Acta virol. **35**: 322-331, 1991

COMPARISON OF DIFFERENT *IN SITU* HYBRIDIZATION TECHNIQUES FOR THE DETECTION OF HUMAN PAPILLOMAVIRUS DNA IN CERVICAL SMEARS

W. ZIMMERMANN[x], T. NAUMANN[x], E. BAUSCHKE[xx], K.-D. JOHANNSMEYER[xxx], H. PROKOPH[x], S. SCHERNECK[x]

Central Institute of Cancer Research, [x]Department of Virology; [xxx]Department of Gynecology; and [xx]Central Institute of Molecular Biology, Berlin-Buch, Germany

Received April 28, 1990

Summary. - Different hybridization methods were used for detection of human papillomavirus (HPV) DNA in cervical smears. The results obtained by filter *in situ* hybridization (FISH) are consistent with most of the reports recently published. To overcome the unsatisfactory limitations of this method, especially the difficulties to distinguish clearly between positive and negative signals, we developed an *in situ* hybridization protocol using a cytospin and ^{35}S-labelled as well as biotinylated DNA-probes. For direct comparison of different methods, the samples were obtained from two groups of patients. One group were women with reiterated Papanicolaou smears III, IV; the other were women with reiterated Pap III, IV and additional histological scoring. In all cases but one, the different methods used have shown the same results. In one case the hybridization on slides using ^{35}S-labelled as well as biotinylated probes gave a negative result, whereas the FISH method using a ^{32}P-labelled probe allowed to detect of HPV 16 and 18 DNA only when more than 1×10^6 cells were present per filter. Our data demonstrate that *in situ* hybridization on slides is a specific and sensitive technique, which enables a clear distinction between positive and negative results using a small number of cells and which, especially with biotinylated probes, is suitable for application in routine work.

Key words: human papilloma virus; in situ hybridization; cervical cancer

Introduction

Genital human papillomaviruses (HPV) have been implicated in the development of premalignant and malignant cervical lesions (Gissmann and Schneider, 1986; McCance, 1986, Gupta *et al.*, 1987; Zur Hausen, 1987). HPV types 6 and 11 are closely associated with benign epithelial proliferations (*Condyloma accuminatum)* and low grade intraepithelial neoplasia (CIN I)

(Sutton *et al.*, 1987; Lorincz *et al.*, 1987), whereas HPV 16 and 18 and less frequently the types HPV 31 and 33 has been shown to be associated with higher grades of dysplasias (CIN II and III) and invasive carcinoma (McCance *et al.*, 1985; Milde and Löning, 1986; Düst *et al.*, 1987). On the basis of these findings, it has been suggested that identification of HPV types in cervical swabs might be important for diagnosis and prognosis of cervical lesions. After introduction of filter *in situ* hybridization (Wagner *et al.*, 1984; Schneider *et al.*, 1985) this became a basis for screening of a large number of cervical swabs enabling to obtain data for a more exact analysis of the role of different HPV types in induction of cervical lesions (Schneider *et al.*, 1986; Gissmann and Schneider, 1986; Schneider *et al.*, 1987). However, the unsatisfactory limitation of the method, the difficulties to distinguish clearly between positive and negative samples in some cases, restricted their application in routine laboratories.

In situ hybridization methods on slides are being increasingly used in viral diagnosis. Most of the relatively complicated techniques have been developed for the detection of viral DNA in tissue sections using radioactive probes (Syrjänen *et al.*, 1986; Ostrow *et al.*, 1987; Collins *et al.*, 1988). Biotinylated probes have been used as an alternative based on the extremely high affinity of the reaction occurring between biotin and streptavidin (Leary *et al.*, 1983; Brigati *et al.*, 1983; Milde and Löning, 1986; Syvänen, 1986; Löning *et al.*, 1987; Teo and Griffin, 1987; Syrjänen *et al.*, 1988).

The present study describes the development of a hybridization protocol for the detection of HPV DNA in cervical swabs on slides using a cytocentrifuge and ^{35}S-labelled as well as biotinylated probes and the comparison of this technique with the filter *in situ* hybridization.

