Detection of Transcripts of Human Papillomaviruses 16 and 18 in Cancer-Derived Cell Lines and Cervical Biopsies by Enzyme Immunoassay for DNA-RNA Hybrids following Solution Hybridization

FRANÇOIS COUTLÉE,^{1†*} KEERTI V. SHAH,² JANET S. RADER,³ JOHN L. CURRIE,³
AND RAPHAEL P. VISCIDI¹

Eudowood Division of Infectious Diseases, Department of Pediatrics,¹ and Department of Gynecology and Obstetrics,³ School of Medicine, and Department of Immunology and Infectious Diseases, School of Public Health,² The Johns Hopkins University, Baltimore, Maryland 21205

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mRNAs of human papillomaviruses (HPV) 16 and 18 were detected in cancer-derived cell lines and genital tract biopsy specimens by a novel hybridization assay. Biotinylated whole genomic HPV DNA probes were hybridized in solution to extracted total nucleic acids. Hybrids between the labeled probes and RNA transcripts were captured on a microplate coated with an anti-biotin antibody. Bound hybrids were incubated with a β -galactosidase-labeled monoclonal antibody to DNA-RNA hybrids and measured by the addition of a fluorogenic substrate. HPV 18 and HPV 16 mRNAs were detected in nucleic acids from 2.3×10^5 HeLa cells and 10^4 SiHa cells, respectively. The specificity of the assay for mRNA was demonstrated by the low reactivity of nucleic acids from SiHa cells after treatment with T_1 RNase and by the selective reactivity of cellular nucleic acids which bound to an oligo(dT) column. With HPV 16 subgenomic probes, E6-E7 transcripts but not L1-L2 transcripts were detected in SiHa cells. Tests of 58 biopsy specimens from 31 patients showed that the detection of HPV 16 and HPV 18 transcripts in tissue specimens was feasible. Analysis of biopsy specimens with subgenomic probes revealed HPV 16 E6-E7 transcripts in all specimens that reacted with the whole genomic probe, while L1-L2 transcripts were found infrequently.

Human papillomaviruses (HPV) are etiological agents of cutaneous and mucosal papillomas (18). HPV 16 and HPV 18 have also been implicated in the genesis of anogenital carcinomas (6, 14, 17). Since these viruses cannot be propagated in cell cultures, the diagnosis of HPV infections is usually established by the detection of viral genetic material in tissue specimens by nucleic acid hybridization techniques. HPV are double-stranded DNA viruses, and the most common hybridization methods used for viral diagnosis are dot blot and Southern blot hybridization methods (29). The detection of HPV RNA, on the other hand, has received little attention, although the presence of specific mRNA has been described in cervical carcinoma cell lines (32, 42) and in a limited number of clinical specimens (13, 34).

mRNA has most commonly been detected by in situ hybridization (7, 37) or mixed-phase hybridization assays (2, 13, 21, 34, 39). The utility of these techniques for routine diagnosis and large epidemiological studies is limited. Both methods are labor-intensive and rely on the use of radioisotopes. The manipulation and disposal of radioactive material are hazardous and inconvenient. In addition, radiolabeled probes have a short functional half-life and therefore must be regenerated regularly. A further disadvantage of mixed-phase assays with nitrocellulose filters is that nucleic acids bound to filters have unfavorable reaction kinetics (15). Assays with filters are also difficult to standardize because

the preparation of filters and their performance characteristics are highly variable. We have recently described a highly sensitive, nonisotopic solution hybridization assay for the detection of RNA which overcomes many of the technical problems inherent in other RNA detection methods (12, 40). In this assay, hybrids between a biotinylated DNA probe and specific RNA sequences are captured on a microtiter plate coated with an antibody to biotin. Bound hybrids are detected with an enzyme-labeled monoclonal antibody against DNA-RNA hybrids (5, 43). We report here the use of this assay for the detection of mRNAs from HPV 16 and HPV 18 in cell lines and biopsy specimens.

