American Journal of Pathology, Vol. 131, No. 3, June 1988

Sensitivity of In Situ *Detection With Biotinylated Probes of Human Papilloma Virus Type 16 DNA in Frozen Tissue Sections of Squamous Cell Carcinomas of the Cervix*

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The sensitivity of human papilloma virus type 16 (HPV-16) DNA detection by DNA *in situ* hybridization using biotinylated probes (bio-DISH) was estimated by performing this technique on snap-frozen tissue sections of 10 cervical squamous cell carcinomas containing increasing amounts of HPV-16 as determined by Southern blot hybridization. A protocol using serial sections for bio-DISH and DNA extraction was used. The number of positively stained cells and the detection limit were strongly dependent on the treatment of the sections with proteinase K prior to hybridization. At low proteinase K concentration (0.1 μg/ml), the detection limit appeared to be 30–40 HPV-16 DNA copies per carcinoma cell, whereas morphology was preserved. A high proteinase K concentration (1–5 μg/ml) often resulted in an increase in the number of positively stained cells but also in a poor morphology. The detection limit was improved to at least 20 HPV-16 DNA copies per carcinoma cell. (Am J Pathol 1988, 131:587–594)

INFECTIONS with certain human papilloma virus (HPV) types, of which types 16 and 18 are considered to be the most important ones, have been postulated to be a high-risk factor for the development of human cervical cancer.[1–6] Besides several blotting techniques,[7] detection of HPV-DNA using DNA *in situ* hybridization (DISH) with radioactive probes has also been reported.[8–12] The applicability of DISH techniques for routine screening is, however, greatly enhanced by the possibility of using nonradioactive biotin label as detection marker.[13–15] In order to evaluate the possibility for the routine application of biotinylated probes for DISH (bio-DISH) for HPV detection, more information is needed about its sensitivity. Therefore, we determined the sensitivity of the bio-DISH technique on snap-frozen sections of 10 cervical squamous carcinomas in which an increasing number of HPV-16 DNA copies per cellular genome was found by Southern blot analysis. Our results show that about 30–40 copies of the HPV genome per cell can be detected in sections with preserved morphology. Still higher sensitivity can be obtained with a more extensive protease treatment of the section prior to hybridization. This treatment, however, generally results in a poor morphology.

Materials and Methods

Material Collection and Tissue Processing

Tumor samples consisted of resection material or biopsy specimens of patients who underwent a total hysterectomy for invasive squamous cell carcinoma

Supported in part by the Dutch Organization for the Fight Against Cancer, "Koningin Wilhelmina Fonds," and the Preventiefonds (nr. 28-1502).

Accepted for publication February 3, 1988.

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of the cervix uteri. Samples were snap-frozen in liquid nitrogen (−180 C) and stored at −80 C until used. Neighboring parts of the tumours used for routine histologic examination were formalin-fixed and paraffin-embedded.

Snap-frozen tissue of 10 squamous cell carcinomas was serially sectioned on a cryostate. The first section (6 μ) was used for hematoxylin eosin (HE) staining, and bio-DISH was performed on the next four 6-μ sections. The first three sections were used for HPV-16 bio-DISH at different proteinase K concentrations and the fourth for (p)HPV-6 at 0.1 μg/ml proteinase K as a control. The following 20 sections of 20 μ were collected (at 0 C) and used for DNA extraction. Finally, four sections of 6 μ were cut for bio-DISH as described above, but with the exception that (p)HPV-18 or PBR322 was used instead of (p)HPV-6 as a control and then another one was cut for HE staining. Sections used for bio-DISH were mounted on gelatin-chrome alum precoated glass slides. The carcinoma portion of the tissue was calculated using a planimetric method[16] on the first and last HE-stained sections.

The human cervical cell line CaSKi was obtained from the American Type Culture Collection and grown in Dulbecco's modified Eagle (DME) medium supplemented with 10% fetal calf serum (FCS). Cells were grown near confluency on glass coverslips, washed with ice-cold phophate buffered saline (PBS), pH 7.4, and fixed in 4% paraformaldehyde in PBS for 20 minutes at room temperature. The coverslips were washed with PBS and stored in 70% alcohol at 4 C until needed. HeLa cell line 229 was obtained from Dr. K. H. Thian, Department of Dermatology, Erasmus University (Rotterdam), and grown in DME with 10% FCS. Near confluency, cells were harvested by trypsinization, spun down, and enclosed in agar. The agar blocks were formalin-fixed, paraffin-embedded, and 6 μ sections were used for bio-DISH.