Materials and Methods

Specimens. Cervical swab samples were taken from 44 patients with reiterated Papanicolaou (Pap) smears III, IV in the Department of Gynecology, Central Institute of Cancer Research, Berlin-Buch. From 20 of the 44 patients cervical biopsy specimens were obtained allowing histological examination. Cells from the swabs were suspended in phosphate buffered saline (PBS), washed two times in PBS and before using for the different hybridization methods the number of cells was estimated. The human cell lines HeLa, CaSki, and SiHa were obtained from the American Type Culture Collection (Rockville, MD) and grown in Dulbecco modified Eagle's medium (Serva, Heidelberg) supplemented with 10 % foetal calf serum (SIFIN, Berlin).

Labelling of the probes. HPV DNA probes of types 16 and 18 were kindly provided by Drs. H. zur Hausen and L. Gissmann, DKFZ, Heidelberg. The papillomavirus DNA was purified from the vector by cleavage with restriction enzymes (*Bam*HI, *Eco*RI) agarose electrophoresis and extraction from the agarose gel (Maniatis *et al.*, 1982). The DNA probes were labelled by nick-translation with (^{32}P) dCTP (110 TBq/mmol) or (^{35}S) dCTP (37 TBq/mmol) (all from Amersham, U.K.) to specific activities of 2–4 x 10^8 cpm/μg. Biotinylation of the probes was performed using Bio-11-dUTP and a nick translation kit according to the protocol provided by the suppliers (BRL, Bethesda, MD). Optimal labelling of the probes were monitored by a dot blot hybridization using standard conditions (Leary *et al.*, 1983). Biotinylated probes allowing the detection of target DNA at the level of 1 pg were used for *in situ* hybridization.

Filter in situ hybridization. Using a filter holder 10^5 - 2 x 10^6 cells were filtered onto a nitrocellulose membrane (diameter 25 mm, pore size 0.45 μm Schleicher and Schüll, Dassel), lysed with 0.5 mol/l NaOH, 1.5 mol/l NaCl and neutralized with 1.5 mol/l NaCl, 1 mol/l Tris-HCl pH 7.0. The filter was baken at 80 ^{0}C for two hr. Prehybridization was performed in 6 x SSC (1 x SSC : 0.15 mol/l NaCl, 0.015 mol/l NaCitrate pH 7.0), 50 % formamide, 5 x Denhardt's solution (Denhardt, 1966), 0.1 % sodium dodecyl sulphate (SDS), and 100 μg Salmon sperm DNA per ml for 5 hr at 42 ^{0}C. Hybridization was performed in the same solution with 10 ng/ml of ^{32}P-labelled HPV 16 + 18 DNA (specific activity 4 x 10^8 cpm/μg) at 42 ^{0}C for 48 hr. After hybridization the filter was washed three times for 30 min in 2×SSC, 0.1 % SDS at 65 ^{0}C and two times in 0.1 x SSC, 0.1 % SDS at 65 ^{0}C. The dry filters were exposed to X-ray film for 4–10 days at -70 ^{0}C with intensifying screens.

In situ hybridization on slides. About 10^4 - 5 x 10^4 cells were centrifuged onto slides coated with the Photo-Flo 200, diluted in PBS 1:100 (Kodak Inc., U.S.A.) using a centrifuge T52 with cytorotor (MLW, Engelsdorf, Germany). After air drying the cells were fixed for 10 min in 4 % paraformaldehyde in PBS, 10 mmol/l $MgCl_2$, washed three times in PBS, 10 mmol/l $MgCl_2$, dehydrated through graded ethanol and stored in 70 % ethanol at 4 ^{0}C until use.

