JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 2000, p. 688–695 Vol. 38, No. 2
0095-1137/00/$04.00+0

A Novel and Rapid PCR-Based Method for Genotyping Human Papillomaviruses in Clinical Samples

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Received 4 May 1999/Returned for modification 11 August 1999/Accepted 22 November 1999

Many human papillomavirus (HPV) genotypes are associated with cervical carcinoma. We demonstrate the utility of an innovative technique for genotyping of HPV in cervical tissue samples. This method provides an accurate means of identification of the specific HPV genotypes present in clinical specimens. By using the MY09-MY11 and the GP5$^+$-GP6$^+$ consensus primer pairs, HPV sequences were amplified by nested PCR from DNA isolated from cervical smear samples. This led to the production of an approximately 140-bp PCR product from the L1 (major capsid) gene of any of the HPVs present in the sample. PCR was performed with a deoxynucleoside triphosphate mixture which resulted in the incorporation of deoxyuridine into the amplified DNA product at positions where deoxythymidine would normally be incorporated at a frequency of about once or twice per strand. Following the PCR, the product was treated with an enzyme mix that contains uracil *N*-glycosylase (UNG) and endonuclease IV. UNG removes the uracil base from the nucleotide, and endonuclease IV cleaves the phosphodiester bond at this newly formed abasic site, producing fragments of various sizes. By having end labeled one of the amplification primers, a DNA ladder which is analogous to a "T-sequencing ladder" was produced upon electrophoresis of the products. By comparing this T-sequencing ladder to the known sequences of HPVs, the genotypes of unknown HPV isolates in samples were assigned. Data showing the utility of this technique for the rapid analysis of clinical samples are presented.

Human papillomaviruses (HPVs) are small double-stranded DNA viruses that infect the human epithelium and cause hyperproliferation (16). There are over 70 genotypes of HPVs; one subset causes common warts and the other subset, which infects anogenital mucosa, causes genital warts (11). Within the anogenital HPVs a subgroup termed the "high-risk" HPVs is associated with greater than 90% of cervical cancers, a leading cause of death by cancer in women worldwide (19). Early detection of preneoplastic and neoplastic lesions in the cervix through the use of Pap smears has led to a reduction in the number of deaths due to cervical cancer in countries where women have good access to health care. With the appreciation of the role of HPVs in cervical cancer, there is a growing appreciation of the potential value for routine screening of female patients not only for cytological abnormalities present in cervical lavage specimens (Pap smears) but also for the presence and type of HPV (2).

Currently, two main approaches are used to screen clinical specimens for the presence of HPV: (i) PCR with consensus primers that amplify a region of the major viral capsid L1 gene that is highly conserved among anogenital HPVs (14) and (ii) the Hybrid II Capture Assay (Digene Diagnostics, Inc.), which detects the presence of either high-risk or low-risk HPVs by formation of DNA-RNA hybrids (17). While consensus primer-based PCR allows the detection of HPV DNA, it gives little information about the genotype of HPV present. The Hybrid II Capture Assay can show the presence of most of the high-risk HPVs by using the high-risk HPV RNA probe mix or low-risk HPVs by using the low-risk HPV RNA probe mix, but it does not give any genotype-specific information.

In order to obtain HPV genotype information from clinical specimens, genotyping techniques have been developed. One approach is to use a pool of genotype-specific primers in a PCR (4). This leads to the amplification of a portion of certain HPVs if they are present in the sample. Because the various HPV genotypes will give amplimer products of different sizes, a genotype can be assigned by measuring the size of a product on an ethidium bromide-stained gel or using Southern hybridization and comparing the amplimer size to what would be expected from the various HPV genotypes. The drawbacks to this method are that it can be labor-intensive, especially if Southern hybridization is performed, and it tests for only a small number of HPV genotypes. A reverse line blot detection method in which PCR is performed with consensus primers has also been developed (7, 8). The product is then used to probe a nylon strip containing immobilized DNAs of several HPV genotypes. While this technique gives an exact genotype, only 27 HPV genotypes are screened by this assay. The "gold standard" for HPV genotyping is sequencing, in which HPV DNA is amplified with consensus primers and the product is cloned into a plasmid and is sequenced. While this technique gives the most conclusive genotype information, it is the most labor-intensive.

We describe here a simple, rapid, one-step PCR-based technique for genotyping of HPVs in clinical samples. The method should be easily adaptable to high-throughput screening with automated sequencing hardware. This technique uses the BESS-T Base Reader Kit (Epicentre Technologies, Madison,

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Wis.) protocol to genotype the HPV DNA present in clinical samples. The protocol involves performance of PCR with samples by using the same HPV L1 consensus primers that are already being used for high-sensitivity HPV detection. The PCR conditions are modified; one of the primers is end labeled with either a radioactive or a fluorescent probe and the BESS deoxynucleoside triphosphate (dNTP) mix, which contains dUTP, is used. The PCR product is digested for a half hour by using an enzyme mixture that cleaves a DNA strand wherever dUTP has been incorporated (9). The cleaved products are run on a sequencing or capillary gel producing a "T-sequencing ladder" that is unique to a specific HPV genotype. The T-sequencing ladder is compared to theoretical T-sequencing ladders in order to assign a specific genotype to the sample.

MATERIALS AND METHODS

DNA preparation from paraffin-embedded tissue. Paraffin-embedded tissue sections (10 μm) were prepared for testing as described below by a modified version of a previously described technique (14). Paraffin was removed from tissue sections that were transferred to microcentrifuge tubes by two extractions with 1 ml of xylenes. Excess xylenes were removed by two extractions with 1 ml of 100% ethanol. Dried tissue samples were resuspended in 100 μl of "K buffer" (50 mM KCl, 10 mM Tris-HCl [pH 8.3], 2.5 mM $MgCl_2$, 100 μg of gelatin per ml, 0.45% IGEPAL, 0.45% Tween 20, 60 μg proteinase K per ml) and were incubated at 55°C for 3 h and then at 95°C for 10 min. The debris was pelleted, and 3 μl of the supernatant was used for PCR.

