Infectious Cycle of Human Papillomavirus Type 11 in Human Foreskin Xenografts in Nude Mice

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We have performed the first molecular analysis of a time course of infection by a papillomavirus. The Hershey isolate of the human papillomavirus type 11 was used to infect human foreskin tissues, which were then implanted under the renal capsules of nude mice. The xenografts were recovered every 2 weeks for 14 weeks, fixed in formalin, and embedded in paraffin. Four-micrometer serial sections were examined by light microscopy for morphological changes, by immunocytochemistry for virion antigen production, and by *in situ* hybridization with ³H-labeled RNA probes for viral DNA replication and expression of the major mRNA species. After a lag period, probes spanning the E4 and E5 open reading frames, which are present in all E region viral mRNAs, generated the first detectable signals at week 4. Signals of other E region probes were minimally detected at week 6. Between weeks 6 and 8, there was an abrupt change in the implant such that cellular proliferation, viral DNA replication, and E and L region mRNA transcription were robust and reached a plateau. By weeks 10 to 12, the experimental condylomata were morphologically and histologically indistinguishable from naturally occurring condylomata acuminata. These findings suggest that cellular hyperproliferation and the morphologic features of condylomata are direct results of viral genetic activities. Unlike other DNA viruses, the E region transcripts increased with cell age and cellular differentiation and persisted throughout the entire experiment. In particular, the mRNA encoding the E1⁺E4 and perhaps E5 proteins remained overwhelmingly abundant. In contrast, viral DNA replication, L region mRNA synthesis, and virion antigen production were restricted to the most differentiated, superficial cells.

Papillomaviruses are a family of small DNA viruses which contain a circular DNA chromosome of approximately 7,900 base pairs. The human papillomaviruses (HPVs) are species specific and are restricted in their tissue tropism. Infection of target cutaneous or mucosal epithelium results in hyperproliferation. The closely related human papillomaviruses type 6 (HPV-6) and type 11 (HPV-11) are usually associated with benign genital warts (condylomata acuminata). One major obstacle to research on human papillomaviruses is the inability to propagate them in cultured cells or to infect tissues of laboratory animals. Nevertheless, it has been demonstrated that infection by a particular HPV-11 isolate, called Hershey, alone is sufficient to generate condylomatous cysts in chips of human foreskin or uterine cervix following implantation under the renal capsule of athymic mice (11, 13). These experimental condylomata have morphologic features identical to those of human lesions. The HPV-11 Hershey isolate has been serially passaged in this system and produces large amounts of virions (10). The tissue specificity of the Hershey isolate has been examined by using skin from different body sites from the same individual, with foreskin and urethral epithelium being most supportive of cellular transformation and viral transcription (12). HPV-11 Hershey has been molecularly cloned. Physical and functional anal-

yses of this isolate have not uncovered any significant differences (8) from the HPV-11 prototype clone (7). The seemingly unique properties of HPV-11 Hershey have been attributed to an unusually high virus titer in the extracts of the patient lesions used to generate the initial experimental condyloma.

HPV-6 and HPV-11 mRNAs from human lesions as well as from the experimental condylomata have been mapped by electron microscopic examination of R-loops (6, 16). The two viruses generate completely analogous families of overlapping mRNAs that are transcribed from several distinct promoters and polyadenylated at one of two sites located at the 3' ends of the E or L genetic regions (Fig. 1). The structures and the coding potentials of the alternatively processed mRNAs have been determined by various methods (6, 14-17, 21). We have prepared whole genomic and subgenomic RNA probes specific for DNA or for different viral mRNA species and performed *in situ* hybridization with serial thin sections of biopsies of patient condylomata associated with HPV-6 or HPV-11 (18, 19). These studies show that both viral DNA replication and mRNA transcription increase with cellular differentiation. Different mRNA species are present in dramatically different relative abundances. The mRNA species *a* encoding an E1⁺E4 (ˆ denotes a splice or fusion) protein (see the legend to Fig. 1 for the change in nomenclature) is by far the most abundant viral RNA, present in nearly all the cells, whereas other messages are expressed at 0.1 to 0.01 times that amount or less, mostly in the more differentiated cells. These results provide snapshots of the viral activity at the times when the biopsies were taken. They do not, however, reveal the time course of