For pretreatment prior to hybridization, the cells were rehydrated in PBS, 10 mmol/l $MgCl_2$ for 10 min and incubated with proteinase K (0.1 mg enzyme/ml in PBS, 10 mmol/l $MgCl_2$) for 10 min at room temperature. Immediately following the proteolytic treatment the reaction was stopped by washing the slides twice for 5 min in PBS containing 2 mg/ml glycine. Slides were given to a postfixation for 5 min in 4 % paraformaldehyde washed twice in PBS containing 2 mg/ml glycine, dehydrated through graded ethanol and air dried. The hybridization mixture for biotinylated probes contained 50 % formamide, 10 % w/v dextran sulphate, 2 x SSC, 2 x Denhardt's solution, 400 μg/ml sonicated salmon sperm DNA. The HPV 16 and 18 DNA was added at a concentration of 1 μg/ml to the hybridization mixture. For ^{35}S-labelled probes the same hybridization mixture supplemented with 1 mmol/l DTT and a probe concentration of 100 ng/ml was used. The hybridization mixture (25 μl) was pipetted onto the cells and the samples were covered with a siliconized coverslip. The target DNA as well as the probe DNA were denatured simultaneously in an oven at 120 ^{0}C for 6–10 min using a plastic tray for the slides. The denaturating temperature of 82 ^{0}C was controlled by a visual temperature indicator (Levermore, London) on the slides. After denaturation the plastic tray with the slides was transferred into an ice bath, the coverslips were sealed with rubber cement and the hybridization was carried out at 38 ^{0}C using biotinylated probes or 42 ^{0}C using ^{35}S-labelled probes for 48 hr. After hybridization the coverslips were removed and the cells were washed three times for 10 min in 2 x SSC, two times for 30 min in 2 x SSC at 60 ^{0}C, once for 10 min in 0.2 x SSC and, finally, twice for 20 min in 0.2 x SSC at 42 ^{0}C. In case of using ^{35}S-labelled probes after the post-hybridization washes the slides were dehydrated through graded ethanol, allowed to dry and dipped in photographic emulsion (K 6, ORWO). The slides were exposed for 4 - 7 days, developed in MH-28 developer (ORWO), fixed in A 300 fixer (ORWO), counterstained with hematoxylin, coverslipped and mounted with Permount. For the detection of the biotinylated probes the slides were equilibrated in buffer A (0.1 mol/l Tris-HCl, pH 7.4, 0.1 mol/l NaCl, 2 mmol/l $MgCl_2$, 0.05 % Triton X-100) for 10 min and then immersed in 5 % BSA in buffer A for 15 min to block non-specific binding sites. The slides were incubated with streptavidin-alkaline phosphatase (Blue Gene, BRL, Bethesda, MD) (1 μg/ml buffer A) for 15 min at room temperature, washed three times for 10 min with buffer A and twice for 5 min with buffer B (0.1 mol/l Tris-Hcl pH 9.5, 0.1 mol/l NaCl, 50 mmol/l $MgCl_2$). Following washing, the slides were incubated with the developing reagent containing 4 μl nitroblue tetrazolium (75 mg/ml in 70 % dimethylformamide) and 3 μl 5-bromo-4-chloro-3-indolyl phosphate (50 mg/ml in dimethylformamide) in 1 ml buffer B for 1–3 hr. The reaction was stopped with water and coverslips were mounted on the slides with 1 mol/l Tris-HCl, pH 7.4/glycerol/1:9 (v/v).