DNA preparation from cervical scrapes. Samples were collected and DNA was prepared as described previously (5, 6). Briefly, a cotton-tipped swab was scraped over the surface of the portio vaginalis. The cells on the swab were collected by placing the swab in a plastic tube containing 1.5 ml of STE (0.1 M NaCl, 10 mM Tris-HCl [pH 8.0], and 1 mM EDTA). The cells were centrifuged, washed twice with phosphate-buffered saline, and incubated at 60°C for 1 h in 100 μl of lysis buffer (10 mM Tris-HCl [pH 7.9], 0.45% Nonidet P-40, 0.45% Tween 20, 60 μg of proteinase K per ml), followed by incubation at 95°C for 10 min. A total of 10 to 25 μl of the solution was used for PCR.

PCR amplification. HPV DNA was amplified by two approaches. Both approaches used the MY09-MY11 consensus primer pair (which amplifies nucleotides 6722 to 7170 in HPV type 6 [HPV-6] and the corresponding regions of the other genital HPVs) and the primer pair $GP5^+$-$GP6^+$ (which amplifies nucleotides 6764 to 6902 in HPV-6 and the corresponding regions of other HPVs) in a nested PCR (3, 14). DNA prepared from paraffin-embedded tissue was placed in a PCR mixture with 1× PCR buffer (Boehringer Mannheim), 200 μM dNTP, 1 μM MY09, 1 μM MY11, 3.5 mM $MgCl_2$, and 0.5 U of *Taq* polymerase (Boehringer Mannheim). Twenty cycles were performed, with denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and polymerization at 72°C for 1 min. A total of 0.5 μl of this PCR product was then used in a subsequent PCR. The conditions were the same as those for the previous PCR, with the exceptions that the dNTP mixture was replaced by the dNTP mixture supplied in the BESS-T Base Reader Kit, which contains a small amount of dUTP, and the MY09-MY11 primer pair was replaced by 5 pmol of $GP6^+$ and 4 pmol of γ-^{32}P-end-labeled $GP5^+$. The PCR was performed as described above for 25 cycles. The cervical scrape samples were amplified by the nested PCR that was described previously (1). A total of 0.5 μl of this PCR product was used in a PCR mixture which was the same as that for the second PCR step described above for the paraffin-embedded tissue samples with $GP6^+$, γ-^{32}P-end-labeled $GP5^+$, and the dNTP mixture containing dUTP. In all PCR experiments, negative and positive controls were included. For negative controls PCR was carried out in the absence of template and in the presence of DNA isolated from the HPV-negative cervical cancer cell line C33A. For positive controls PCR was carried out with recombinant plasmids containing the HPV-16, HPV-18, or HPV-31b genomes and DNAs isolated from the HPV-positive cervical cancer cell lines Caski and SiHa.

γ-^{32}P end labeling of $GP5^+$. $GP5^+$ was γ-^{32}P end labeled in a 25-μl kinase reaction with 1 μl of 50 μM $GP5^+$, 2.5 μl of 10× polynucleotide kinase (Boehringer Mannheim), 2.5 μl of 1 U of polynucleotide kinase (Boehringer Mannheim) per μl, 2.5 μl of [γ-^{32}P]ATP (3,000 Ci/mmol), and 16.5 μl of double-distilled H_2O. The reaction mixture was incubated at 37°C for 30 min, followed by an incubation at 75°C for 10 min. The resulting solution was passed through a Quick Spin Column (Sephadex G-25; Fine; Boehringer Mannheim).

Sequencing of PCR samples. Samples were PCR amplified as described above by using cold $GP5^+$-$GP6^+$. Sequencing was then performed with this product by cycle sequencing. Two microliters of the PCR amplimer was mixed with 4 μl of ABI PRISM BigDye Terminator Ready Reaction Mix (Perkin-Elmer), 4 μl of dilution buffer (400 mM Tris-HCl [pH 8.3], 10 mM $MgCl_2$), 3.2 μl of 1 μM primer ($GP5^+$ or $GP6^+$), and 6.8 μl of double-distilled H_2O. The 20-μl reaction mixture was treated for 5 s at 96°C, followed by 25 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. The products were then passed through CENTRI-SEP Spin Columns (Princeton Separations) and dried in a Speed-Vac. The samples were resuspended in 4.5 μl of resuspension solution (1 part loading dye [25 mM EDTA {pH 8.0}, 50 mg of blue dextran per ml] and 8 parts of deionized formamide). Two microliters of this was then run on an ABI 377 instrument and analyzed with Lasergene99 (DNASTAR Inc.) software.

PCR with fluorescently labeled primers. The PCR mixture contained 1 ng of HPV plasmid DNA or 3 to 5 μl of cells in lysis buffer from cervical scrapes, 1× PCR buffer, 1 U of MasterAmp *Taq* DNA polymerase (Epicentre Technologies), 2.5 mM $MgCl_2$, 200 μM BESS-T Base Reader dNTP mix, 10 pmol of MY09, and 10 pmol of MY11, which was 5′ end labeled with 6-FAM. 6-FAM-labeled oligonucleotides (obtained from Operon Technologies, Inc., Alameda, Calif.) were synthesized by the beta-cyanoethyl phosphoramidite method with 6-FAM phosphoramidite. Cycling conditions were 94°C for 2 min, followed by 29 cycles of 94°C for 1 min, 58°C for 1 min, and 73°C for 1 min and a final extension of 73°C for 4 min.

