuine messages, whereas extremely rare species might be processing intermediates. Three different putative promoters and two alternative polyadenylation sites were used, as suggested by the 5' and 3' ends of the R-loops. If more remote 5' leader segments were present, they would have been very short to have escaped detection, for a 41-nt-long, 5' exon in the adenovirus transcripts was reproducibly observed (6, 8) when similar electron microscopic mapping techniques were applied. Inspection of the HPV-1 DNA sequence revealed no consensus eucaryotic promoterlike sequence in the E7 ORF near the site to which we mapped the predominant 5' end of the mRNAs. Neither BPV-1 nor cottontail rabbit papillomavirus seems to have a promoter in E7, but they do have a comparable promoter at the beginning of the E6 ORF. In addition, BPV-1 also has promoters in the URR at somewhat different locations (37, 44; C. Baker, M. Botchan, and P. Howley, personal communications). The existence of a putative promoter near nt 4000 was not certain, as species j of Fig. 1 was seen very rarely and could have been a result of degradation from species g or h. The 3' cleavage and polyadenylation signal AAUAAA is found at nt 3983 (E region) and at nts 7380 and 7426 (L region), near the locations where we mapped the polyadenylation sites.

The splice patterns of HPV-1 transcripts relative to the ORFs were rather similar to the complex families of RNAs present in anogenital warts associated with HPV-6 or HPV-11 (L. T. Chow, M. Nasser, S. M. Wolinsky, and T. R. Broker, submitted for publication). The predominant RNA species in cutaneous and mucosal warts was the same, i.e., species a. The corresponding cDNA from an HPV-11 condyloma has been cloned and sequenced (M. Nasser, R. Hirochika, T. R. Broker, and L. T. Chow, submitted for publication). It encodes an E1-E4 fusion protein. Conserved potential splice sites can be found in HPV-1, and we believe that the major RNA (species a) also encodes an E1-E4 protein. BPV-1 and cottontail rabbit papillomavirus mRNAs containing the same splice have been reported (14, 37, 44). However, in the animal papillomaviruses, the 5' exon contains the entire E6 and E7 ORF, in addition to the beginning of the E1 ORF.

A doublet of 16- and 17-kilodalton E4 proteins has been detected as the most abundant protein in plantar warts (11, 16). Their localization in plantar warts has been determined by immunocytochemistry in serial sections of warts (F. Breitburd, O. Croissant, and G. Orth, *Cancer Cells*, vol. 5, in press). They are most abundant in the middle strata but are present in cells from the suprabasal layer to the uppermost stratum corneum, where the predominant species are a 10- and 11-kilodalton doublet that also reacts with the antibody raised against the larger E4 proteins. Two interpretations are possible and are not mutually exclusive: alternative splicing of the E1 donor to two or more of the potential acceptors (resulting in the doublets) and proteolytic cleavage or other differences in posttranslational modification during keratinocyte maturation (resulting in the substantially different-sized, but antigenically related proteins). Only cDNA characterization or protein sequencing is likely to resolve these issues.

Two of the RNA species (b and e) we have mapped could encode the E6 protein. The E7 protein could be made through internal reinitiation of translation from the same mRNA or from rare mRNA which we did not detect. A spliced species fusing the E6 and E7 ORFs has been identi-

fied in BPV-1 (1, 37, 44). Spliced E6 mRNAs have also been reported in HeLa cells and CaSki cells that harbor integrated HPV-18 and HPV-16 DNA, respectively, and in carcinomas associated with HPV-16 (32, 34, 36). We found no such species in this study. An examination of the DNA sequences revealed no consensus splice sites comparable to those in BPV-1, HPV-16, or HPV-18.

One of the interesting results of this study is that the putative mRNAs for the major capsid protein encoded by the L1 ORF had two exons from the early region (species d and f). Species d is rather similar to L1 RNAs of HPV-6 or HPV-11 (Chow et al., submitted), whereas species f is somewhat similar to the L1 mRNA for BPV-1, which also has two exons, one from URR and the other from the E region (C. Baker and P. Howley, personal communication). This phenomenon of a combination of E and L region exons has not been observed in other virus systems. Most intriguing is the observation that the more abundant of the two putative L1 mRNAs, species d, had the same 5' exon and, hence, presumably originated from the same promoter as did the putative E1-E4 mRNA, which is at least 10 times more abundant. E4 and related proteins are found throughout the plantar wart starting from the suprabasal layer (Breitburd et al., in press). In contrast, the major capsid protein was detected only in the more differentiated upper layers of mature keratinocytes (11, 16). Therefore, the regulation of genetic expression appears to reside in the choice of two alternative polyadenylation sites or mRNA splice sites.

The L1 protein in purified virions has a molecular mass of 55,000 daltons in sodium dodecyl sulfate-polyacrylamide gels (16), corresponding to the predicted coding capacity of the L1 ORF. We do not know whether the two 5' exons are translated in the putative L1 mRNAs (species d and f) and subsequently cleaved off or whether initiation starts in the 3' main body of the RNAs. E5 and L2 ORF proteins might be translated through internal reinitiation from RNA species that have an exon extending from nt 3200 to 7400 (species g and h), although it is possible that these are processing intermediates for the putative L1 mRNAs (species d and f), as is species i. L2 might also be translated from species j, if it is a genuine mRNA, rather than a fragment derived from species g or h.

Our results show that the HPV-1 RNAs from infected keratinocytes are a subset of those found in warts. The major transcripts, species a, d, and g, are the same from both sources. It is not likely that the absence of activity of the putative promoter in the URR was a major cause for the failure of the keratinocyte cultures to support the growth of the virus, as its activity was extremely low in warts. Instead, we believe that two other factors were responsible, both related to the fact that keratinocytes in culture do not differentiate as they do in vivo. First, mRNA transcription was rather inefficient in the keratinocyte cultures, as judged from the absence of HPV-1-specific bands in Northern blots. In contrast, multiple HPV-1 RNA species from warts were present in the same blots (data not shown). Second, S. Reilly and L. Taichman (*Cancer Cells*, vol. 5, in press) recently demonstrated that HPV-1 DNA was underreplicated, relative to the cellular DNA in keratinocyte cultures. Therefore, cultured keratinocytes must lack cellular factors correlated with cellular differentiation in vivo.

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