MATERIALS AND METHODS

Probes and specimens. The complete genomes of HPV 16 (pg16) and HPV 18 (pg18) cloned in pGEM2 were provided by M. H. Stoler (37). Two subgenomic HPV 16 probes (24a) constructed in the Bluescript vector (Stratagene, San Diego, Calif.) were used: T16EP for the E6-E7 open reading frame (ORF) (1.3-kb *Pst*I-*Eco*RI fragment) and T16SB for the L1-L2 ORF (1.7-kb *Bam*HI-*Stu*I fragment). The human cervical cancer lines SiHa and CasKi were obtained from the American Type Culture Collection (Rockville, Md.). SiHa, HeLa, and Vero cells were maintained in Eagle's minimum essential medium supplemented with 10% fetal calf serum. Genital tract biopsy specimens from female patients (57 specimens from 30 patients) and a laryngeal papilloma biopsy specimen from a child, collected from 1986 to 1988 and stored at -70°C , were processed for RNA detection. On the basis of a previous virological investigation of portions of some of these biopsy specimens by Southern hybridization

* Corresponding author.

† Present address: Department of Microbiology and Infectious Diseases, Notre-Dame Hospital, 1560 Sherbrooke East, Montreal, Quebec H2L 4M1, Canada.

and/or ViraPap (a dot blot screening test from Life Technologies for HPV 6, 11, 16, 18, 31, 33, and 35), the specimens were characterized as containing HPV 16 or HPV 18 (diagnosis by Southern hybridization, 5 specimens), containing other HPV types (positive by ViraPap but negative for HPV 16 and HPV 18 by Southern hybridization, 13 specimens), not containing HPV (15 specimens), and not tested for HPV (25 specimens).

Nucleic acid extraction. Tissue culture cells were washed twice in phosphate-buffered saline (PBS), resuspended in 0.1% 5-diethylpyrocarbonate-treated water (DEPC-H₂O) containing 1.0% sodium dodecyl sulfate (SDS) as an RNase inhibitor, and lysed by three cycles of rapid freezing and thawing. Proteinase K was added to a final concentration of 250 µg/ml, and preparations were incubated at 55°C for 2 h. Samples were extracted once with a 1:1 mixture of buffered phenol and chloroform-isoamyl alcohol (24:1) and once with chloroform-isoamyl alcohol. Nucleic acids were precipitated in 95% cold ethanol–0.3 M sodium acetate at –70°C for 1 h (23). After centrifugation at 10,000 × *g* for 15 min at 4°C, samples were air dried and resuspended in DEPC-H₂O containing 0.5% SDS. Biopsy specimens were thawed, minced, and ground. The specimens were resuspended in 300 µl of 10 mM Tris (pH 7.4)–1 mM EDTA (TE) containing 1% SDS and processed as described above for tissue culture cells. Total nucleic acids were quantitated by spectrophotometry at 260 nm, and measured amounts of nucleic acids were quantitated by spectrophotometry at 260 nm, and measured amounts of nucleic acids were tested in the hybridization assays.

Total RNA from cultured cells was prepared by the single-step acid guanidinium thiocyanate-phenol-chloroform extraction method (8). In brief, 10⁶ cells were lysed in 1 ml of 4 M guanidinium thiocyanate (Sigma Chemical Co., St. Louis, Mo.)–25 mM sodium citrate (pH 7.0)–0.5% sodium sarcosyl–0.1 M 2-mercaptoethanol (solution D). The solubilized cells were mixed with 1 ml of water-saturated phenol (pH 5.0)–0.1 ml of 2 M sodium acetate (pH 4)–0.2 ml of chloroform-isoamyl alcohol (24:1). The suspension was vortexed for 10 s, cooled on ice for 15 min, and centrifuged for 20 min at 10,000 × *g* and 4°C. The aqueous phase was retrieved, mixed with an equal volume of isopropanol, and incubated at –20°C for 1 h. After centrifugation at 10,000 × *g*, the RNA pellet was resuspended in 0.3 ml of solution D and precipitated with 3 volumes of 95% cold ethanol for 1 h at –70°C. After a final centrifugation at 10,000 × *g*, the RNA pellet was dissolved in DEPC-H₂O and treated for 30 min with 2 U of RNase-free DNase I (Promega, Madison, Wis.). The mixture was boiled for 3 min, cooled rapidly on ice, and adjusted to 0.5% SDS. Poly(A)⁺ RNA was obtained by chromatography on oligo(dT)-cellulose (1). The nonadsorbed material was collected by continuous washing. The material retained on the column was eluted with buffers of reduced ionic strength. A total of 0.5 µg of tRNA was added to the bound and unbound RNA fractions, and the mixtures were precipitated with 0.3 M sodium acetate–95% cold ethanol at –70°C for 2 h.