BESS-T Base Reader Kit reactions. Ten microliters of the final PCR product was incubated with 1.2 μl of 10× excision buffer and 1.0 μl of BESS-T Excision Enzyme Mix for 30 min at 37°C. The reaction was stopped by adding 6 μl of stop-loading dye. ^{32}P-labeled samples were incubated at 75°C for 5 to 10 min, and 2 μl was loaded and run on an 8% denaturing polyacrylamide gel. The banding patterns were visualized with a PhosphorImager (Molecular Dynamics) and by radiography. Fluorescently labeled samples were run on an ABI 310 automated sequencer and analyzed with GeneScan software (10).

Genotyping with genotype-specific primers. Cervical scrape samples which were positive for HPV DNA were genotyped with genotype-specific primers as described previously (4).

RESULTS

The goal of this study was to test the feasibility of using the BESS-T Base Reader Kit methodology for genotyping of the HPV DNA present in clinical samples. The BESS-T Base Reader Kit methodology (Fig. 1) makes use of bacterial enzymes (uracil *N*-glycosylase and endonuclease IV) to cleave a polydeoxyribonucleic acid chain at the positions of uracils (9). HPV DNA was amplified by PCR. dUTP was added to the PCR mixture, leading to its infrequent incorporation into the newly synthesized DNA products at positions where dTTP is normally incorporated. Treatment of the amplified product (the amplimer) with the enzymes mentioned above led to removal of the uracil base wherever it was incorporated and consequent chain breakage. Thus, for each strand, a set of cleavage products reflective of the positions where T residues normally reside were obtained. Because one of the primers used in the PCR was end labeled, one could selectively visualize the products from this strand by autoradiography or a nonradioactive detection method. The pattern of the products generated upon their electrophoresis on a denaturing polyacrylamide gel or capillary gel were equivalent to the T-sequencing ladder one would generate for the same DNA segment by performing a Sanger sequencing reaction with ddTTP.

To genotype HPVs in clinical samples we combined use of this BESS-T Base Reader Kit methodology and PCR amplification of segments of the HPV genomes using consensus primers. PCR is the most sensitive technique available for the detection of HPV DNA in clinical samples (14). The standard approach is to perform PCR with pairs of consensus oligonucleotides designed to direct amplification of a segment of the viral genomes of most HPV genotypes. MY09-MY11 is such a consensus primer pair; these primers direct amplification of a portion of the viral L1 capsid gene (14). The $GP5^+$-$GP6^+$ primer pair, which amplifies a region contained within the MY09-MY11 amplimer, is commonly used together with the MY09-MY11 primer pair in nested PCRs to yield information regarding the HPV status of clinical samples (5). We compared the predicted T-ladder banding pattern for all known anogenital HPVs for the region amplified by the $GP5^+$-$GP6^+$ primer pair (Fig. 2). The T-ladder banding pattern was unique for each HPV genotype compared. As one might predict, similarities in banding patterns reflected the previously ascribed phylogenetic relationships among these HPVs (15). This analysis indicated the potential utility of the BESS-T Base Reader Kit for genotyping of the anogenital HPVs.

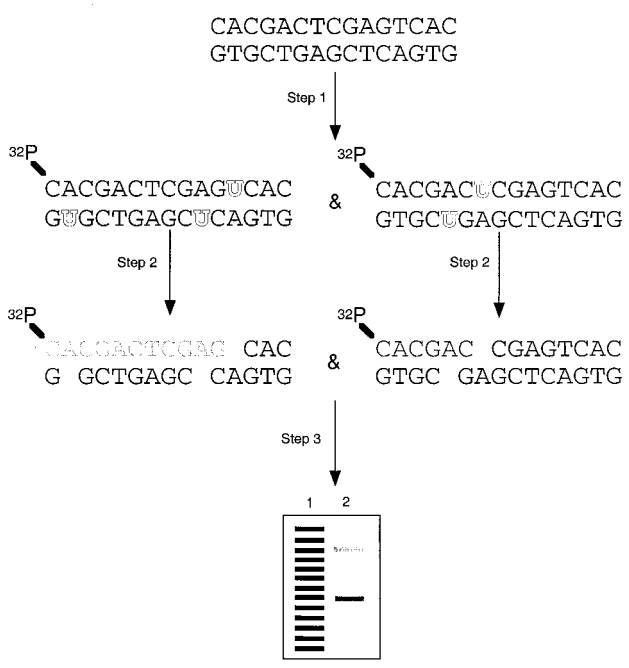

FIG. 1. Principle behind use of BESS-T Base Reader Kit method for genotyping of HPVs. A schematic outlining the key steps in the BESS-T Base Reader Kit method is shown. In step 1, a DNA template is amplified by the PCR method. Standard PCR conditions were modified in two essential ways: (i) one of the primers is ^{32}P end labeled and (ii) dNTP is spiked with dUTP. The resulting PCR products have dUTP randomly incorporated into each strand in place of dTTP at an average frequency of one to two dUTP residues per DNA molecule. In step 2, the PCR products are treated with the uracil *N*-glycosylase and endonuclease IV, causing the cleavage of DNA strands at deoxyuridine (U) residues. In step 3, the cleaved, ^{32}P-end-labeled PCR products (shaded typeface) are made single stranded and are electrophoretically resolved on a denaturing polyacrylamide gel, and the labeled products are visualized by autoradiography or with a PhosphorImager (Molecular Dynamics). Shown at the bottom is an idealized autoradiographic result. In lane 2 are resolved the two ^{32}P-end-labeled cleavage products displayed in step 2. Lane 1 represents a DNA ladder in which each DNA fragment differs by one nucleotide in length. The banding pattern generated with the BESS-T Base Reader Kit is equivalent to that generated by performing a Sanger sequencing reaction with ddTTP.