DNA Isolation

Tumor 20-μ sections were collected in ice-cold 50 mM Tris HCl with 50 mM EDTA, pH 8.5, and lysed by addition of sodium dodecyl sulphate (SDS) to a final concentration of 1%. The lysate was treated for 30 minutes at 37 C with proteinase K (100 μg/ml) and extracted two to three times with phenol/chloroform. Nucleic acids were ethanol precipitated and redissolved in 10 mM Tris HCl with 1 mM EDTA, pH 7.5 (TE), and treated with RNAse (20 μg/ml) for 30 minutes at 37 C. The solution was extracted once with phenol/chloroform, DNA was precipitated with ethanol and the pellet was dissolved in TE.

Southern Blot Analysis

Southern blot analysis[17] for HPV detection was carried out with 5 μg DNA aliquots digested with the restriction endonuclease PstI according to recommendations of the manufacturer (Boehringer, Mannheim). DNA samples were subjected to electrophoresis in 0.8% agarose gels, denatured, and transferred to nitrocellulose membranes. The filters were hybridized with nick-translated[18] ^{32}P-labeled (specific activity 10^8 cpm/μg) HPV-16 DNA. Hybridization was carried out at 68 C in 10% dextran sulphate, 6× SSC (SSC: 0.15 M NaCl, 0.015 M Na citrate, pH 6.8), 0.1% SDS, and 50 μg sheared salmon sperm DNA per ml (Tm −30 C). Filters were washed at Tm −10 C.

Probes

HPV-6, −16, and −18 DNAs cloned in the BamHl site of pBR322 (pHPV DNAs) were kindly provided by Dr. H. zur Hausen (Heidelberg). HPV insert DNAs were purified by BamHl cleavage and agarose electrophoresis.

In Situ Hybridization

In situ hybridization was carried out with insert HPV-DNA or pHPV-DNA. Biotinylated probes were made by incorporation of Bio-11-dUTP (Bethesda Research Laboratories) by nick-translation[19] and purified by Sephadex G50 gelfiltration. Bio-11-dUTP–labeled probes with a mean size of 500 bp as determined by alkaline gel electrophoresis[20] were used throughout these experiments. One picogram of such a probe could be visualized by direct spotting. Filter hybridization with these probes could detect 3 pg of spotted homologous HPV-DNA.[21]

Frozen sections were air-dried during 30 minutes for optimal attachment of the sections on the precoated glass slides and fixed for 15 minutes with 4% paraformaldehyde in PBS (pH 7.4). After washing in PBS, sections were incubated with proteinase K (Boehringer Mannheim, nuclease-free). A proteinase K concentration range from 0.1 to 5 μg/ml in 20 mM Tris-HCl buffer, pH 7.4, with 2 mM $CaCl_2$ used for 30 minutes at 37 C, followed by washing with PBS with 0.2% glycine and subsequently PBS. After the sections were dehydrated with graded alcohol and dried, they were prehybridized at 37 C for 60 minutes in a hybridization solution, containing 50% formamide, 10× Denhardt's (0.2% ficoll, 0.2% BSA, 0.2% polyvinylpyrrolidon), 2× SSC, 10% dextran sulfate and 250 μg/ml denatured salmon sperm DNA. After prehybridization the sections were washed two times with