Solution hybridization assay and enzyme immunoassay for DNA-RNA hybrids. The hybridization assay and enzyme immunoassay were performed as described previously (11, 12). HPV 16 and HPV 18 plasmids were labeled with bio-11-dUTP (Enzo Biochem Inc., New York, N.Y.) in a standard nick translation procedure (12, 20). Extracted nucleic acids in DEPC-H₂O containing 0.5% SDS were mixed with 40 ng of a biotinylated HPV DNA probe in a total volume of 200 µl of hybridization buffer containing 2× SSC

(1× SSC is 150 mM NaCl plus 15 mM sodium citrate [pH 7.0]), 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 7.4), 1 mM EDTA, and 0.1% SDS. The mixture was heated to 100°C for 3 min and hybridized overnight at 78°C. After the addition of 20 µl of 10% Triton X-100, samples were added to triplicate wells (50 µl per well) of a black microtiter plate (MicroFluor B 96-well U-microplate 011-010-7201; Dynatech Laboratories, Chantilly, Va.). The plate had been previously coated overnight at 4°C with 50 µl of polyclonal goat anti-biotin antibody (Sigma) per well at a concentration of 1 µg/ml in 0.06 M carbonate buffer (pH 9.6) and washed six times before use with PBS–0.05% Tween 20 (PBST). After the addition of samples, the plate was incubated at 37°C for 2 h. After six washes with PBST, 50 µl of a solution containing the Fab' fragment of a monoclonal antibody against DNA-RNA hybrids labeled with β-D-galactosidase (0.024 µg/ml in PBST–0.5% gelatin plus 0.5% mouse serum) was added to the wells, and the plate was incubated for 1 h at 37°C. The monoclonal antibody was kindly provided by Robert J. Carrico (Miles Laboratories, Elkhart, Ind.). After six washes with PBST, 50 µl of 0.1 mM 4-methylumbelliferyl-β-D-galactoside (Sigma) per well in PBS buffer, 1 mM MgCl₂, and 50 µg of bovine serum albumin per ml were added. The amount of fluorescent 4-methylumbelliferone generated by the enzymatic degradation of the substrate was measured after 2 h of incubation at room temperature in a Dynatech MicroFluor microtiter plate fluorometer. A sample was considered positive if the mean fluorescence units (fu) exceeded the mean fu of the negative controls plus 3 standard deviations.

Isotopic hybridization for viral RNA. A dot blot procedure (4) for RNA detection was performed as follows. Plasmid pg16 was labeled by a nick translation reaction with [³²P]dCTP to a specific activity of 2 × 10⁸ cpm/µg (27). RNA was extracted by the single-step guanidinium method, diluted in 8× SSC containing 3.7% formaldehyde, and incubated at 60°C for 15 min. The samples were applied to nitrocellulose filters with a manifold apparatus (Slot Blotter; Schleicher & Schuell). The filters were baked for 2 h at 80°C and prehybridized for 4 h at 42°C in a solution of deionized formamide–5× Denhardt's solution–3× SSC–0.1% SDS–0.1 mM EDTA–1 mM sodium PP_i–10 mM HEPES buffer (pH 7.0)–100 µg of salmon sperm DNA per ml. The hybridization solution was identical to the prehybridization solution, except for the inclusion of 10% dextran sulfate as a volume excluder and 10⁷ cpm of a heat-denatured ³²P-labeled HPV 16 probe per ml. After incubation overnight at 42°C, the filters were washed three times, first at room temperature in 2× SSC, then at 60°C in 0.1× SSC–0.1% SDS, and finally at room temperature in 0.1× SSC. The filters were exposed to X-ray film for 2 days at –70°C with one intensifying screen.

Statistical analyses. Statistical analyses were done with the unpaired Student *t* test (41).