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FIG. 1. Genetic organization and message-specific probes of HPV-11. The circular genome of 7,933 base pairs is represented in a linear fashion from a *Bst*I site in the upstream regulatory region. The ORFs deduced from the DNA sequence are represented by open boxes. Vertical dotted lines mark the locations of the first AUG codon in each ORF. Viral messages are depicted as arrows in the 5' to 3' direction, with gaps indicating introns (6, 14, 16). The dots at the 5' ends of the mRNAs represent proven or putative promoters. The presumptive L2 mRNA (species *k*) has not been observed frequently enough to predict its promoter. Coding potentials, as deduced from the cDNA sequences (16), are listed, with the exception of the E5 protein(s), which could potentially be translated from any or all of the E region mRNAs or from an mRNA yet to be defined. Shaded boxes indicate regions spanned by exon-specific subgenomic clones in pGEM or BRL19 vectors (19). Pre, Putative precursor. The E1ⁱE4 protein has previously been designated E1ⁱE4 protein. We now introduce this new terminology, with "i" signifying translation initiation, to distinguish this protein, which derives only the initiation methionine and four additional amino acids from the E1 ORF, from other fusion proteins with substantial E1 domains (C.-M. Chiang, T. R. Broker, and L. T. Chow, unpublished results).

infection and the relationship of viral gene expression to the development of a condyloma. Taking advantage of the availability of infectious HPV-11 Hershey virions and the reproducibility with which they induce experimental condylomata, we systematically surveyed viral DNA replication and the synthesis of the major mRNA species at different times after the implantation of infected human xenografts. This report provides the first molecular analysis of the time course of a productive infection by a papillomavirus. A time course of cyst development in skin grafts infected by bovine papillomavirus has been analyzed for morphological changes (9).

MATERIALS AND METHODS

Infection of human foreskin chips and subrenal implantation. Human foreskin was obtained from a routine neonatal circumcision at an area hospital. Split-thickness skin chips (0.5 by 2.0 by 2.0 mm) were cut with a scalpel and incubated either with HPV-11 Hershey extracted from previous experimental condylomata or with control saline for 1 h at 37°C. They were then inserted beneath the renal capsules of nude mice, as previously described (11, 13). Female athymic mice, NIH strain, 4 to 6 weeks old, were used as hosts. Two mice were used for each time point. Each mouse received one infected implant and one control implant.

In situ hybridization with HPV-11 message-specific riboprobes. Two mice were sacrificed every 2 weeks for 14 weeks after implantation. The kidneys were removed, and

the xenografts were fixed in 10% neutral-buffered formalin and then embedded in paraffin. Serial sections (4 μ m) were mounted onto polylysine-coated microscope slides. The control specimens were examined histologically. Sections of each of the two sets of the infected xenografts were hybridized to ³H-labeled, asymmetric whole genomic or message-specific riboprobes (Fig. 1) in two separate experiments, each with a set of probes of the same specific activity. The probes have been described previously (18, 19). A probe for E5 open reading frames (ORFs) (nucleotides 3901 to 4557) which does not react with mRNA *j* was also used (Fig. 1). Probe concentrations were normalized according to their lengths. Autoradiograms were developed after a 4-week exposure. Therefore, the intensities of the signals generated were proportional to relative copy numbers of the target molecules. Negative controls included hybridization of whole genomic and subgenomic sense-strand riboprobes to specimens without prior heat denaturation of the viral DNA. We define sense-strand probes to be those of the same polarity as the viral mRNAs. These controls were uniformly negative (data not shown). All slides from one of the experimental groups were photographed with bright-field illumination for histological examination and immunocytochemistry and with dark-field illumination for optimal ³H signal detection.

