DETECTION OF HUMAN PAPILLOMA VIRUS

A study of normal cells, cervical intraepithelial neoplasia and cancer of the uterine cervix

AKADEMISK AVHANDLING

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A study of normal cells, cervical intraepithelial neoplasia and cancer of the uterine cervix.

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ABSTRACT
Human papillomavirus (HPV) infections of the genital tract are now recognized to be among the most prevalent sexually transmitted diseases and also a contributing factor to some cancers of the lower genital tract of women and men. Presence of HPV in a clinical specimen is confined to detection of the HPV genome by DNA hybridization techniques.

In this thesis, the commonly used DNA hybridization techniques Southern blot and filter in situ hybridization (FISH), were first used for detection of genital HPV infection. In order to increase and simplify the detection of HPV in clinical specimens a more sensitive technique, the polymerase chain reaction (PCR) was subsequently utilized.

For type-specific amplification of HPV 6, 16, 18 and 33 by PCR, oligonucleotide primers located in the E6 and E7 regions of the HPV genome were selected. They were found to specifically amplify the four types. To be able to amplify a broad spectrum of genital HPV types, general primers located in the E7 and E1 region of the HPV genome, were designed and evaluated. They were found to amplify a wide range of genital HPV types. To further increase the sensitivity and specificity, a two-step PCR using general primers, was assembled and evaluated against a one-step PCR on cervical scrapes from young women in a population-based study. The two-step PCR increased the sensitivity about three-fold compared to the one-step PCR.

By Southern blot and FISH, 46% of women with abnormal Papanicolaou (Pap) smears were shown to carry HPV DNA. Of the women analysed by Southern blot, 39% harboured HPV DNA and 25% proved HPV 16 positive. Of the samples analysed with FISH, 27% contained HPV DNA, compared to 11% of samples from a group of reference women with normal cytology. With the Southern blot technique, HPV DNA was detected in 66% of women with cervical intraepithelial neoplasia grade III (CIN III) lesions. Fifty-four percent of the women with CIN III lesions were positive for HPV 16 DNA.

By type-specific PCR, 12 out of 13 women with cervical squamous carcinoma were shown to carry HPV 16 and/or 18. Among women with adenosquamous carcinoma of the cervix, HPV 18 was the most prevalent type (26%) but HPV 16 was also found in a proportion of the women (15%). Nine of 13 premenopausal cases with cervical adenocarcinoma were HPV positive compared to only 2 of 13 postmenopausal cases (p<0.015). HPV 16 DNA was detected in 48% of women with cervical intraepithelial neoplasia (CIN), by the use of type-specific PCR.

Three different groups of women with normal cytology were studied. Among women attending a family planning clinic in Kenya, 19% were shown to carry HPV virus, by the use of general primers. HPV 16 was found in 5.2% of these women and HPV 18 in 3.9%. In another group of women, attending the gynecological department in Umeå, HPV 16 DNA was detected in 21% by type-specific PCR. However, if consideration was taken to the medical status of the women, only 10% of women without any medical history were HPV 16 DNA positive, versus 54% of women with diseases and women with a relative progesterone dominance. Finally, by use of a two-step PCR using general primers, 20% of young women from Umeå taking part in a population-based study were demonstrated to carry HPV DNA. The most prevalent types were HPV 6 (2.0%) and HPV 16(2.7%). Among the women in this study with normal cytology, 19% were HPV positive.

Keywords: HPV/PCR/General primers/Genital cancer

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1. ABSTRACT

Human papillomavirus (HPV) infections of the genital tract are now recognized to be among the most prevalent sexually transmitted diseases and also a contributing factor to some cancers of the lower genital tract of women and men. Presence of HPV in a clinical specimen is confined to detection of the HPV genome by DNA hybridization techniques.

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2. PAPERS IN THIS THESIS
This thesis is based on the following articles. Hereafter these publications will be referred to in roman numerals I-IX.


3. INTRODUCTION

3.1. HISTORY

Transmissibility of canine (McFadyean and Hobday, 1898) and human (Ciuffo, 1907) warts by cell free extracts was shown already at the turn of the century. The first papillomavirus was described in 1933, when Shope recognized the cottontail rabbit papillomavirus (CRPV) as the etiological agent responsible for cutaneous papillomatosis in the cottontail rabbit (Shope and Hurst, 1933). A role of papillomavirus was then established in the induction of malignant tumors in rabbits (Syverton and Barry, 1935). In the 1970s, when techniques for the molecular cloning of viral DNA became available, the remarkable plurality of human and animal papillomaviruses was recognized (zur Hausen et al., 1974; Gissman et al., 1977; Orth et al., 1977).

Characterization of viral DNAs cloned from individual lesions and DNA hybridization studies have led to the identification of more than 60 different HPVs and to the recognition of characteristic differences of the lesions produced by infection with the different types. HPV infections of the genital tract are now recognized to be among the most prevalent sexually transmitted diseases; furthermore, infection by some of these viruses is now clearly recognized as a causative factor in some cancers of the lower genital tract of women and men. Some genital HPV types, HPV 6 and HPV 11, transmitted at birth from infected mothers to offspring, produce respiratory papillomas of juvenile onset. Some cutaneous HPVs, HPV 5 and HPV 8, play an active role in the development of squamous cell carcinomas that arise in the warty lesions of a rare dermatological disorder, epidermodysplasia verruciformis (EV).