RESULTS

Initial experiments were aimed at determining the sensitivity and specificity of the solution hybridization assay–enzyme immunoassay for the detection of HPV 16 and HPV 18 transcripts from cancer-derived cell lines. Integrated HPV genomes in cancer-derived cell lines have been shown to actively transcribe mRNA (21, 24, 32, 42). Total nucleic acids were extracted from SiHa or HeLa cells, which contained HPV 16 or HPV 18, respectively, and were serially diluted in DEPC-H₂O containing 0.5% SDS. To maintain a total of 1 µg of nucleic acid per assay, we added

FIG. 1. Detection of HPV 16 mRNA in SiHa cells. Nucleic acids were prepared by the phenol-chloroform extraction procedure and precipitated in ethanol. Twofold dilutions of nucleic acids extracted from SiHa cells were prepared with a fixed amount of nucleic acids extracted from Vero cells (5×10^5 cells per test). Specific RNA was quantitated in the solution hybridization assay-enzyme immunoassay. Samples were hybridized to an HPV 16 probe (●), an HPV 18 probe (+), or the pGEM2 vector without an insert (▲). The results, expressed in fu, are means of triplicate determinations. Vertical bars represent 1 standard deviation. probe, Reactivity of each probe with nucleic acids extracted from 5×10^5 Vero cells. ↓, Detection cutoff (mean reactivity of the HPV 16 probe plus five standard deviations). When reacted with 1.6×10^5 SiHa cells ($1.54 \mu\text{g}$ per test), the HPV 18 probe gave a signal of 47 ± 2 fu, while the pGEM2 probe gave a signal of 55 ± 2 fu. The reactivities of $1 \mu\text{g}$ of nucleic acids extracted from SiHa, HeLa, or Vero cells per assay in the absence of a probe were 2 ± 1 , 2 ± 1 , and 3 ± 1 fu, respectively.

nucleic acids extracted from 5×10^5 Vero cells, which did not contain HPV genomes, to each dilution. Samples from each cell line were tested in separate solution hybridization assay-enzyme immunoassay reactions with biotinylated pg16 or pg18 probes. Optimal conditions for the hybridization reaction and enzyme immunoassay were selected on the basis of previous work (9, 12, 44). The titration curves for nucleic acids from SiHa cells and HeLa cells are shown in Fig. 1 and 2, respectively. HPV 16 and HPV 18 transcripts were detected in total nucleic acids from 10^4 SiHa cells ($0.096 \mu\text{g}$) with a signal of 74 ± 4 fu and from 2.3×10^3 HeLa cells ($0.023 \mu\text{g}$) with a signal of 113 ± 3 fu, respectively. Reactions with the heterologous probe served as controls for the type specificity of each assay. The reactivity of pg16 with $1 \mu\text{g}$ of nucleic acids from HeLa cells was 52 ± 2 fu, and that of pg18 with $1 \mu\text{g}$ of nucleic acids from SiHa cells was 47 ± 2 fu.

HPV 16 RNA detection by the solution hybridization assay-enzyme immunoassay was also compared with that by a standard dot blot procedure with a ^{32}P -labeled probe. Total RNA was extracted from SiHa cells by the single-step acid guanidinium-phenol method, which yields nucleic acid preparations enriched for RNA. The RNA was treated with DNase I to ensure that residual HPV 16 DNA would not react in the dot blot assay. Twofold dilutions of total RNA, starting with $2.53 \mu\text{g}$ (10^6 SiHa cell equivalents), were prepared with a fixed amount of total RNA derived from 5×10^5 Vero cells (approximately $1.25 \mu\text{g}$). The results of the two assays are shown in Fig. 3. The detection limit for HPV 16 RNA in both assays was similar ($0.039 \mu\text{g}$ of RNA, or 1.6×10^4 SiHa cell equivalents).

FIG. 2. Detection of HPV 18 mRNA in HeLa cells. Procedures were as described in the legend to Fig. 1, except that nucleic acids were extracted from HeLa cells (20 copies of HPV 18 DNA per cell). Samples were hybridized to an HPV 16 probe (+), an HPV 18 probe (●), or the pGEM2 vector without an insert (▲). ↓, Detection cutoff. Results are means of triplicate determinations. Vertical bars represent 1 standard deviation. When hybridized with nucleic acids extracted from 1.4×10^5 HeLa cells ($1.6 \mu\text{g}$ per test), the HPV 16 and pGEM2 probes gave signals of 52 ± 2 and 50 ± 4 fu, respectively.