RESULTS

Kidneys from two mice, each with one infected and one control foreskin implant, were harvested every 2 weeks after

implantation. Uninfected human foreskin xenografts formed squamous epithelial cysts and have never shown koilocytosis or viral antigen. Serial thin sections of HPV-infected xenografts from both sets of animals were hybridized with whole genomic or subgenomic ^3H -labeled riboprobes in separate experiments, as described in Materials and Methods. Because the amounts of probe used were normalized against the probe size, the intensity of the ^3H signals reflects the relative abundances of individual RNA species. Essentially identical results were obtained with the two parallel experiments. Only one set of sections was photographed and presented in this report. Due to the necessity of taking individual time points from different animals, there were occasional small deviations in the trend of viral activities over the 14-week period. However, at any one time point, consistent assessments were obtained with regard to histology, viral DNA replication, and transcription of individual mRNA species. We point out that it takes perhaps 1 or 2 weeks for the keratinocytes to migrate from the parabasal layer to the surface. Accordingly, the topography of the epithelium itself presents an ongoing time course. The salient features of the time course of infection are summarized below.

Replication of viral DNA. Whole genomic RNA probe with the same polarity as the viral messages was used to detect viral DNA replication following heat denaturation of DNA in the specimens. Viral DNA was not detectable for the first 4 weeks, and the signals were barely detected in the week 6 sample (Fig. 2A and B). By week 8, most of the cells in the foreskin epithelium cells were positive, including cells in the lower stratum spinosum (Fig. 2C and D). The strongest signals were in the upper stratum spinosum and stratum granulosum. Thereafter, DNA signals were restricted to the more differentiated cells (Fig. 2E to H). Relative to week 8 or week 12 specimens, the week 10 xenograft appeared less active in both DNA replication and in mRNA transcription (data not shown). We attributed these lower activities to a somewhat slower rate with which the 10-week graft was established in the particular animal.

Expression of individual mRNA species. The locations of the message-specific subgenomic probes (19) are presented in Fig. 1. With the exception of the E4-E5 and E5 probes, each probe is complementary to a region (E6-E7, E1, E2, L2, and L1) unique for the target mRNA. The E4-E5 probe overlaps the carboxyl-terminal half of the E2 ORF, which encodes the E2-C protein, and also hybridizes to the L1 mRNA species *j* (Fig. 1). The E4-E5 and the E5 probes also hybridize to all the other E region mRNAs. The E2-C mRNA is extremely rare in patient lesions and in the experimental condylomata (6, 16) and therefore accounts for a very small fraction of the abundant signals detected, except perhaps in a subpopulation of the cells (see Discussion). The amount of the E1i⁺E4 mRNA species *a* can be deduced from the difference between the cytoplasmic signals generated from the E4-E5 probe and those specific for the other E region messages and for the L1 mRNA. This adjustment makes only a very small difference as, all together, these other

transcripts amount to but a small fraction of the E1i⁺E4 mRNA (see Fig. 4). By using these message-specific RNA probes, we examined adjacent thin sections for the expression of each mRNA for each of the time point specimens.

The two probes that span the E4-E5 and E5 region first generated marginally detectable signals at week 4 in the relatively undifferentiated, basallike cells (data not shown), and the signals clearly increased in strength in the week 6 specimen (Fig. 3B and D). From week 8 on, when cellular proliferation and condylomatous differentiation were evident, E4 and E5 signals were detected in the basal cells and increased dramatically with cellular differentiation (Fig. 4D and E). They were overwhelmingly predominant and virtually indistinguishable from signals generated by the whole genomic probe for total viral RNA (data not shown). This high relative abundance persisted through week 14 (Fig. 5C), indicating that the E1i⁺E4 mRNA species *a* is the most abundant viral message throughout the infection.