Using recombinant HPV type 16, 18, and 31 plasmid DNAs as templates and the GP5$^+$-GP6$^+$ primer pair (GP5$^+$ was ^{32}P end labeled), we compared the patterns of the labeled products obtained by the BESS-T Base Reader Kit method (Fig. 3A) to the predicted T-ladder pattern (Fig. 2). A perfect correlation was observed. The individual bands obtained with the BESS-T Base Reader Kit were equivalent in their intensities throughout the region amplified, indicating that the method permits facile identification of all T positions in a sequence. This experiment provided proof of principle.

We next tested the utility of this BESS-T Base Reader Kit method for genotyping of the HPV DNAs present in clinical samples. For this purpose we analyzed 30 coded samples obtained from an ongoing prospective study of European women in which the incidence of HPV-related diseases is being monitored (12, 13). The samples obtained were DNA samples from cervical scrapes that had been subjected to nested PCR with the MY09-MY11 plus GP5$^+$-GP6$^+$ primer pairs. Twenty-eight of the 30 samples had been demonstrated by this PCR methodology to be positive for HPV DNA. Two of the 30 samples were known to be negative for a detectable HPV-specific PCR product. The 28 HPV-positive samples had been analyzed initially for HPV genotype by using genotype-specific primer pairs specific for a limited number of common anogenital HPVs (HPV types 6, 11, 16, 18, and 33). Fifteen of these 28 HPV-positive samples were determined to be positive for at least one of these common HPVs; the other 13 HPV-positive samples did not yield PCR amplimers with this limited set of genotype-specific primer pairs (Table 1). We subjected all 30 coded samples to testing with the BESS-T Base Reader Kit using the GP5$^+$-GP6$^+$ primer pair in which the GP5$^+$ primer was ^{32}P end labeled. As predicted, 28 of the 30 samples yielded ^{32}P-labeled PCR products. Cleavage of the amplimers with uracil *N*-glycosylase and endonuclease IV led to the generation of multiple products for each of the 28 positive samples, yielding specific banding patterns upon their electrophoresis on a denaturing polyacrylamide gel (Fig. 3B). These banding patterns were compared to the predicted T-ladder banding patterns for individual anogenital HPVs (Fig. 2). From this comparison, specific genotypes could be ascribed to 26 of the 28 HPV-positive samples (Table 1). The two HPV-positive samples that could not be genotyped (samples 23 and 28) appeared to have a mixture of at least two different HPV genotypes, as deduced by (i) the presence of two full-length products evident toward the top of the lanes in Fig. 3B for each of these samples, (ii) the complexities of the banding patterns in these two lanes, and (iii) the complexity of the banding pattern seen by sequence analysis of the GP5$^+$-GP6$^+$ amplimer (data not shown). Mixtures of uncleaved products were also evident in samples 1 and 7; however, for these samples one product predominated over the other, allowing the successful genotyping of the isolates in those samples. The results that were obtained from the BESS-T Base Reader Kit analysis and that are illustrated in Fig. 3B were confirmed by duplicate analysis of these samples (data not shown), demonstrating the reproducibility of this technique.

Once the genotypes were assigned for the 30 samples, the code was broken and the genotype information obtained from the BESS-T Base Reader Kit was compared to the HPV status and HPV genotype information obtained from prior analyses of these samples (Table 1). The same 28 samples that were HPV positive in the initial nested PCR analysis were HPV positive by analyses with the BESS-T Base Reader Kit. Among the 15 HPV-positive samples for which PCRs with genotype-specific primers had yielded specific genotype information, there was good concordance with the genotypes assigned to these samples by the BESS-T Base Reader Kit method. Discordance was seen for only 2 of these 15 samples. Sample 20 was found to contain HPV-35 on the basis of the BESS-T Base Reader Kit analysis but had been determined to be HPV-16 positive on the basis of the use of genotype-specific primer pairs. We sequenced the GP5$^+$-GP6$^+$ amplimer from this sample and found it to be identical to the HPV-35 sequence, confirming the result obtained with the BESS-T Base Reader Kit. The isolate in the second sample with a discordant result, sample 28, which had been found to contain HPV-18 on the basis of genotype-specific PCR, could not be genotyped successfully by the BESS-T Base Reader Kit analysis; it was one of the two samples for which there appeared to be evidence that a mixture of at least two HPV genotypes was present in the sample, precluding its successful analysis by genotyping. Of the 12 samples that yielded genotype information by BESS-T Base Reader Kit analysis but that had not yielded genotype information by the genotype-specific PCR analysis (samples 6 to 11, 14, 15, 19, 21, 23, 25, and 29), 3 were determined to contain genotypes that should have been detected by the latter technique (samples 10 and 11 were found by BESS-T Base Reader Kit analysis to contain HPV-16, and sample 29 was found by the BESS-T Base Reader Kit analysis to contain HPV-33;