Several experiments demonstrated the specificity of the assay for RNA (Table 1). Nucleic acids extracted from 2.5×10^5 SiHa cells by the single-step acid guanidinium-phenol method showed a reactivity of 135 ± 10 fu with the pg16 probe. After treatment with 1,000 U of T_1 RNase at 37°C for 2 h, the signal was 16 fu, below the background reactivity (34 ± 2 fu) of nucleic acids extracted from 10^5 Vero cells. The specificity of the assay for HPV 16 RNA was shown by reactions of nucleic acids from 10^6 SiHa cells with the pg18 probe (34 ± 2 fu) or biotinylated pGEM2 plasmid without an insert (30 ± 4 fu). To demonstrate that the signal originated from a reaction with mRNA, we filtered total RNA extracted from 10^7 SiHa cells on an oligo(dT) column. Bound and unbound RNA fractions from the column were tested in the nonisotopic hybridization assay. One microgram of the unbound RNA fraction plus tRNA gave a signal of 47 ± 3 fu with the pg16 probe, while $1 \mu\text{g}$ of the bound RNA fraction plus tRNA gave a signal of 332 ± 15 fu. Having shown that the assay was specific for mRNA, we used subgenomic probes for HPV 16 E6-E7 (T16EP) and L1-L2 (T16SB) ORFs to examine the expression of different segments of the HPV 16 genome in SiHa cells. Total RNA extracted from 5×10^5 SiHa cells gave a clear signal above the background with the T16EP probe (197 ± 10 fu) but not with the T16SB probe (35 ± 5 fu).

Next, we evaluated the performance of the solution hybridization assay-enzyme immunoassay for the detection of HPV 16 and HPV 18 mRNAs in clinical specimens. Fifty-seven genital biopsy specimens from 30 women and a laryngeal papilloma biopsy specimen from a child were tested for HPV 16 or HPV 18 mRNA. Virological data were available for 33 of these specimens. Results of the solution hybridization assay-enzyme immunoassay for HPV 16 and HPV 18 mRNAs in these 33 biopsy specimens are shown in Fig. 4. The reactivities of nucleic acids extracted from 15 specimens that were negative for HPV were 52 ± 20 fu (range, 20 to 90 fu) with the pg16 probe and 55 ± 20 fu (range, 21 to 88 fu) with the pg18 probe. From these results,

FIG. 3. Comparison of the solution hybridization assay-enzyme immunoassay and a ³²P-based slot blot assay for the detection of HPV 16 mRNA. Total cellular RNA was extracted from SiHa cells by the acid guanidinium-phenol method and treated with 2 U of RNase-free DNase I for 30 min at room temperature. Twofold dilutions were prepared in DEPC-H₂O containing 0.5% SDS with a fixed amount of RNA extracted from 5 × 10⁵ Vero cells per test. The presence of HPV 16 mRNA was detected by the solution hybridization assay-enzyme immunoassay (right) or the slot blot assay with a ³²P-labeled HPV 16 probe (specific activity, >10⁸ cpm/μg) (left). Results for the former assay are given as means ± 1 standard deviation for triplicate determinations. probe, Reactivity of the HPV 16 probe with RNA extracted from 5 × 10⁵ Vero cells. A weak signal was visible on the original slot blot down to 3.2 × 10⁴ SiHa cells.

the detection cutoff values (mean reactivity of samples devoid of HPV sequences plus 3 standard deviations) were calculated for the HPV 16 (>112 fu) and HPV 18 (>115 fu) assays. The reactivities of nucleic acids extracted from 13 specimens that were positive for HPV other than HPV 16 and HPV 18 were 63 ± 20 fu (range, 32 to 100 fu) with the pg16 probe and 48 ± 25 fu (range, 21 to 110 fu) with the pg18 probe. These reactivities did not differ significantly from those of the HPV-negative specimens for both probes (*P* > 0.1). Nucleic acids extracted from 10⁵ Vero cells showed a reactivity of 49 ± 3 fu with the pg16 probe. The three specimens that were positive for HPV 16 DNA were also positive for HPV 16 mRNA and showed reactivities of 354, 500, and 194 fu. One sample also reacted positively with the pg18 probe (133 fu). Of the two specimens that were positive for HPV 18 DNA, only one was positive for HPV 18 mRNA (918 fu).