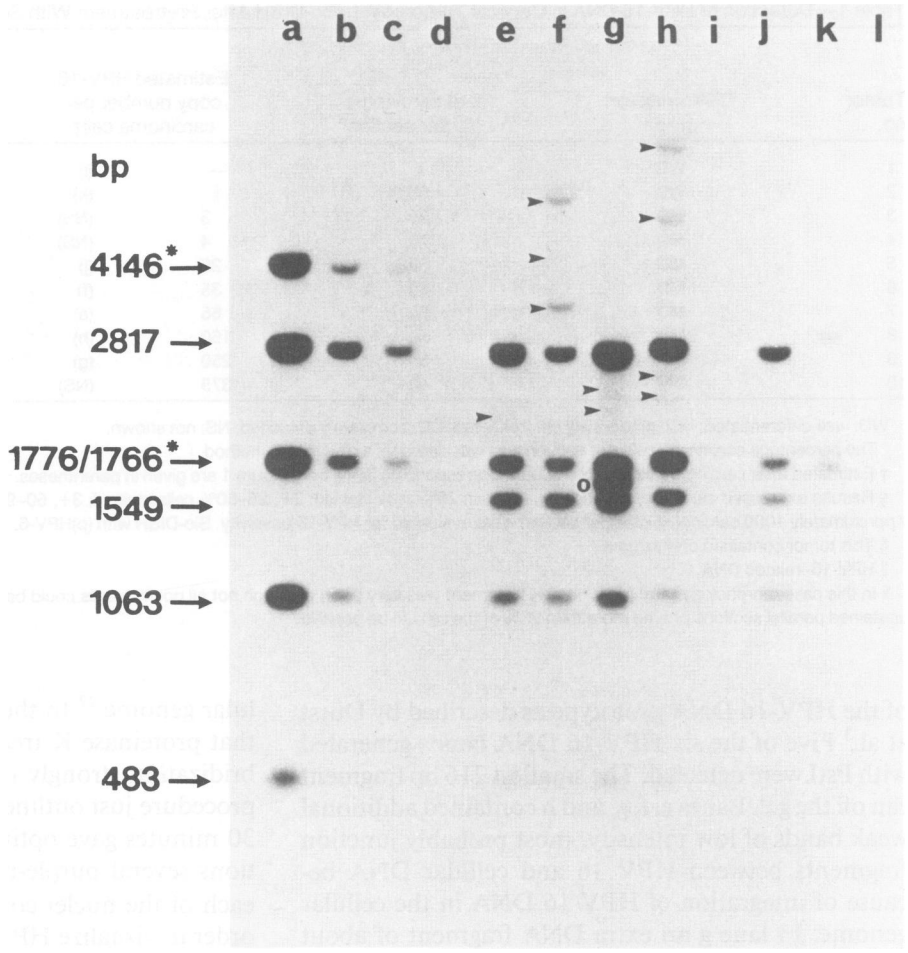

Figure 1—Southern blot analysis of tumors of the cervix uteri. Cellular DNA was extracted from 20 μ sections of snap-frozen cervical tumors (lanes e–k) digested with PstI and subjected to electrophoresis. A reconstruction containing 1000, 100, and 10 pg, respectively, of pHPV-16 DNA added to 5 μg human placenta DNA corresponding to about 100, 10, and 1 HPV-DNA equivalents per cell (lane a, b, c) and 5 μg of human placenta DNA only (lane d and l) were digested with PstI and included. DNA was transferred to nitrocellulose for hybridization with a ^{32}P-labeled purified HPV-16 DNA fragment. Numbers at the left refer to the molecular weights of the PstI fragments of pHPV-16 DNA. *HPV and pBR322 containing fragments. The extra viral band is marked with O. The weak bands are marked with arrowheads. The number of HPV-16 DNA copies per carcinoma cell was estimated from the band signals on the original autoradiograms (Table 1). The 1549 bp band present in most tumor samples represents the internal HPV-16 PstI fragment, which contains the BamH1 site that was used to clone the HPV-16 insert in pBR322.

2× SSC, dehydrated and dried again. Ten microliters of hybridization solution containing 20 ng biotinylated probe was subsequently added to the sections, and a glass coverslip was mounted. The DNA probe and the target DNA in the tissue section were denatured together by heating at 100 C for 5 minutes. Thereafter hybridization was carried out for 18 hours at 37 C in a sealed humidified glass container. The sections were subsequently washed for 15 minutes at 37 C in 2× SSC, three times in 0.1× SSC with 50% formamide at 37 C (Tm −10 C), and two times in PBS at room temperature. Visualization of the biotinylated DNA hybrids was performed using a streptavidin-biotinylated polyalkaline phosphatase staining kit with BCIP and NBT as substrate[22] as indicated by the manufacturer (BRL). Finally, slides were washed with 10 mM Tris and 10 mM EDTA, pH 7.5, and then PBS, and mounted in glycerol/gelatin.