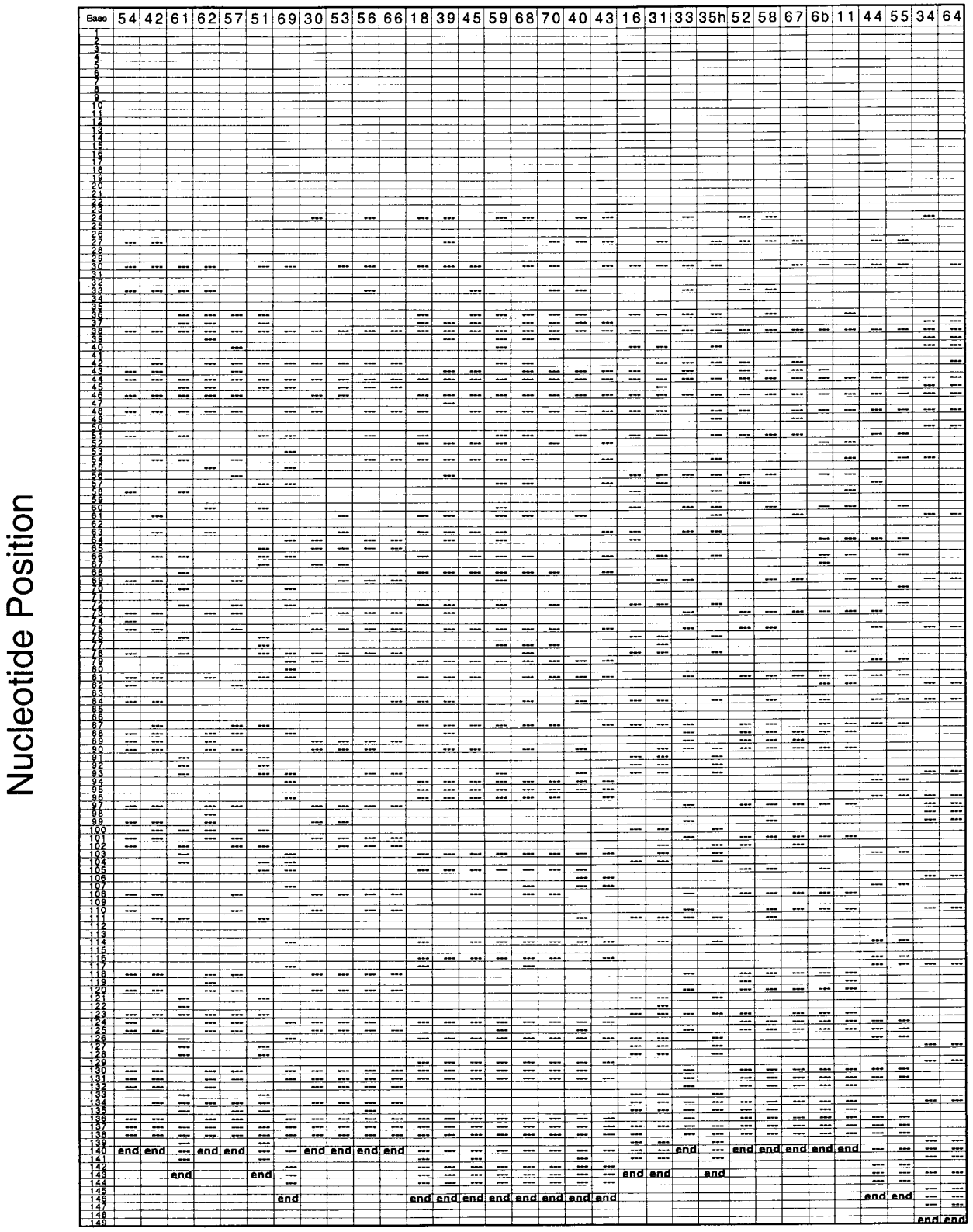

FIG. 2. T-ladder banding pattern for known anogenital HPVs. For each anogenital HPV, the sequence within the region that would be amplified by the degenerate primers $GP5^+$ and $GP6^+$ was analyzed to deduce the hypothetical banding patterns for all DNA fragments generated by cleavage at thymidine residues. Shown are the results for the strand amplified by $GP5^+$. Each column represents the banding pattern for an individual HPV genotype (designated at the top). HPV genotypes were grouped by their phylogenetic relatedness. Each row indicates a specific nucleotide position in the DNA sequence amplified by $GP5^+$-$GP6^+$. The three hyphens in a cell designate the position of a hypothetical DNA band (i.e., the location of a T residue at that position in the DNA sequence). Note the similarities in banding patterns between closely related HPVs.