An additional 25 biopsy specimens, for which virological data were not available, were also tested in the solution hybridization assay-enzyme immunoassay for HPV 16 and HPV 18 mRNAs (Fig. 5). Seven samples were positive for HPV 16 mRNA (122 to 332 fu). Five samples were positive for HPV 18 mRNA (133 to 833 fu). The remaining 13 samples

TABLE 1. Specificity of the solution hybridization assay-enzyme immunoassay for DNA-RNA hybrids for the detection of HPV 16 mRNA in SiHa cells^a

No. of SiHa cells/assay (10 ⁵)	Probe	Treatment	fu (mean ± SD)
2.5	pg16	None	135 ± 10
2.5	pg16	RNase	16 ± 3
10	pg18	None	34 ± 2
10	pGEM2	None	30 ± 4
10	None	None	7 ± 1
1.2	pg16	Oligo(dT), unbound fraction	47 ± 3
		Oligo(dT), bound fraction	332 ± 15
1.2	pg18	Oligo(dT), unbound fraction	32 ± 2
		Oligo(dT), bound fraction	34 ± 2
5	T16EP	None	197 ± 10
5	T16SB	None	35 ± 5

^a Total RNA was extracted from SiHa cells and tested in the solution hybridization assay-enzyme immunoassay without further processing, after treatment with T₁ RNase, or after separation on an oligo(dT) column. Biotinylated DNA probes were prepared from recombinant plasmids containing whole genomic HPV 16 (pg16) or HPV 18 (pg18), the HPV 16 E6-E7 ORF (T16EP) or L1-L2 ORF (T16SB), or the pGEM2 plasmid without an insert. The detection cutoff for pg16 was 51 fu (mean reactivity of the probe with nucleic acids extracted from 5 × 10⁵ Vero cells plus 5 standard deviations [36 + (5 × 3) = 51 fu]).

showed reactivities of 62 ± 20 fu (range, 30 to 90 fu) with the pg16 probe and 57 ± 13 fu (range, 41 to 94 fu) with the pg18 probe.

Six samples positive for HPV 16 mRNA with the full genomic probe and 16 negative samples were tested in the solution hybridization assay-enzyme immunoassay with sub-genomic probes (Fig. 6). Samples negative for HPV 16 mRNA showed reactivities of 14 to 60 fu with the T16EP probe for the E6-E7 ORF (mean, 38 ± 13 fu), resulting in a detection cutoff for the assay of 75 fu. Samples positive for

FIG. 4. Detection of HPV 16 and HPV 18 transcripts in biopsy specimens characterized for the presence of HPV DNA. Nucleic acids were extracted from 33 biopsy specimens and tested in the solution hybridization assay-enzyme immunoassay with an HPV 16 probe or an HPV 18 probe. The horizontal line is the cutoff (mean fu + 3 standard deviations for biopsy specimens negative for HPV DNA = 112 fu) for the HPV 16 mRNA assay, and the vertical line is the cutoff (115 fu) for the HPV 18 mRNA assay. The specimens were previously characterized as containing HPV 16 (■), HPV 18 (●), or other HPV (□) or as negative for HPV (○). Results are means of triplicate determinations.

FIG. 5. Detection of HPV 16 and HPV 18 transcripts in biopsy specimens not characterized for the presence of HPV DNA. Nucleic acids were extracted from 25 biopsy specimens and tested in the solution hybridization assay-enzyme immunoassay with an HPV 16 probe or an HPV 18 probe. The horizontal line is the cutoff (mean fu + 3 standard deviations for biopsy specimens negative for HPV DNA = 112 fu) for the HPV 16 mRNA assay, and the vertical line is the cutoff (115 fu) for the HPV 18 mRNA assay. Results are means of triplicate determinations.