Results

Detection of HPV DNA in cervical cell lines

Sensitivity of FISH depends on the number of cells filtered onto the nitro-

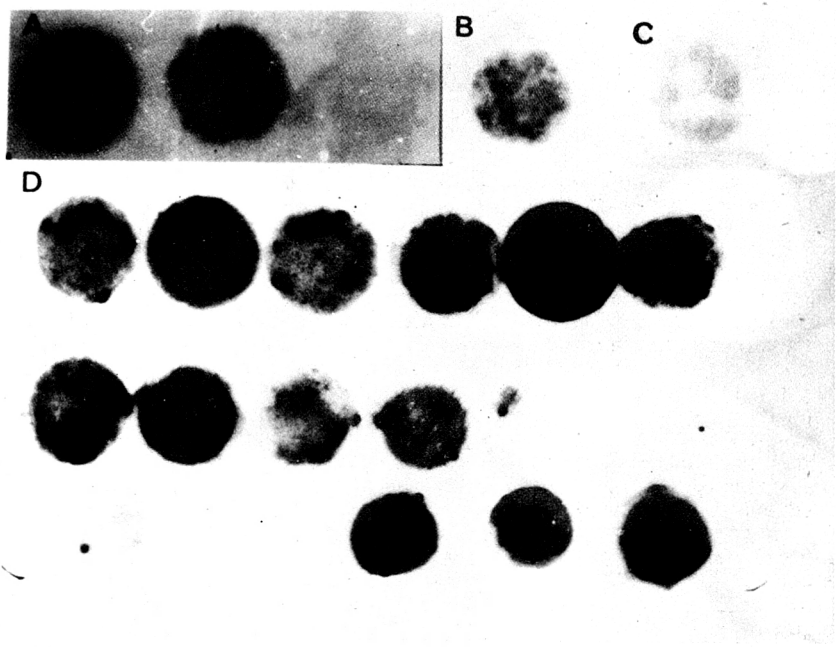
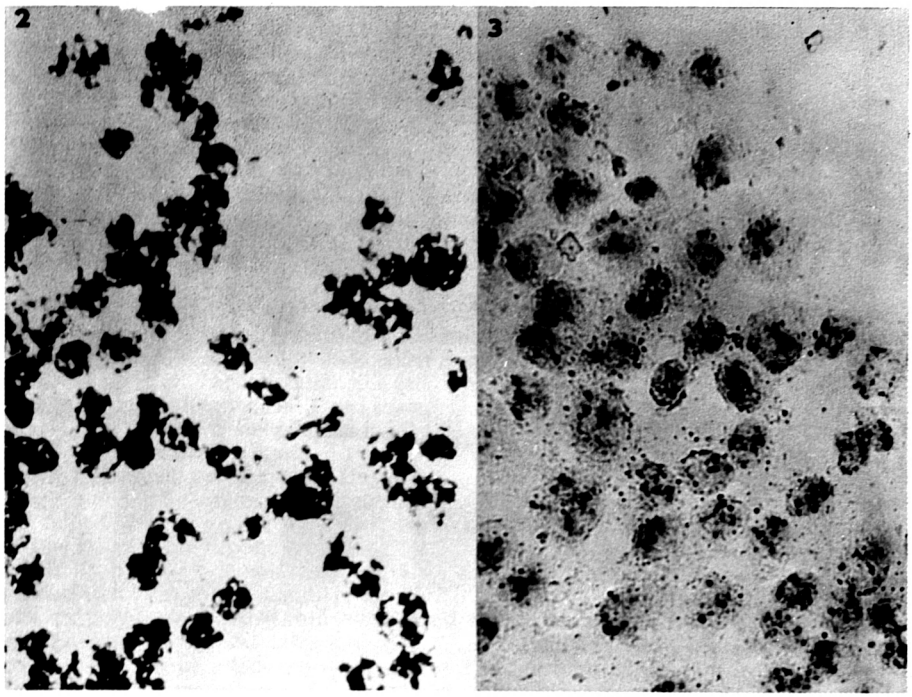


Fig. 1

Autoradiographs after filter *in situ* hybridization of different cell lines and cervical swabs
A. - filter with 10^5, 5 x 10^4, and 10^4 CaSki cells;
B. - 5 x 10^5 HeLa cells on a filter;
C. - filter with 10^6 SiHa cells;
D. - a collection of cervical swabs hybridized with HPV 16 and 18 DNA probes.

cellulose membrane. No signal was obtained below 5 x 10^4 CaSki cells (~500 copies HPV 16 DNA/cell) (Yee *et al.*, 1985), while a weak signal was visible after hybridization of 5 x 10^5 HeLa cells (20 copies HPV 18 DNA/cell) (Schwarz *et al.*, 1985) and 10^6 SiHa cells (1 - 10 copies HPV 16 DNA/cell) (Baker *et al.*, 1987), respectively (Fig. 1). Longer exposure of the autoradiographs did not result in an increased sensitivity, but in an increased background. Application of more cells to the nitrocellulose filter increases the sensitivity only in a limited range, and more than 2 x 10^6 cells (filter diameter 25 mm) influence the signal to noise ratio negatively. HPV DNA was also detected using biotinylated probes in all cell lines examined using *in situ* hybridization on slides (Figs. 2-4). The staining pattern displayed multiple discrete signals within the nuclei. The strongest signal was found in the CaSki cell line, where almost all cells showed several intranuclear dark (purple) spots (Fig. 2). Most of the HeLa cells had a positive reaction with the same colour intensity, but with a reduced