An identical procedure was performed for CaSKi cells except that after paraformaldehyde fixation cells were treated with 0.2 M HCl (15 minutes, 22 C) prior to proteinase K treatment. Paraffin sections of HeLa cells were pretreated with 1 mg proteinase K per ml prior to hybridization.

Results

Human Papilloma Virus Type 16 DNA in Patient Material

To study the sensitivity of the bio-DISH, 10 of 27 cervical squamous cell carcinomas were selected for increasing amounts of HPV-16 DNA. For this purpose we used a protocol for an accurate correlation of Southern blot and bio-DISH regarding HPV copy number. The protocol involves using sequential neighboring sections for carcinoma:stroma ratio determination, DISH, and DNA extraction.

Figure 1 shows the analysis of the HPV type and virus genome copy number in seven of 10 selected tumors using PstI digestion and ^{32}P-labeled HPV-16 insert DNA as a probe. The PstI cleavage pattern of the viral sequences in five of the seven tumor DNAs shows a striking similarity to the PstI digestion pattern

Table 1—Detection of HPV-16 DNA in Cervical Tumors With Bio-DISH After Pretreatment With Several Proteinase K Concentrations

Tumor no.	Differentiation grade	% of carcinoma cells per section*	Estimated HPV-16 copy number per carcinoma cell†		Bio-DISH-HPV-16 proteinase K concentration (μg/ml)‡		
					0.1	1	5
1	WD	§	—	(i)	–	–	–
2	WD	90	‖	(k)	–	–	–
3	MD	80	3	(NS)	–	–	–
4	MD	70	4	(NS)	–	–	–
5	MD	50	20	(j)	–	–	4+¶
6	MD	80	35	(f)	2+	4+	4+
7	MD	80	65	(e)	4+	4+	CD
8	MD	50	150	(h)	2+	4+	CD
9	MD	50	250	(g)	4+	CD	CD
10	MD	40	375	(NS)	3+	4+	4+

WD: well-differentiated; MD: moderately differentiated; CD: completely detached; NS: not shown.

* The percentage carcinoma cells per section was calculated by a planimetric method.

† Estimated after carcinoma:stroma correction. Corresponding lanes from Figure 1 are given in parentheses.

‡ Results expressed as: –, no staining; +, less than 25% cells stained; 2+, 25–50% cells stained; 3+, 50–95%; and 4+, >95% carcinoma cells stained. Approximately 1000 carcinoma cells per section were evaluated for HPV-16 positivity. Bio-DISH with (p)HPV-6, 18, and PBR322 were negative.

§ This tumor contained only stroma.

‖ HPV-16–related DNA.

¶ In this case morphology after proteinase K treatment was very poor; although not all positive cells could be counted exactly, comparison with intact but unstained parallel sections proved more than 95% of the cells to be positive.

of the HPV-16 DNA prototype as described by Dürst et al.[3] Five of the six HPV-16 DNA bands generated with PstI were detected. The smallest 216 bp fragment ran off the gel. Lanes e, f, g, and h contained additional weak bands of low intensity, most probably junction fragments between HPV 16 and cellular DNA because of integration of HPV 16 DNA in the cellular genome. In lane g an extra DNA fragment of about 1600 bp was observed. Recent experiments show that this fragment is of viral origin (unpublished results). In lane i no signal was detected. By examining the HE-stained section of this tumor it appeared that only the fibroblastic stroma component was present. In lane k no HPV-16 specific digestion pattern was observed, which means that an HPV-16 related type was present. Hybridization with the purified ^{32}P-labeled HPV-6 or -18 DNA fragments was negative for all tumors excluding the presence of HPV-6 or -18 DNA in the tumors. By comparing the hybridization signals of the PstI fragments in the tumor DNA with reconstructions (a, b, c) and after correction for the carcinoma: stroma ratio, the mean HPV copy number per carcinoma cell (Table 1) was estimated. The first and the last HE sections, which enclose the tumor part from which DNA was isolated, hardly show any difference in the proportion of malignant cells and stroma.