HPV 16 mRNA showed reactivities with the T16EP probe ranging from 83 to 152 fu (mean, 112 ± 27 fu). The HPV 16 mRNA-positive samples were significantly more reactive in the assay with the T16EP probe than were the HPV 16 mRNA-negative samples ($P < 0.001$). Samples negative for HPV 16 mRNA showed a low reactivity in the assay with the T16SB probe for the L1-L2 ORF (mean, 15 ± 6 fu; detection cutoff, 35 fu); two samples that were positive for HPV 16 mRNA gave signals of 50 and 65 fu, and four samples gave signals between 17 and 30 fu.

FIG. 6. Detection of subgenomic HPV 16 transcripts in biopsy specimens. Nucleic acids were extracted from biopsy specimens and tested in the solution hybridization assay-enzyme immunoassay with a probe for the HPV 16 E6-E7 ORF or the HPV 16 L1-L2 ORF. The vertical line is the cutoff (mean fu + 3 standard deviations for biopsy specimens negative for HPV 16 mRNA = 35 fu) for the HPV 16 L1-L2 mRNA assay, and the horizontal line is the cutoff (75 fu) for the HPV 16 E6-E7 mRNA assay. Specimens were previously characterized as containing HPV 16 mRNA (●) or as negative for HPV 16 mRNA (○). Results are means of triplicate determinations.

TABLE 2. Frequency of HPV 16 and HPV 18 transcripts as determined by histological diagnoses

Diagnosis	No. of specimens with the following transcript(s):			
	None	HPV 16	HPV 18	HPV 16 and HPV 18
Low-grade squamous cell lesions, vulva and cervix ^a	7	0	4	0
High-grade squamous cell lesions, vulva and cervix ^b	5	8	0	1
Other ^c	5	0	0	0

^a Viral changes, condyloma, cervical intraepithelial neoplasia (CIN)-1, and vaginal intraepithelial neoplasia (VIN)-1.

^b VIN-2, VIN-3, CIN-2, CIN-3, and invasive cancer.

^c Normal tissues, endometrial cancer, transitional cell carcinoma of the vagina, lymph nodes, and laryngeal papilloma.

Next, we compared the results of the solution hybridization assay-enzyme immunoassay for HPV 16 or HPV 18 mRNA with the histological diagnoses for 30 of the patients (Table 2). HPV 16 transcripts were found in a majority of high-grade squamous cell lesions of the lower genital tract. HPV 18 transcripts were detected in one high-grade and four low-grade squamous cell lesions of the lower genital tract. Five tissues with pathological diagnoses not associated with HPV 16 or HPV 18 were negative for both transcripts.

DISCUSSION

This report describes a novel nucleic acid detection method for measuring HPV mRNA in cell lines and biopsy specimens. The method involves two steps, a hybridization reaction in solution with a biotinylated DNA probe and an enzyme immunoassay to quantitate specific, biotinylated DNA-RNA hybrids. By measuring the products of nucleic acid hybridization reactions in an enzyme immunoassay format, the method provides more objective results than do membrane-based systems, which are usually analyzed by subjective, visual interpretations. Unlike filter or in situ hybridization, the method can be readily applied to the development of quantitative assays. We have previously shown that the solution hybridization assay-enzyme immunoassay for DNA-RNA hybrids is reproducible and linear over $>3 \log_{10}$ dilutions of target RNA and can be applied to the accurate quantitation of interleukin 1 (IL-1) and IL-2 transcripts in lymphoid cell lines (10). In addition, the use of a nonisotopic label avoids the biohazard, inconvenience, and cost associated with the use of a radioactive label while achieving a sensitivity comparable to those of ^{32}P -based methods. It should be noted that although a fluorogenic substrate was used in these experiments, equal sensitivity can be achieved with an alkaline phosphatase-labeled antibody and a colorimetric substrate (11). The low background reactivity of the double-stranded DNA probe in the absence of an RNA target demonstrates the specificity of the monoclonal antibody for DNA-RNA hybrids. The molecular basis for this specificity most likely resides in the difference between the A-form double helix, which is the structural form of DNA-RNA hybrids, and the B-form double helix, which is generally believed to represent native double-stranded DNA (28).