Figs. 2–3

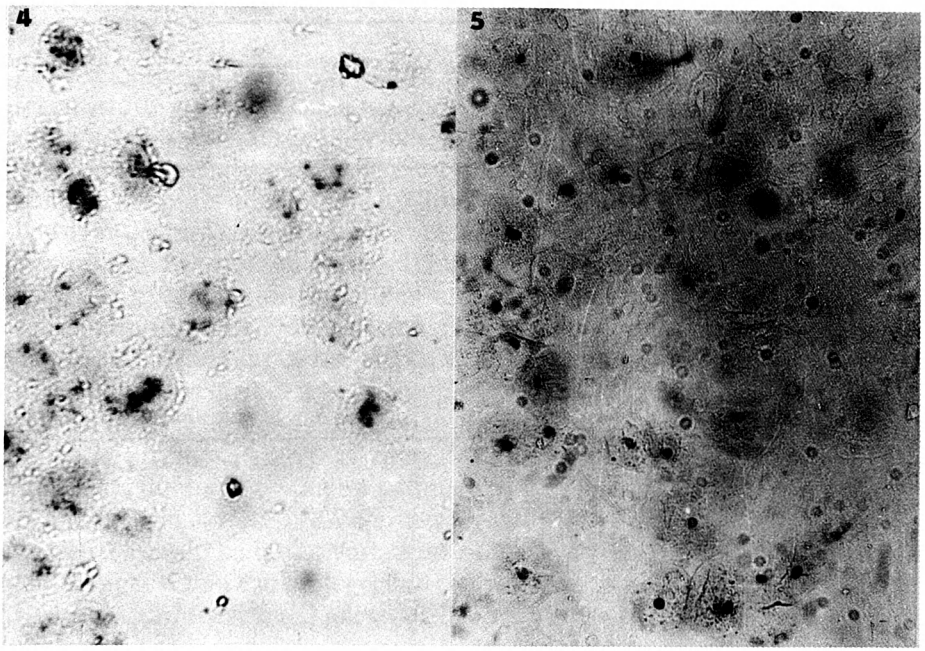
Fig. 2. CaSki cells hybridized with the biotinylated HPV 16 DNA probe. All cells show a positive hybridization reaction (several intranuclear spots) (Magn. x 344; reduced).

Fig. 3. HeLa cells spotted by cytospin and hybridized with the biotinylated HPV 18 DNA probe. A positive hybridization reaction (intranuclear dark spots) is visible in the most of cells (Magn. x 400; reduced).

number of intranuclear spots (Fig. 3). In SiHa cells only a few nuclei were definitely positive for HPV 16 DNA. This result suggest a detection limit of as few as 10 copies per cell. In all three cell lines tested HPV DNA could also be detected using ^{35}S-labelled probes (data not shown).

Detection of HPV DNA in cervical smears

Three different methods were used for detection of HPV 16 and 18 DNA in cervical swabs collected from 44 women. An example of a typical result of the FISH is shown in Fig. 1. The autoradiograph illustrates clearly the limitation of the method to distinguish strictly between positive and negative samples in some cases. Repeated hybridization and reiterated independent analysis of the data enabled, however, an acceptable result. On the contrary, *in situ* hybridization on slides using biotinylated probes allowed a clear discrimination between



Figs. 4–5

Fig. 4. SiHa cells hybridized with the biotinylated HPV 16 DNA probe. A positive hybridization signal is present in a few cells (Magn. x 400; reduced)

Fig. 5. Cervical epithelial cells from a patient with CIN III spotted by cytospin on slide, and hybridized with biotinylated HPV 16 and HPV 18 DNA probes. Positive cells show a discrete stained nucleus (Magn. x 250; reduced)

positive and negative samples. Positive cells showed a discrete stained nucleus without background staining of the cells and the slide surface, whereas in negative cells the nucleus appeared absolutely unstained (Fig. 5). In consequence of the scattering of the signal using ^{35}S-labelled probes, the relation between signal and attached cell was difficult to establish (Fig. 6). Analysis of the results summarized in Table 1 shows that 8 out of the 20 smears from patients with reiterated Pap grade III and IV and with histological examination were positive for HPV 16 and 18 DNA with all three methods used. The number of positive smears in this group varied from 4 out of 13 samples in patients with normal histology to 2 out of 3 samples in patients with CIN III/CIS.

In the group of patients with reiterated Pap III, IV only 12 of the 24 smears were positive for HPV DNA using *in situ* hybridization on slides with ^{35}S-labelled and biotinylated probes, respectively. Using the FISH technique 13 smears were positive for HPV DNA. In latter case a high number of cells (1.8×10^6) was applied onto the filter but only a single swab was additionally positive.