RNA transcripts containing E6 and E7 ORFs (Fig. 1, species *d*, *e*, and *f*) were not detected at week 4. Marginal signals comparable to those in Fig. 3A were present at week 6, and by week 8, the RNA became much more abundant (Fig. 4A). The messages first appeared in the parabasal layer to the midepithelium and increased dramatically in the more differentiated cells. The relative abundance of the E6-E7 messages seemed to decrease somewhat in subsequent weeks (Fig. 5A). In a similar fashion, E1 and E2 probes first generated marginal signals in the week 6 specimen (Fig. 3A). They were strong from week 8 onward (Fig. 4B and C; 5B). Unlike the diffuse pattern of cytoplasmic and nuclear distribution observed with other probes, the majority of these signals were restricted to the nucleus. The strong nuclear signals observed with the E1 and E2 probes are interpreted to be residual intron sequences derived from the abundant E1i⁺E4 RNA and other E region messages. Accumulation of E1 and E2 mRNAs in the cytoplasm was very low, even in the more differentiated cells, which is consistent with the role of their translation products in regulating DNA replication and RNA transcription (4). Such proteins and their mRNAs are typically generated in very low quantities. Both the nuclear and the cytoplasmic signals from the E1 and E2 regions were somewhat reduced at later weeks and were largely confined to the superficial cells (Fig. 5B).

Probes specific for L2 and L1 ORFs produced no signal at week 6 (Fig. 3C). At week 8, cytoplasmic L2 and L1 signals were strong and were present only in the superficial, most differentiated cells (Fig. 4F, G, and H). Thereafter, the mRNAs for the capsid proteins remained high in the superficial cells (Fig. 5D and E), except for week 10, which had lower signals for all probes. The L2 probe, and the L1 probe to a lesser extent, also generated signals in the nuclei of cells in the mid-epithelial strata (Fig. 4F, G, and H).

Morphology and immunocytochemistry. The implants remained small and showed little or no growth for the first 4 weeks. By week 6, some cell proliferation was evident (Fig. 3). In the next 2 weeks, there was an abrupt change in the xenografts, with robust cell proliferation and marked condy-

FIG. 2. Time course of HPV-11 DNA replication in human foreskin xenografts. ^3H -labeled whole genomic RNA probes of the same polarity as the viral messages were used to probe sections of formalin-fixed xenografts that were heat denatured. Viral DNA signals, not detectable in the nuclei for the first 4 weeks, were marginal at week 6 and strong from week 8 onward. (A and B) 6 weeks; (C and D) 8 weeks; (E and F) 12 weeks; (G and H) 14 weeks. (A, C, E, and G) Photographed with bright-field illumination; (B, D, F, and H) photographed with dark-field illumination. In panel A, arrowheads delineate the human foreskin implant. In panels C, E, and G, arrowheads point to the basal cells. Viral DNA, presumably in mature virions, is also present in the cornified cells and in the desquamified cells at weeks 12 and 14; several such examples are circled in panels E and G.

FIG. 3. HPV-11 RNA transcription at 6 weeks postimplantation. ^3H -labeled RNA probes were separately hybridized to serial sections of the xenograft as described in Materials and Methods. The implant clearly shows cellular proliferation but exhibits little, if any, condylomatous tissue differentiation. (A) E1 probe showing marginal signal; the same results were obtained with the E6-E7 and E2 probes. (B and D) E4-E5 probe showing low, yet definitive, signals; the same result was obtained with the E5 probe and the whole genomic probe. (C) L1 probe showing no signal; the same result was obtained with the L2 probe. (A, B, and C) Dark-field illumination; (D) bright-field illumination. In panel D, arrowheads point to the basal cells. Also clearly visible in panel D are desquamified cells at the upper right corner.

lomatous tissue differentiation (Fig. 2 to 5). By week 10, the implants were morphologically and histologically indistinguishable from naturally occurring condylomata acuminata, exhibiting marked epithelial acanthosis and cellular koilocytosis. Papillomatosis was evident at weeks 12 and 14. Immunocytochemically detectable papillomavirus group-specific L1 antigen correlated with the presence of the L1 message in the most differentiated cells from week 8 onward (Fig. 4G and H; 5E and F). As has been described in naturally occurring lesions (19), only a small subset of the cells that express L1 message are antigen positive. Both viral DNA and L1 antigen persisted in the desquamified cells, which created a cyst enveloped by the foreskin epithelium (Fig. 2E to H; 5F). We interpret this result to mean that mature and stable virions accumulate in the cysts.