In Situ Hybridization for HPV-16 DNA

To standardize HPV-DNA detection by *in situ* hybridization, experiments were set up with the CaSKi cell line, which is known to contain approximately 500 copies of integrated HPV-16 DNA per diploid cellular genome.[22] In these experiments it became clear that proteinase K treatment of the cells prior to hybridization strongly influenced the signal. Using the procedure just outlined, 1 μg proteinase K/ml during 30 minutes gave optimal results. Under these conditions several purple-blue granular spots localized in each of the nuclei could be observed (Figure 2d). In order to visualize HPV-16 DNA in frozen tumor sections this protocol was also applied on the 10 selected tumors but without HCl pretreatment, which considerably decreased the quality of cell morphology. A proteinase K concentration range of 0.1–5 μg/ml during pretreatment of the sections was used. Figure 2a shows the HE staining of the first section of tumor no. 9. The ratio of carcinoma cells to stroma appeared to be about equal. The tumor was found to contain at least 125 HPV-16 DNA copies per cellular genome (Figure 1, lane g) as demonstrated by Southern blot analysis. Figure 2b shows a uniform staining of all malignant cells after bio-DISH for HPV-16 at low proteinase K concentration (0.1 μg/ml). Several spots can be seen in the nuclei of the epithelial cells (Figure 2c), whereas bio-DISH with (p)HPV-6, (p)HPV-18, or pBR322 DNA probes gave negative results under identical conditions (not shown). In a separate experiment the specificity of the reaction was also confirmed by DNAse I treatment (0.01 mg/ml, 30 minutes, 37 C) of a section before hybridization. This treatment completely prevented the staining. In Table 1 the amount of HPV-16 DNA per carcinoma cell, estimated after Southern blot analysis and stroma correction, and the results of bio-DISH with increasing pro-