Table 1. Detection of HPV 16 and 18 DNA by different in situ hybridization methods

Origin of cervical swabs	Number of samples	Positives on slides with biotinylated probe	Positives on slides with ^{35}S-labelled probe	FISH
Patients with reiterated Pap. III/IV	24	12	12	13
Patients with reiterated Pap. III/IV and histological data	20	8	8	8
- normal histology	13	4	4	4
- CIN I	2	1	1	1
- CIN II	2	1	1	1
- CIN III/CIS	3	2	2	2

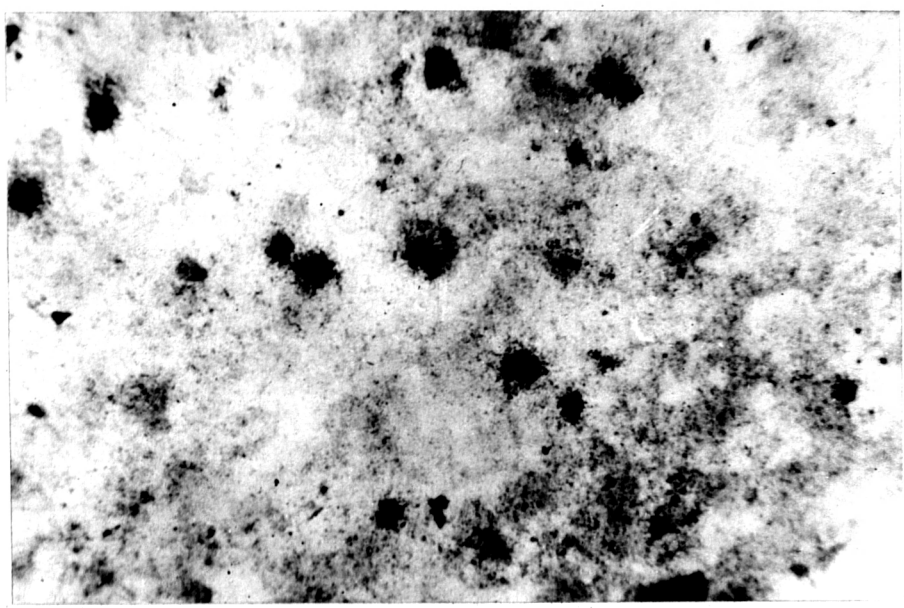


Fig. 6
Cervical epithelial cells from a patient with CIN III hybridized with ^{35}S-labelled HPV 16 and HPV 18 DNA probes (Magn. x 344)

Discussion

The sensitivity of the different *in situ* hybridization techniques used in the present study was assessed using cervical cancer cell lines on the basis of their estimated copy number of HPV DNA. By the FISH a weak, but clearly positive signal was visible after hybridization of 10^6 SiHa cells (1 - 10 copies HPV 16/ cell). On the basis of the capacity limit of 2 x 10^6 cells per nitrocellulose filter (diameter 25 mm) a reproducible detection of less than 10 copies of a HPV genome could be demonstrated. HPV 16 DNA was also detected in SiHa cells by *in situ* hybridization on slides using biotinylated as well as ^{35}S-labelled probes, although the hybridization signal was visible in a few cells only. The basis for the detection of the HPV DNA in SiHa cells was an optimal labelled probe especially in the case of the biotinylated probe.

Crum *et al.* (1986) concluded that *in situ* hybridization with nonradioactive probes is not as sensitive as using ^{35}S-labelled probes. On the other hand, recent data demonstrate an equal sensitivity of the different probes (Löning *et al.*, 1987; Burns *et al.*, 1987; Syrjänen *et al.*, 1988). Possible reason for these differences could be in the variations of the hybridization protocols used. Our results suggest that the biotinylated probes were as sensitive as the ^{35}S-labelled probes to demonstrate the presence of HPV DNA. In addition, possibly higher sensitivity of ^{35}S-labelled probes is limited in practice by the background problems frequently occurring especially on cytological smears.

We used different hybridization methods for detection of HPV 16 and HPV 18 DNAs in servical swabs collected from 44 women. In the group of patients with reiterated Pap III/IV and additional histological examination, 8 of the 20 smears were positive for HPV 16 and HPV 18 DNAs. Although we tested only a limited number of patients, these results and the data from the different subgroups are comparable with the data obtained by others (Wagner *et al.*, 1984; Schneider *et al.*, 1985; Cornelissen *et al.*, 1988). In the group of patients with reiterated Pap III/IV only 12 of the 24 samples were positive for HPV 16 and HPV 18 DNA using the *in situ* hybridization on slides with biotinylated and ^{35}S-labelled probes, respectively, whereas 13 smears were positive for HPV DNA using FISH. One possible explanation of this result could be the high number of cells (1.8 $\times$ 10^6 cells) applied onto the filter in latter case.