DISCUSSION

We have performed the first molecular analysis of a time course of papillomavirus infection. There appeared to be an initial incubation period of 4 weeks before viral activity could be detected. This lag may reflect the time needed for vascularization of the implants to allow optimal nutrient delivery and hormonal stimulation. The state of the virions or viral DNA during this period is not known, but the DNA is stably maintained in the basal stem cells. Probes that detect the E region RNAs in general and E4 and E5 mRNAs in particular produced the first detectable signal at week 4. Over the next 4 weeks, there was a dramatic transition from a near absence of viral DNA replication and mRNA transcription to maximal activities in both (compare Fig. 3 and

FIG. 4. HPV-11 RNA transcription at 8 weeks postimplantation. ^3H -labeled, message-specific RNA probes were individually hybridized to serial sections of the xenograft. All viral RNA signals increased with tissue differentiation. (A) E6-E7 probe, showing cytoplasmic and nuclear signals that start in parabasal cells; (B and C) E1 and E2 probes, respectively, showing signals that are predominantly nuclear; (D and E) E4-E5 and E5 probes, respectively, showing cytoplasmic and nuclear signals that first appear in the basal cells; (F) L2 and (G and H) L1 probes, respectively, showing cytoplasmic and nuclear signals that are restricted to the superficial cells; some purely nuclear signals were also present midepithelium. (A through G) Dark-field illumination; (H) bright-field illumination. White (A to G) and black (H) arrowheads point to the basal cells.

FIG. 5. HPV-11 RNA transcription and L1 capsid antigen at 14 weeks postimplantation. (A) E6-E7 RNA probe. (B) E1 RNA probe; the same result was obtained with the E2 RNA probe. (C) E4-E5 RNA probe; the same results were obtained with the E5 RNA probe and whole genomic probe. (D and E) L1 RNA probe; the same result was obtained with the L2 RNA probe. (F) L1 antigen probe; abundant L1 antigens are present in the cyst, which consists of desquamified cells. A few cells in the superficial layer were also positive. (A, B, C, and D) Dark-field illumination; (E and F) bright-field illumination. In panel F, arrowheads point to the basal cells. Some examples of the L1 antigen signals are circled.

4). These viral activities were accompanied by an abrupt change in the xenografts, with cell proliferation and condylomatous tissue differentiation. By week 10, the infected xenografts were morphologically and histologically indistinguishable from naturally occurring condylomata acuminata. Thus, E region gene expression preceded cellular transformation by at least 2 to 4 weeks. These findings are consistent with the interpretation that cellular hyperproliferation and

the morphologic features of condylomata are direct results of viral genetic activity.

Viral DNA replication increased with cellular differentiation. Maximal viral DNA replication was observed in the week 8 specimen (Fig. 2). Viral DNA was detectable in cells just above the basal layer (lower stratum spinosum) and became abundant in the more mature cells of the mid- and upper epithelium (Fig. 2C and D). The DNA molecules in the

less differentiated cells can only serve as templates for mRNA expression, not for packaging into virions, because the same cells were negative for capsid mRNAs (compare Fig. 2C and D; 4F to H). The replacement generations of these parabasal cells were not similarly permissive for DNA replication. At and beyond week 10, DNA replication was restricted to the more differentiated cells (Fig. 2E to H). We interpret this pattern to represent the vegetative reproduction phase where accumulation of viral DNA, L region mRNA transcription, capsid protein synthesis, and virion assembly are evident. Presently, we are not sure whether the week 8 specimen truly represented an unusual phase of the viral DNA replication or was an adventitious result of tangential sectioning of the xenograft. Examination of the other week 8 specimen from a different mouse did not reveal the same phenomenon. Additional experimentation is needed to resolve this issue.