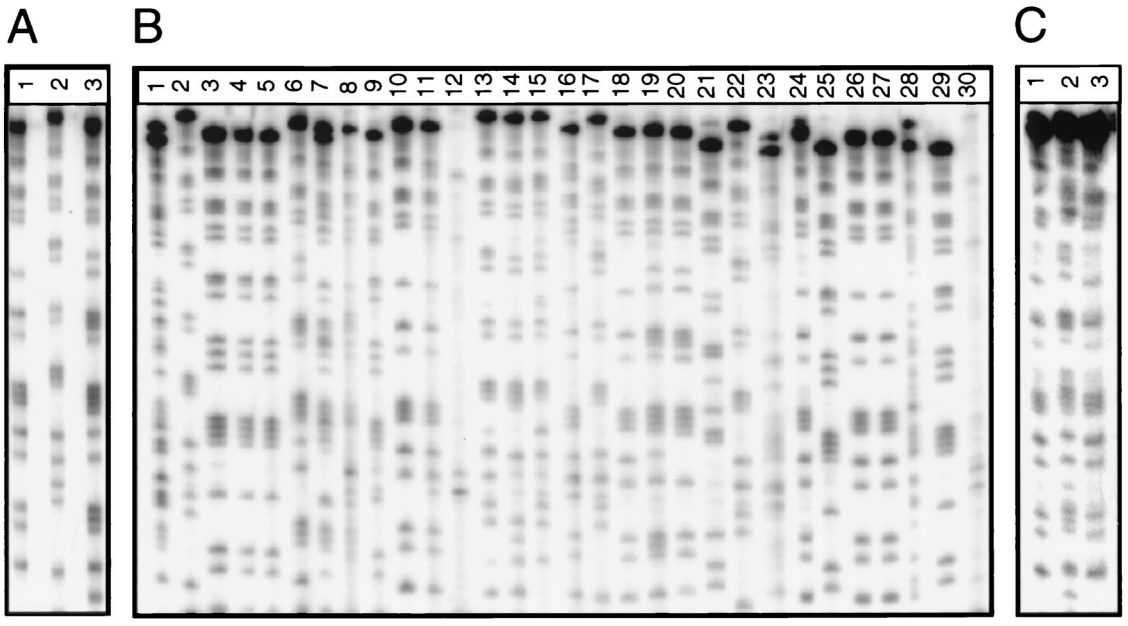

FIG. 3. BESS-T Base Reader Kit analyses. Shown are autoradiographic images of denaturing polyacrylamide gels on which were resolved products from BESS-T Base Reader Kit reactions that used $GP5^+$-$GP6^+$ primer pairs ($GP5^+$ only was ^{32}P end labeled). (A) BESS-T Base Reader Kit reactions performed with recombinant HPV type 16 (lane 1), 18 (lane 2), and 31 (lane 3) DNAs. (B) BESS-T Base Reader Kit reactions performed with 30 coded DNA samples from cervical scrapes. To assign genotypes to the HPVs in each of these 30 samples, the banding pattern for each sample shown in this autoradiographic image was compared to the T-ladder banding patterns deduced for the anogenital HPVs (see Fig. 2). Genotype assignments for these 30 samples are provided in Table 1. (C) BESS-T Base Reader Kit reactions performed with three HPV-positive archival biopsy specimens from women with cervical cancer. See the text for the genotype assignments for these three samples.

Table 1). Sequence analysis of the amplimer generated from these three samples with $GP5^+$-$GP6^+$ confirmed the accuracy of BESS-T Base Reader Kit analysis (Table 1). Of the remaining nine samples whose isolates were genotyped successfully only by BESS-T Base Reader Kit analysis, seven HPV genotypes not tested for with the genotype-specific primer pairs were detected (HPV type 31, 35, 45, 51, 56, 58, and 59).

We also tested the utility of BESS-T Base Reader Kit analysis for genotyping of isolates in archival material. DNA was extracted from seven paraffin-embedded cervical cancer biopsy specimens and subjected to HPV-specific PCR with the MY09-MY11 primers. Three samples positive for HPV DNA were then analyzed by the BESS-T Base Reader Kit method (Fig. 3C). Isolates in all three samples could be genotyped. Samples 1 and 3 were found to contain HPV-16; sample 2 was found to contain HPV-31.

Application of this new genotyping method to the analysis of large numbers of clinical samples would require fast-throughput screening capabilities. To this end we tested the utility of using an automated capillary DNA sequencer to resolve BESS-T Base Reader Kit cleavage products. BESS-T Base Reader Kit reactions were performed with recombinant HPV type 16, 18, and 31 plasmid templates by using the MY09-MY11 primer pair. The MY11 primer was fluorescently labeled. The amplimer was subjected to cleavage with the uracil *N*-glycosylase and endonuclease IV mixtures, and the products were resolved on an ABI automated DNA sequencer. The readout from the ABI apparatus for these three reactions is shown in Fig. 4A to C. Each sample gave a peak pattern that was consistent with the deduced T-ladder banding pattern predicted for the regions of the three genomes analyzed. This experiment proved the potential value of using automated DNA sequencing technology for automation of the BESS-T Base Reader Kit HPV genotyping methodology. Using this

TABLE 1. Comparison of genotyping information gathered by BESS-T Base Reader Kit analysis versus genotype-specific PCR

Sample no.	HPV type detected by the following methods:		
	BESS-T Reader Kit analysis	Genotype-specific PCR	Direct sequencing
1	6, mix	6 or 11	
2	18	18	
3	33	33	
4	33	33	
5	33	33	
6	31	Other	
7	51-like, mix	Other	
8	Probable 51	Other	51
9	58	Other	
10	16	Other	16
11	16, polymorph	Other	16, polymorph
12	Negative	Negative	
13	18	18	
14	59	Other	
15	45-like	Other	45
16	16, polymorph	16	16
17	18	18	
18	16	16	
19	31, polymorph	Other	31, polymorph
20	35h	16	35h
21	56	Other	56
22	18	18	
23	Mix	Other	Mix
24	16	16	
25	33	Other	33
26	16	16	
27	16	16	
28	Mix	18	Mix
29	33	Other	33
30	Negative	Negative	