The advantage of the slightly higher sensitivity of the filter *in situ* hybridization method using an optimal number of cells is compensated by the difficulties to distinguish clearly between positive and negative results in some cases. The results presented here show that the *in situ* hybridization on slides is a specific and sensitive technique which enables a clear distinction between positive and negative cells using a small number of cells on the slide. Biotinylated probes give results which are comparable or even better in the sensitivity to results obtained using ^{35}S-labelled probes. The method with biotinylated

probes is more rapid, devoid of any health hazards and is especially suitable for an application in routine screening programmes.

Acknowledgements. The authors thank Dr. M. zur Hausen and Dr. L. Gissmann, Deutsches Krebsforschungszentrum, Heidelberg, for providing the HPV DNA probes. The skillful technical assisstance of Miss Helga Zeidler and Miss K. Wuske is gratefully acknowledged.

References

Baker, C. C., Phelps, W. C., Lindgren, V., Braun, M. J., Gouda, M. A., and Howley, P. M. (1987): Structural and transcriptional analysis of human papillomavirus type 16 sequences in cervical carcinoma cell lines. *J. Virol.* **61**, 962–971.

Brigati, D. J., Myerson, D., Leary, J. J., Spalholz, B., Travis, S. Z., Fong, C.K.Y., Hsiung, G. D., and Ward, D. C. (1983): Detection of viral genomes in cultured cells and paraffin-embedded tissue sections using biotin-labeled hybridization probes. *Virology* **126**, 32–50.

Burns, J., Graham, A. K., Frank, C., Fleming, K. A., Evans, M.F., and McGee, J.O.D. (1987): Detection of low copy human papilloma virus DNA and messenger RNA in routine paraffin sections of cervix by non-isotopic *in situ* hybridization. *J. clin. Pathol.* **40**, 858–864.

Collins, J. E., Jenkins, D., and McCance, D. J. (1988): Detection of human papillomavirus DNA sequences by *in situ* DNA-DNA hybridization in cervical intraepithelial neoplasia and invasive carcinoma: a retrospective study. *J. clin. Pathol.* **41**, 289–295.

Cornelissen, M.T.E., van der Velden, K. J., Walboomers, J.M.M., Briet, M. A., Smits, H. L., van der Noordaa, J., and ter Schegget, J. (1988): Evaluation of different DNA-DNA hybridization techniques in detection of HPV 16 DNA in cervical smears and biopsies. *J. med. Virol.* **25**, 105–114.

Crum, C. P., Nagai, N., Levine, R. U., and Silverstein, S. (1986): *In situ* hybridization analysis of HPV 16 DNA sequences in early cervical neoplasia. *Am. J. Pathol.* **123**, 174–182.

Denhardt, D. T. (1966): A membrane-filter technique for the detection of complementary DNA. *Biochem. Biophys. Res. Commun.* **23**, 641–646.

Dürst, M., Dzarlieva-Petruseoska, R. T., Bonkamp, P., Fusenig, N. E., and Gissmann, L. (1987): Molecular and cytogenetic analysis of immortalized human primary keratinocytes obtained after transfection with human papillomavirus type 16 DNA. *Oncogene* **1**, 251–256.

Gissmann, L., and Schneider, A. (1986): Human papillomavirus DNA in preneoplastic and neoplastic genital lesions, pp. 217–224. In Peto, R., zur Hausen, H. (Eds): *Viral Etiology of Cervical Cancer,* Cold Spring Harbor Laboratory.

Gupta, J. W., Gupta, P. K., Rosenshein, N., and Shah, K. V. (1987): Detection of human papillomavirus in cervical smears. – A comparison of *in situ* hybridization, immunocytochemistry and cytopathology. *Acta cytol.* **31**, 387–396.

Leary, J. J., Brigat, D. J., and Ward, D. C. (1983): Rapid and sensitive colorimetric method for the visualizing biotin-labeled DNA probes hybridized to DNA or RNA immobilized on nitrocellulose: Bioblots. *Proc. natn. Acad. Sci. U.S.A.* **80**, 4045–4049.

Lorincz, A. T., Temple, G. F., Kurman, R. J., Jenson, A. B., and Lancaster, W. D. (1987): Oncogenic association of specific human papillomavirus types with cervical neoplasia. *J. natn. Cancer Inst.* **79**, 671–677.