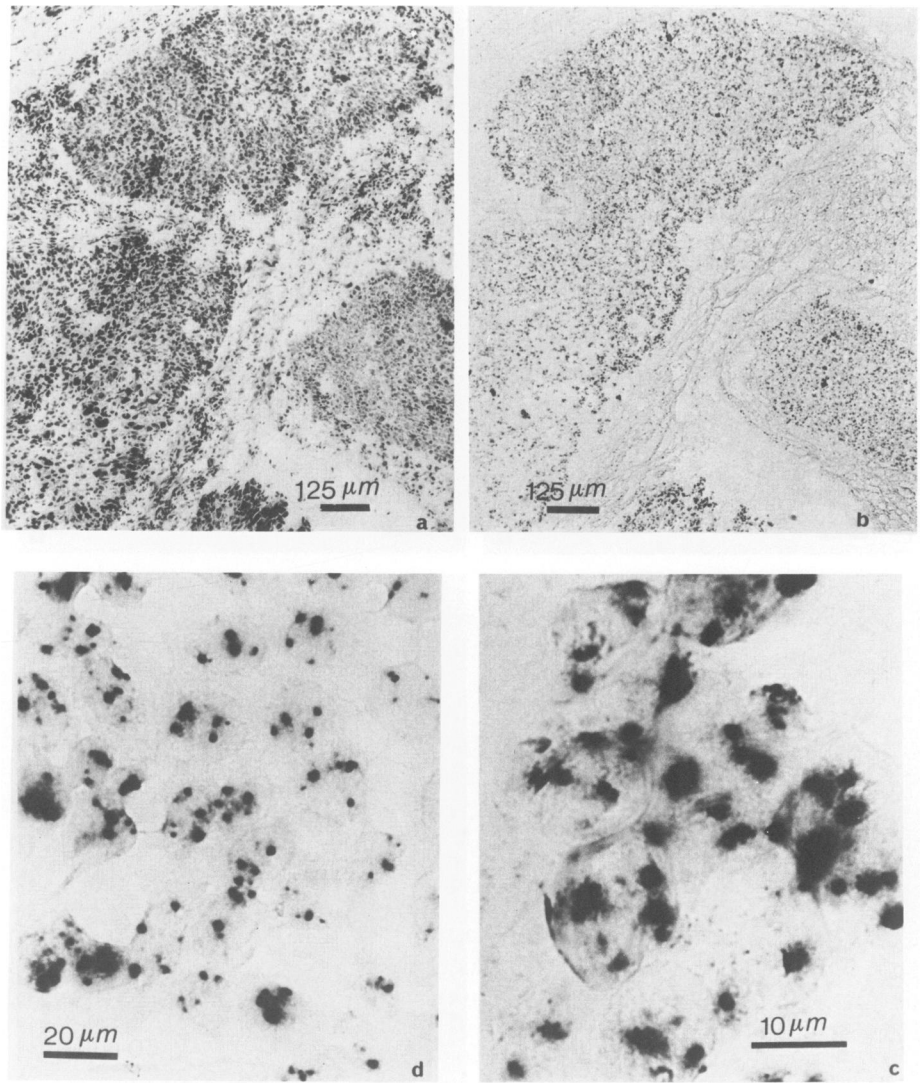

Figure 2—*In situ* hybridization of cervical squamous carcinoma no. 9 and the CaSKi cell line with a purified biotinylated HPV-16 DNA probe. **a**—Low-power photomicrograph of an HE-stained section of the tumor. **b**—Serial section showing granular staining of the malignant infiltrative part of the tumor after DISH with a biotinylated HPV-16 probe; stroma is negative. **c**—High magnification detail of B illustrates granular staining in the nuclei of the malignant cells. **d**—Hybridization of CaSKi cells with HPV-16. Note the well-preserved morphology and the granular staining pattern. In the case of CaSKi cells the protocol was slightly different to obtain better probe penetration into intact cells. Briefly, after fixation cells were sequentially incubated in 0.2 M HCl and proteinase K (1 μg/ml) for 30 minutes before hybridization.

teinase K concentrations are compared for the ten tumors.

At low proteinase K concentration (0.1 μg/ml) tumor samples 1 through 5, which contained up to 20 HPV-16 DNA copies per carcinoma cell, were all negative in bio-DISH. More than 95% of the malignant epithelial cells in tumor nos. 7 and 9 which contained about 60 and 250 copies of HPV-16 DNA per cell, respectively, were positive. In tumor nos. 6, 8, and 10 positive and negative cells could be observed. However, when the proteinase K concentration was increased to 1 μg/ml during pretreatment it could be shown that almost all the malignant cells contained HPV-16 DNA. Figure 3a shows a bio-DISH of tumor no. 6 after pretreatment with 1 μg/ml proteinase K. In contrast to low proteinase K treatment again more than 95 percent of the carcinoma cells are positive and yet morphology is reasonably preserved. Figures 3b and 3c show the bio-DISH results of tumor 8, treated with low (0.1 μg/ml) and high (1 μg/ml) concentrations, respectively, of proteinase K. In this case the morphology is well conserved at 0.1 μg/ml, and up to 50% of the carcinoma cells are positive. At 1 μg/ml proteinase K practically all carcinoma cells are stained, although morphology is poor. Tumor no. 5, containing 20 HPV-16 DNA copies per cell, only stained positively after the very high proteinase K concentration of 5 μg/ml. Under these conditions morphology was strongly deteriorated (not shown) and in many cases sections were detached from the slides. To verify the ability to detect less than 50 HPV-DNA copies per cell, we applied bio-DISH with HPV-18 on the HeLa cell line 229. At 1 mg of proteinase K per ml (5 minutes) we scored positive hybridization (Figure 3d). HPV-18 DNA copy number was approximately 20 as determined by dot blot hybridization.[7]