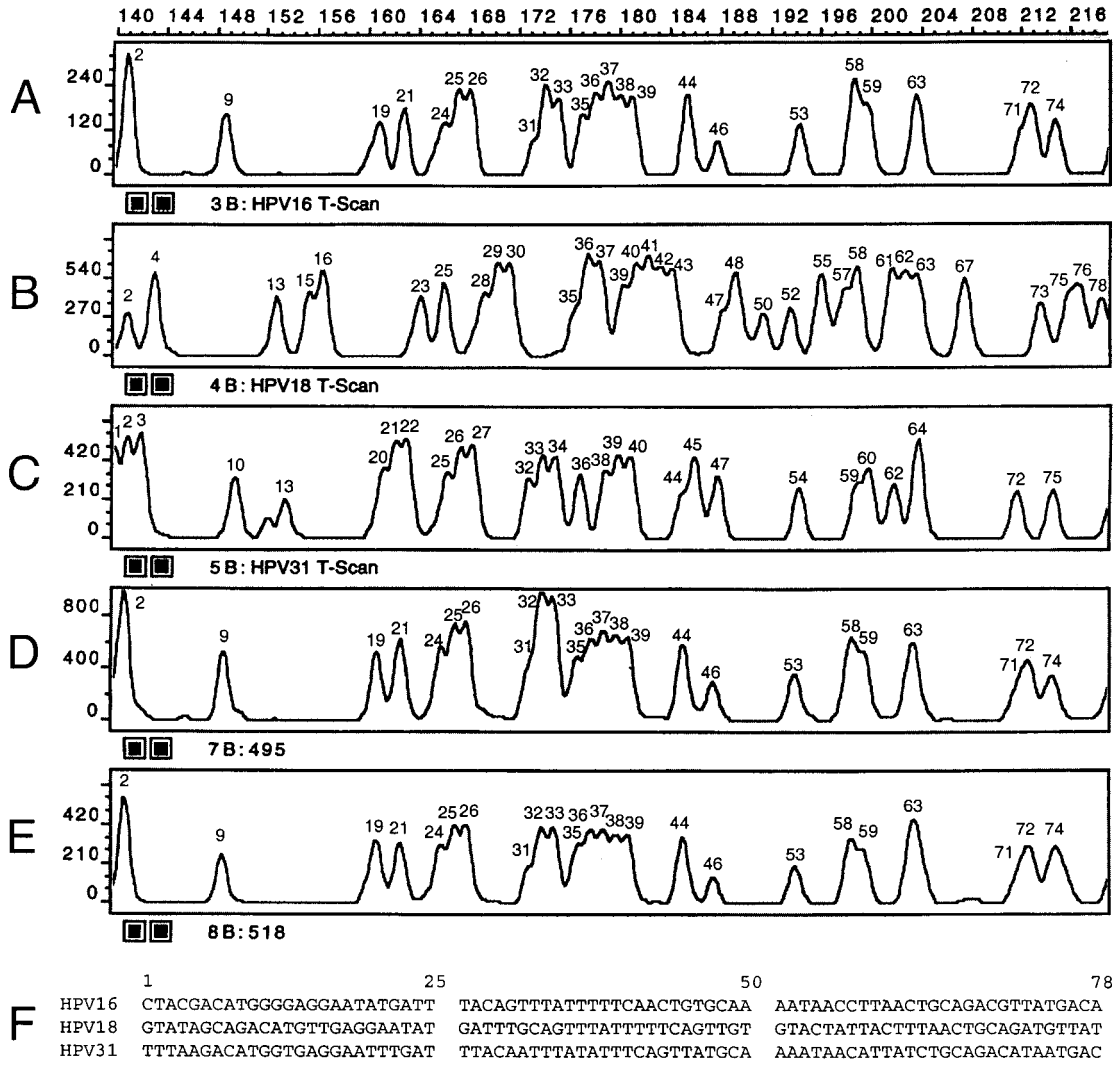

FIG. 4. Automated analysis of BESS-T Base Reader Kit reactions. Shown are the peak patterns obtained when BESS-T Base Reader Kit reactions were run on an ABI 310 automated sequencer. For this experiment, the BESS-T Base Reader Kit reactions were performed with the MY09-MY11 primer pair. The MY11 primer was fluorescently labeled. Shown are the peak patterns generated with recombinant HPV-16 (A), HPV-18 (B), and HPV-31 (C) DNAs as templates. For each HPV, a distinct peak pattern that was identical to the predicted T-ladder pattern was obtained. (F) The sequence that corresponds to the peak patterns in panels A, B, and C. (D and E) Peak patterns from the analysis of two clinical samples (samples 495 and 518, respectively) taken from an ongoing prospective study of North American women. Peak numbers in panels A through E correspond to the nucleotide positions in panel F.

capillary sequencing system, we analyzed 20 additional clinical samples from an ongoing study of HPV prevalence in North American women. Two examples of this analysis are provided in Fig. 4D and E; both clinical samples were determined to contain HPV-16 DNA. Complete concordance was found between the genotype information gained with the ABI sequencer compared to that obtained by the manual analysis of the same samples according to the experiments described in Fig. 3 (data not shown). That these automated analyses were carried out by single-round PCR, without the need for using nested PCR primers, increases the utility of this technique for large-scale analyses.

DISCUSSION

Establishment of HPV infection status and identification of the HPV genotype in clinical samples are gaining ground as important prognostic indicators in the clinical screening of women and management of those found to be at risk for the development of cervical cancer (2). Combining the existing Pap smear analysis protocol with HPV screening has been proposed as a more definitive method for assessing the risk of an individual for developing cervical cancer. The risks for developing cervical cancer differ greatly between individuals infected with low-risk anogenital HPV genotypes and those infected with high-risk anogenital HPV genotypes (11). For this reason, it will be valuable to know the exact genotype present in clinical samples found to be HPV positive. To date, HPV genotyping studies have largely been limited to epidemiological studies and have used a variety of technologies, some of which are of relatively low resolution and others of which, while of high resolution, are limited in the genotypes that they can identify. In this study we describe a facile method for efficient genotyping of the HPV present in clinical samples.

A value of the BESS-T Base Reader Kit methodology is that it reflects an extension of a preexisting method routinely used

for determination of the HPV infection status of clinical samples, i.e., PCR screening with the consensus L1-specific primers MY09-MY11 and/or $GP5^+$-$GP6^+$. The BESS-T Base Reader Kit can be adapted to the analysis of any region of the HPV genome depending upon the choice of primers used in the PCR. The sensitivity and reliability of the BESS-T Base Reader Kit method are the same as those of PCR, the technology upon which it is built. The primary limitation of the BESS-T Base Reader Kit method is in resolving the identities of genotypes in samples containing a mixture of two or more HPVs. When one HPV genotype predominates, its identity but not that of the other genotype(s) present can be determined with the BESS-T Base Reader Kit (e.g., see Table 1, samples 1 and 7). In a mixed sample where no individual genotype predominates, results are ambiguous (e.g., Table 1, samples 23 and 28). The frequency with which clinical samples are found to have mixed infections has not been extensively investigated. Among the 30 coded clinical samples from a prospective study and the 3 archival samples chosen for analysis in this study, 12% (4 of 33) had mixed infections. It would be important that such samples be further analyzed, perhaps by a technique like the line probe assay, if not BESS-T Base Reader Kit analysis of multiple individually cloned HPV DNAs from a given sample.