Löning, T., Henke, R.-P., Reichart, P., and Becker, J. (1987): *In situ* hybridization to detect Epstein-Barr virus DNA in oral tissues of HIV-infected patients. *Virchows Arch.* **A 412**, 127–133.

Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982): In: *Molecular Cloning – A Laboratory Manual,* pp. 164–166. Cold Spring Harbor Laboratory.

McCance, D. J. (1986): Human papillomavirus and cancer. *Biochim. Biophys. Acta* **ii**, 237–240.

McCance, D. J., Campion, M. J., Clarkson, P. K., Chesters, P. M., Jenkins, D., and Singer, A. (1985): The prevalence of human papillomavirus type 16 DNA sequences in cervical intraepithelial neoplasia and invasive carcinoma. *Br. J. Obstet. Gynaecol.* **92**, 1101–1105.

Milde, K., and Löning, T. (1986): Detection of papillomavirus DNA in oral papillomas and carci-

nomas; application of *in situ* hybridization with biotinylated HPV 16 probes. *J. oral. Pathol.* **15**, 292–296.

Ostrow, R. S., Manias, D. A., Clark, B. A., O Kagahi, T., Twiggs, L. B., and Faras, A. J. (1987): Detection of human papillomavirus DNA in invasive carcinomas of the cervix by *in situ* hybridization. *Cancer Res.* **47**, 649–653.

Schneider, A., Kraus, H., Schumann, R., and Gissmann, L. (1985): Papillomavirus infection of the lower genital tract: Detection of viral DNA in gynecological swabs. *Int. J. Cancer* **35**, 443–448.

Schneider, A., Schumann, R., de Villiers, E.-M., Knauf, W., and Gissmann, L. (1986): Clinical significance of human papillomavirus (HPV) infections of the lower genital tract. *Geburtsh. Frauenheilk.* **46**, 261–266.

Schneider, A., Sawada, E., Gissmann, L., and Shah, K. (1987): Human papillomaviruses in women with a history of abnormal Papanicola smears and in their male partners. *Obst. Gynecol.* **69**, 554–562.

Schwarz, E., Freese, U. K., Gissmann, L., Mayer, W., Roggenbuck, B., Stremlan, A., and zur Hausen, H. (1985): Structure and transcription of human papillomavirus 18 and 16 sequences in cervical carcinoma cells. *Nature (London)* **314**, 111–114.

Sutton, G. P., Stehman, F. B., Ehrlich, C. E., and Roman, A. (1987): Human papillomavirus deoxyribonucleic acid in lesions of the female genital tract: Evidence for type 6/11 in squamous carcinoma of the vulva. *Obstet. Gynecol.* **70**, 564–568.

Syrjänen, S., Syrjänen K., Mäntyjärvi, R., Parkkinen, S., Väyrynen, M., Saarikoski, S., and Castren, O. (1986): Human papillomavirus (HPV) DNA sequences demonstrated by *in situ* DNA hybridization in serial paraffin-embedded cervical biopsies. *Arch. Gynecol.* **239**, 39–48.

Syrjänen, S., Partanen, P., Mäntyjärvi, R., and Syrjänen, K. (1988): Sensitivity of *in situ* hybridization techniques using biotin- and ^{35}S-labeled human papillomavirus (HPV) DNA probes. *J. virol. Meth.* **19**, 225–238.

Syvänen, A.-C. (1986): Nucleic acid hybridization: From research tool to routine diagnostic method. *Med. Biol.* **64**, 313–324.

Teo G. G., and Griffin, B. E. (1987): Epstein-Barr virus genomes in lymphoid cells: Activation in mitosis and chromosomal location. *Proc. natn. Acad. Sci. U.S.A.* **84**, 8473–8477.

Wagner, D., Ikenberg, H., Brehm, N., and Gissmann, L. (1984): Identification of human papillomavirus in cervical swabs by deoxyribonucleic acid *in situ* hybridization. *Obstet. Gynecol.* 64, 767–772.

Yee, C., Krishnan-Hewlett, I., Baker, C. C., Schlegel, R., and Howley, P. M. (1985): Presence and expression of human papillomavirus sequences in human cervical carcinoma cell lines. *Am. J. Pathol.* **119**, 361–366.

Zur Hausen, H. (1987): Papillomaviruses in human cancer. *Cancer* **59**, 1692–1696.