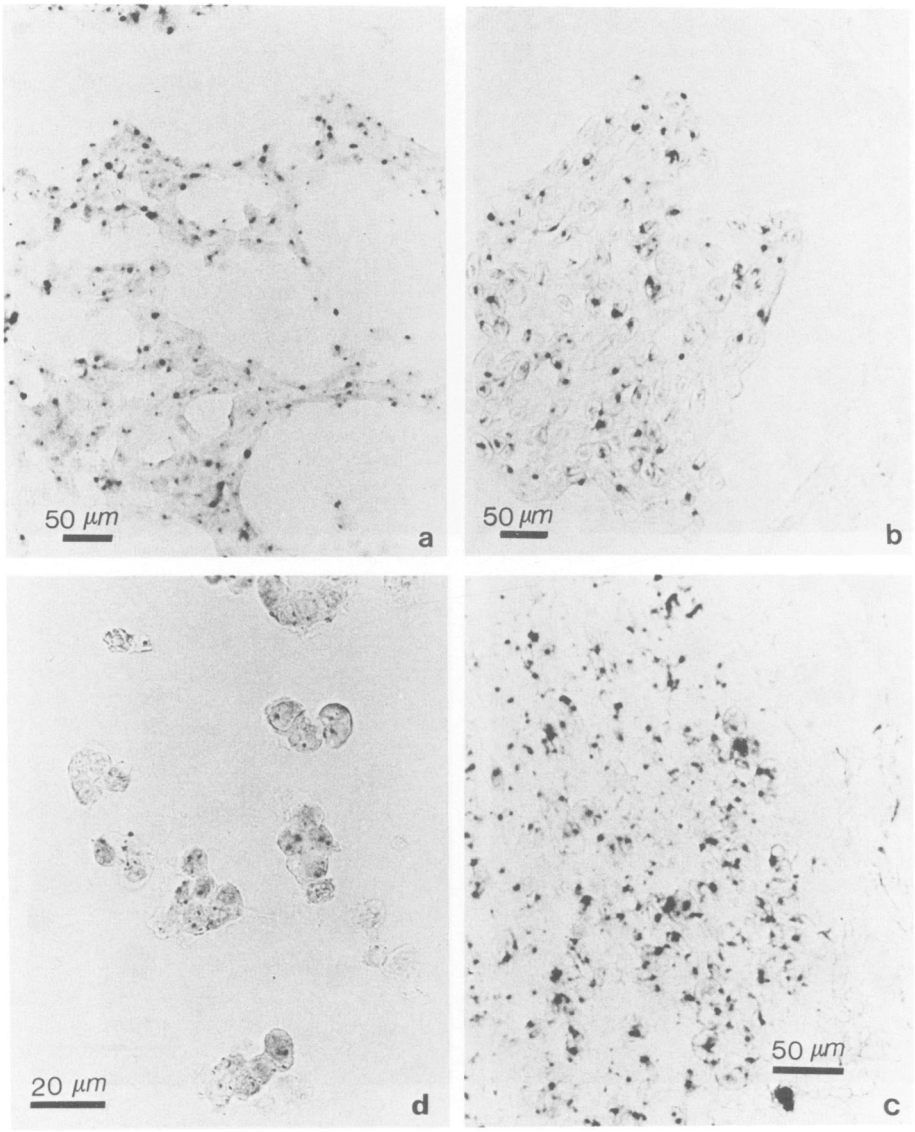

Figure 3—Influence of proteinase K treatment on bio-DISH. **a**—Tumor no. 6 contains 35 HPV-16 DNA copies per carcinoma cell. Treated with 1 μg/ml proteinase K, morphology is reasonably well conserved and almost all cells are equally stained. **b**—Tumor no. 8. contains 150 HPV-16 DNA copies per carcinoma cell. Treated with 0.1 μg/ml proteinase K, positive and negative stained carcinoma cells are present with good morphology. **c**—Frozen section and higher magnification of similar region of tumor no. 8 as in B. Treated with 1 μg/ml of proteinase K, practically all carcinoma cells show positive staining with poor morphology. **d**—Bio-DISH of HeLa cells 229 with HPV-18. Paraffin sections were used. Bio-DISH with (p)HPV-6 and PBR322 were negative.

Discussion

The purpose of this study was to determine and improve the sensitivity of HPV detection with bio-DISH on HPV-16 DNA containing cervical carcinomas. Our approach of using sequential cryostate sections for the carcinoma:stroma ratio determination, DNA isolation, and bio-DISH allows a reasonable approximation of the HPV-16 copy number per malignant cell. The tumors, the CaSKi, and HeLa cell lines analyzed in this investigation contained different amounts of HPV DNA, which made them suitable to study the sensitivity and specificity of the hybridization method. Technically, the bio-DISH procedure can be divided in several main steps: preparation of the probe, tissue fixation and pretreatment to allow penetration of the probe, hybridization, staining, and microscopic evaluation. To study the influence of all these steps on the ultimate results, we initiated our experiments on the CaSKi cell line.[23] Biotinylated HPV DNA probes were standardized with regard to molecular weight, incorporation of biotin, and hybridization capacity. From most fixatives used in routine pathology, paraformaldehyde appeared to be superior. Pretreatment of cells with 0.2 M HCl and proteinase K reduced background staining and increased the number of positive cells. Denaturation temperature, hybridization, and washing conditions were optimalized to reach a maximal specificity and sensitivity. Specificity was further evaluated by Southern blot hybridization, DNAse treatment, and the use of probes for other HPV types and pBR322, which resulted in a negative staining. The CaSKi cell line contains about 500 HPV-DNA copies per cellular genome.[23] By using the described method the cells showed an average of