In comparing the results obtained by the BESS-T Base Reader Kit analysis to those obtained with genotype-specific primers (Table 1), there were several cases of discordance. Consideration of these cases of discordance may provide insights into the strengths and limitations of various genotyping methodologies. In one case, BESS-T Base Reader Kit analysis led to the identification of HPV-35 in sample 20. HPV-16 was detected in the same sample by using genotype-specific primers (Table 1). Sequence analysis of the $GP5^+$-$GP6^+$ amplimer confirmed the BESS-T Base Reader Kit result (Table 1). Perhaps a low-level presence of HPV-16 in the clinical sample in which HPV-35 could be detected with the genotype-specific primers may not have been detectable in the BESS-T Base Reader Kit analysis given the relative abundance of the other viral genome (HPV-35). Were this the case, it points to the previously raised concern that in using the BESS-T Base Reader Kit method with consensus primers, one might not detect HPVs present in patients with mixed infections (an issue of clear relevance to the second case of discordance [sample 28]). Alternatively, HPV-16 may be present at high levels in sample 20, but the region of the viral genome analyzed by the BESS-T Base Reader Kit was not present in the HPV-16 genomes in this clinical sample, for instance, were the HPV-16 genome to have integrated into the cellular genome. In cervical cancers in which high-risk HPV genomes are found to be integrated, deletions within the late region are not uncommon (18). This potential caveat points to the value of analyzing more than one region of the papillomavirus genome by the BESS-T Base Reader Kit method, a viable option given the availability of degenerate primer pairs specific for regions of the HPV genome other than L1 (4, 6). There were three cases of discordance (Table 1, samples 10, 11, and 25) in which the genotype-specific PCR failed to detect HPVs tested for by this technique but in which they were detected by the BESS-T Base Reader Kit method (and confirmed by sequence analysis). The apparently higher sensitivity of the BESS-T Base Reader Kit method may simply reflect the use of nested PCR in the original amplification of the L1 region with degenerate primers compared to the use of a single round of PCR with genotype-specific primers.

The BESS-T Base Reader Kit analysis that we performed led to the detection of single base substitutions at T residues in clinical isolates of certain HPVs. Two examples of this that were confirmed are provided in the cases of samples 11 and 19 (Table 1). In both samples polymorphisms were detected in HPV-16 and HPV-31 on the basis of deviations in the banding pattern observed by BESS-T Base Reader Kit analysis of these samples (Fig. 3B) compared to the deduced T-ladder pattern for the canonical HPV genomes (Fig. 2). These polymorphisms were confirmed upon direct sequence analysis of the amplimer generated with $GP5^+$-$GP6^+$ (Table 1). There is an increasing interest in understanding differences in the pathogenicities of subtypes of high-risk HPV types. The capacity, using our technique, to identify polymorphic markers that distinguish HPV subtypes could be useful in further evaluating the pathogenic properties of subtypes in the context of large-scale prospective or retrospective studies.

The basic advantage of the BESS-T Base Reader Kit method over other genotyping methodologies is that one can assess the genotypes of papillomaviruses in clinical samples in a single-step PCR analysis. This analysis can be achieved by either nested (e.g., Fig. 3) or unnested (Fig. 4) PCR. The BESS-T Base Reader Kit effectively allows one to establish the pattern of T residues in a given sequence (9). In the region of the papillomavirus genomes chosen for analysis in this study, all anogenital papillomaviruses had distinct patterns of T residues on the strand analyzed (Fig. 2), thereby allowing one to determine genotypes unequivocally. As discussed above, verification of the genotype can be provided by BESS-T Base Reader Kit analysis of other regions of the papillomavirus genomes for which there exist degenerate oligonucleotide pairs that permit detection of multiple genotypes. Alternatively, one can perform BESS-T Base Reader Kit analysis of the same region but analyze the opposite strand (i.e., by end labeling the other oligonucleotide, e.g., $GP6^+$ in this study) or use the BESS-G Base Reader Kit, a similar methodology that allows one to establish the G-residue pattern in a given sequence (10). These extensions of the basic methodology described in this study could increase the precision of this genotyping approach where necessary.

The application of the BESS-T Base Reader Kit method or any other method or the genotyping of large numbers of HPV isolates in clinical samples would be greatly enhanced were the method automated. This should be easily achieved with the BESS-T Base Reader Kit method. We were able to make use of an automated DNA sequencing apparatus to generate peak patterns for BESS-T Base Reader Kit reactions of recombinant HPV DNAs cloned into plasmids (Fig. 4A to C). These peak patterns were equivalent to the deduced T-ladder patterns for these test genomes. We also performed a similar automated genotype analysis with clinical isolates. The peak patterns generated with the automated sequencer led to the identification of the same genotypes (for examples, see Fig. 4D and E) identified by the unautomated procedure (data not shown). An added advantage of the automated procedure is the absence of the need to use radioactivity. Preliminary efforts indicate that modification of the ABI sequence analysis program should permit computer-assisted identification of genotypes in clinical samples.

ACKNOWLEDGMENTS

We acknowledge Angie Buehl for preparing histopathological sections and Gary Dahl for critical reading of the manuscript.

This study was supported by grants from the American Cancer Society (grant VM164) and the National Institutes of Health (grant CA22443).

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