The viral E region mRNAs increased with the degree of cellular differentiation, and such elevated expression persisted with time. We suspect that the increase in transcription upon differentiation from lower to upper spinous cells results from two effects. First, as the cells differentiate, there is a change in host transcription factors or their concentrations, triggering an alteration in the viral transcription program toward a productive infection, much like an induction of a bacterial prophage into the lytic phase. The altered viral gene expression in turn allows viral DNA to begin to replicate beyond the maintenance state in the basal cells. Second, transcription is further elevated once there is an increase in gene dosage upon vegetative DNA synthesis. The fact that L1 and L2 mRNAs encoding the morphogenic proteins were synthesized only in the superficial, most differentiated cells (Fig. 4 and 5) also attests that a certain cellular environment associated with terminal differentiation must be present for their synthesis. These patterns of viral transcriptional activity resemble what has been observed in patient biopsies (19), further validating this experimental system. Perhaps because of better nutrient delivery or, alternatively, impaired immune surveillance in nude mice, the infected xenografts had overall higher viral activities than most patient specimens (19), leading to higher levels of virion production (10).

The E6 and E7 proteins of HPV-16 and HPV-18 have been described as stimulating the proliferation of cells not yet committed to differentiation (1, 20). It is therefore puzzling that the E6 and E7 mRNAs are expressed in dramatically higher abundance in the more differentiated but nondividing cells than in the dividing basal cells, as we have also noted in patient biopsies (Fig. 4 and 5) (2, 19). We suggest that the E6 and E7 proteins have primary functions related to viral DNA replication. For instance, they might reactivate the transcription of host genes encoding replication proteins or recruit and stabilize these proteins in the absence of cellular DNA replication.

The signals generated by the E4 and E5 probes were practically indistinguishable from those generated by the whole genomic probe at all time points (data not shown). Their signals, but not those of other E region probes, first appeared in the xenograft at week 4. Because the E4-E5 and E5 probes are complementary to all E region mRNAs, we are not certain whether the signals originated from mRNA species *a* or *h* or were the result of many E region mRNA species, the individual signals from which were too low to be detected at this early time point. Based on the transcription repression function of the E2-C protein, hypothesized to maintain viral activities at a low level in the basal cells (5), it

is probable that at least a fraction of this temporally and topologically early signal from the E4-E5 and E5 probes represents the E2-C mRNA (species *h*). The bulk of the E4 and E5 probe signals, especially in the midepithelium and the more differentiated cells above from 8 weeks onward, clearly originates from mRNAs encoding the E1i'E4 and the E5 proteins, because other E region probes produced only low signals. R-loop analyses have shown that mRNA *a* is the predominant species in patient biopsies as well as in a 300-day experimental condyloma (6, 14, 16). Abundant E4 protein, the function of which is yet to be determined, has been demonstrated in these infected xenografts by Western immunoblots (3). By using immunocytochemical methods, we recently detected abundant E4 and E5a proteins in the cytoplasm of such implants (T. Ho, M. Chin, D. Strike, T. Broker, and L. Chow, unpublished results).

We attribute the nuclear signals generated by the E1 and E2 probes to relatively undergraded intron sequences excised from the abundant E1i'E4 mRNA (species *a*) and other E region transcripts (Fig. 1), as has been hypothesized previously (19). Occasional nuclear signals from L2 and L1 in the midepithelium (Fig. 4F and G) presumably represent run-on transcription past the E region polyadenylation site (14) that fails to span the entire L region. These sequences become nuclear by-products after cleavage and polyadenylation at the E region poly(A) site. Successful transcription of the L region mRNAs is presumably dependent on host cell factors present only in the most differentiated granular keratinocytes. It is curious that there is an equal abundance of the L2 and L1 mRNAs that encode the minor and major capsid proteins, respectively. Possibly the L2 mRNA is translated much less efficiently.

In summary, we have demonstrated that in the infection program of a human papillomavirus, the onset of E region transcription precedes cell proliferation and vegetative viral DNA replication both in time after infection and in the degree of cellular differentiation. The E region mRNAs remain at high abundance at late times after infection, and their transcription increases with cell age, as reflected by their location in the stratified epithelium and the degree of differentiation. The L region is truly late by these same criteria. The E region mRNA transcription pattern is distinct from that of other DNA viruses, in which early transcripts remain at a low level or are turned off upon activation of distinct late promoters. Together with the fact that the L1 mRNA is derived from the same promoter as the E1i'E4 mRNA (6), these observations prompt us to refrain from referring to the messages as being early or late in the conventional sense.

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