four heavy spots per nucleus. Applying this method to the tumors, but with omitting HCl treatment and adaptation of proteinase K concentration, about 30–40 HPV-DNA copies per cell could be detected with good preservation of cell morphology. This sensitivity appeared to be equal to the sensitivity of DISH using radioactive RNA probes[9] and is much higher than that described by Crum et al[24] using bio-DISH. However, in this latter study virus-producing cells and an inadequate Carnoy fixation (own observation) was used. Our results are more in agreement with data recently obtained by Burns et al[25] who reported a sensitivity of *in situ* hybridization with biotinylated HPV probes on paraffin-embedded tissue of about 10 HPV copies per cell. However, this information was calculated by extrapolation of the signal obtained with a 2.1 Kb human DNA probe unrelated to HPV. In analogy we could also detect 10 copies of a 7.0 Kb EcoRi-SalI fragment (pES) of human cytomegalovirus with bio-DISH. This fragment was intergrated tandem in a rat cell line.[26] In addition, we were also able to detect HPV-18 DNA in the HeLa cell line 229, which contains about 20 DNA copies per cell, with bio-DISH using paraffin-embedded cells and a high proteinase K concentration during pretreatment. This indicates that it is possible to detect less than 50 HPV DNA copies per cell. In the present study, the results were obtained by a direct analysis of well-defined tumors.

In studying the distribution of HPV-16 DNA in the carcinoma cells, an important observation was made. Some tumors with low but also with high copy numbers showed positive as well as negative cells (Figure 3b), which was also reported by others.[8,10] A more aggressive pretreatment of these tumors resulted in a strong increase in the number of positively stained cells, a higher detection limit, but also in poor morphology (Figure 3c). This means that apart from the HPV copy number and cutting artifacts, the accessibility of the target DNA in the cell is an important factor and reflects the heterogeneity of the tumor cell population. This observation is consistent with a possible etiologic role of HPV-16 in cervical squamous cell carcinomas.

Because our detection limit of HPV-16 DNA appeared to be about 20 copies per cell, the presence of small amounts of HPV-16 DNA in the stroma component of the tumors could not be excluded. However, when using more sensitive radioactive-labeled HPV probes, the presence of HPV-DNA and RNA was exclusively found in the malignant epithelial cells.[27] This is also in agreement with our observation that in one of the tumors analyzed (which accidentally contained stroma only) no HPV-DNA could be detected (no. 1, Table 1).

In conclusion, we could detect HPV-16 DNA by bio-DISH on frozen sections of squamous cell carcinomas of the uterine cervix with a detection limit of about 30–40 copies per cell, whereas morphologic aspects of the tissue remained unimpaired. This limit could be lowered to 20 copies per cell by using rather destructive proteinase K concentrations. Simultaneously, the number of HPV positive carcinoma cells was increased to about 100%. Positive staining could only be seen in the malignant cells and showed a generally uniform, granular staining pattern. The optimal proteinase K concentration appeared to vary from sample to sample.

We are currently investigating alternative tissue section pretreatments, other microscopic detection systems as reflection-contrast microscopy, and the use of gold labeling,[28] laser microscopy,[29] and biotinylated RNA probes.[27] We expect that in the near future the detection level of nonradioactive DISH for HPV type detection in tissue sections and in cervical smears will further improve.

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Acknowledgments

The authors thank Drs. J. Lindeman and P. Herbrink for critically reading the manuscript, M. Polak and H. Volkers for expert technical assistance, Joke Ambagtsheer for excellent secretarial assistance in the preparation of the manuscript, and G. J. Oskam and J. T. van Veldhuysen for their excellent photography.