Transcripts from integrated HPV in cell lines have been characterized previously by Northern (RNA) blotting and cDNA sequencing (19, 22, 30, 31, 36). Transcripts were

shown to be derived mainly from the early genes, especially E6 and E7. Studies with cell lines containing HPV allowed us to establish the sensitivity and the specificity of the solution hybridization assay-enzyme immunoassay. HPV 16 and HPV 18 mRNA transcripts could be detected in total nucleic acids from 10^4 SiHa cells and 2.3×10^3 HeLa cells, respectively. The elimination of the signal by treatment with RNase and the selective reactivity of polyadenylated nucleic acids showed that the assay is specific for mRNA. The low reactivity of nucleic acids extracted from samples containing one viral type in assays with a heterologous probe or a control probe derived from plasmid sequences showed that the assays for HPV 16 and HPV 18 mRNAs are specific for the respective viral types. In addition, biopsy specimens containing HPV other than HPV 16 and HPV 18 were negative in the assays for HPV 16 and HPV 18 mRNAs.

In a preliminary analysis of the feasibility of using the solution hybridization assay-enzyme immunoassay for the detection of HPV 16 and HPV 18 transcripts in biopsy specimens, stored specimens from 57 genital tract lesions and a laryngeal papilloma were screened with this method. These specimens were collected routinely over 2 years and were not uniformly tested for HPV. Nevertheless, the results of the solution hybridization assay-enzyme immunoassay with these specimens indicated that the method has potential for the detection of viral RNA in biopsy specimens. For the five specimens previously found positive for HPV 16 or HPV 18 DNA, corresponding transcripts were found in four. The possibility that RNA degradation was responsible for the negative results cannot be excluded, since these specimens had been kept frozen for 2 years. Although continued transcription is not required for carcinogenesis (21, 24), most investigators have found HPV mRNA in all lesions of the cervix harboring HPV DNA (13, 34, 39). Both HPV 16 and HPV 18 transcripts were detected in one of these four specimens, although it was previously shown to contain only HPV 16 DNA. Using in situ hybridization techniques, Stoler and Broker described specimens that were positive for HPV mRNA but not for HPV DNA (37). ViraPap-negative specimens and ViraPap-positive specimens found negative for HPV 16 and HPV 18 by Southern hybridization were uniformly nonreactive in the solution hybridization assay-enzyme immunoassay. For the 25 biopsy specimens not previously tested for HPV, HPV 16 transcripts were found in 7 and HPV 18 transcripts were found in 5. The HPV 16 early-region probe was positive in all instances when the HPV 16 full genomic probe was positive. The detection of HPV transcripts from the early genes in nucleic acids extracted from human genital tract specimens has been described previously (13, 21, 32, 34). HPV 16 transcripts from the late region were found in two of the biopsy specimens, but it was not possible to interpret the significance of this observation because the biopsy specimens were not tested for the presence of capsid antigens or for late transcripts by in situ hybridization assays. In a study analyzing mRNA of HPV 16 in precancerous lesions of the vulva by in situ hybridization, a strong hybridization signal was shown in the apical portion of the epithelium of a few lesions with subgenomic probes for the late region (13). Whether this transcriptional pattern is specific for HPV in vulvar carcinoma needs to be clarified by further studies. The high frequency of HPV 16 mRNA detection in specimens with high-grade squamous cell lesions of the lower genital tract is consistent with other reports in the literature (21, 34–36, 39), but a more systematic study is required to

evaluate this technique for its usefulness in the study of clinical material.

RNA detection methods may help resolve controversies about the possible role of HPV in squamous cell carcinomas of the lower genital tract. The link between HPV and cancer is based on laboratory studies demonstrating the transforming properties of these viruses (3) and on epidemiological studies reporting the detection of HPV DNA in 80 to 90% of cervical cancer biopsy specimens (25, 26). However, interpretation of the epidemiological evidence is confounded by the variable frequency of HPV infections in noncancerous lesions. The reported variation in the detection of DNA of HPV 16, the viral type most commonly associated with cervical cancer, is between 12 and 38.5% for women without cytological abnormalities, depending on the study populations and detection techniques (16, 25, 26, 38). Recent studies with highly sensitive techniques, such as the polymerase chain reaction, have reported even higher prevalence rates for HPV 16 (33, 45). If HPV infections are widespread among sexually active individuals, the detection of HPV DNA may not be a useful marker for individuals who are at risk of developing genital cancer. On the other hand, the detection of HPV genomes that are transcriptionally active may be of predictive value, and future studies should address this possibility.

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