3.2. GENERAL CHARACTERISTICS OF HPV

Papillomaviruses are small, nonenveloped, icosahedral DNA viruses that replicate in the nucleus of squamous epithelial cells. The virion particles consist of a single molecule of double-stranded, circular, covalently closed, supercoiled DNA, contained within a capsid with an icosahedral symmetry, composed of 72 capsomers. The papillomavirus particles are 52-55 nm in diameter (Fig. 1). The capsid consists of at least two structural proteins. The viral DNA is complexed with low-molecular-weight histones of cellular origin (Favre et al., 1975; Pfister et al., 1977). The viral genomes are approximately 8,000 base pairs in size and have a molecular weight of $5.2 \times 10^6$. 
daltons. The G + C content of the human papillomaviruses varies between 36.5 - 50.1% (Pfister and Fuchs, 1987; Hirsch-Behnam et al., 1990), the extreme cases being HPV 16 (36.5%; Seedorf et al., 1985) and HPV 57 (50.1%; Hirsch-Behnam et al., 1990). The DNA constitutes approximately 12% of the virion by weight, accounting for the density in cesium chloride of approximately 1.34 g/ml (Crawford LV and Crawford EM, 1963).

3.3. CLASSIFICATION OF PAPILLOMAVIRUSES

Characterization of the biological and biochemical properties of papillomaviruses has been impeded by the failure of these viruses to grow in tissue culture. Fortunately, recombinant DNA technology has helped to circumvent these limitations and analysis of the HPV has become possible. The current classification of HPV is based on nucleic acid homology. The comparison of HPV genomes is made by hybridization in liquid phase followed by separation of the remaining single strand by hydroxyapatite chromatography or by S1 nuclease digestion, (Pfister, 1984; Pfister and Fuchs, 1987). A new isolate is considered an independent type if it shows less than 50%
cross hybridization with the known virus types. If the new isolate shows more than
50% cross hybridization but cross hybridization is incomplete, the type is regarded as
a subtype. If the homology is close to 100% and the isolate differs only in a few
restriction enzyme cleavage sites the isolate can be regarded as a variant strain.
However, the cross hybridization does not reflect exactly the actual nucleic acid
sequence homology between different papilloma virus types as emphasized by Pfister
and Fuchs (1987). A good example of this is the comparison of HPV 6 and HPV 11.
Each of these is associated with similar clinical entities, namely condyloma
acuminatum and laryngeal papillomas. By solution hybridization analyses, they have
been reported to have 25% sequence homology (Gissman et al., 1982a). Comparison
of the complete nucleotide sequence for each of these genomes, however, reveals
82% identity (Dartmann et al., 1986).

The number of recognized HPV types has grown exponentially since the first HPV
types were cloned in plasmid vectors for propagation with viral DNA in vitro, (Danos
et al., 1980; Heilmann et al., 1980). To date more than 60 distinct HPV types have
been characterized (Table 1).

It is possible to classify HPVs into distinct subgroups, such as viruses found in
epidermodysplasia verruciformis (Orth, 1987), in anogenital proliferations (zur
Hausen, 1989), in addition to certain skin HPV infections which seem to form distinct
subgroups (e.g. HPV 1; HPV 4, 60 and 65; or HPV 7, 40) (Hirt et al., 1991). Within
the subgroups the viruses are more closely related (Pfister, 1984; Pfister and Fuchs,
1987). The anogenital HPVs have also been arranged in low-, intermediate- and high-
-risk oncogenic viruses based on the results from both epidemiological studies and data
from the ability to transform human keratinocytes in vitro (Lörincz et al., 1991;
Schiffman et al., 1991; Schlegel, 1991). According to these results, HPV 6, 11 and
42-44 belong to the low-risk viruses, HPV 31, 33, 35, 51, 52 and 58 to the
intermediate-risk viruses and HPV 16, 18 and 45 to the high risk viruses.

3.4. GENOME ORGANIZATION AND GENE FUNCTIONS

The overall genetic organization of the papillomaviruses is very similar (Fig 2). All
open reading frames (ORF) are located on one strand (Giri and Danos, 1986). The
viral genome is divided into an early region (about 4.5 kb) that is necessary for
transformation, a late region (about 2.5 kb) that codes for the capsid proteins, and a
<table>
<thead>
<tr>
<th>HPV type</th>
<th>Location</th>
<th>Associated with</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cutaneous</td>
<td>Verruca plantaris</td>
</tr>
<tr>
<td>2</td>
<td>Cutaneous</td>
<td>Verruca vulgaris;&lt;br&gt;Verruca plantaris</td>
</tr>
<tr>
<td>3</td>
<td>Cutaneous</td>
<td>Verruca plana</td>
</tr>
<tr>
<td>4</td>
<td>Cutaneous</td>
<td>Verruca vulgaris;&lt;br&gt;verruca plantaris</td>
</tr>
<tr>
<td>5</td>
<td>Cutaneous</td>
<td>EV (benign)&lt;br&gt;EV (squamous cell carcinoma)</td>
</tr>
<tr>
<td>6</td>
<td>Genital mucosa</td>
<td>Condyloma acuminata&lt;br&gt;CIN&lt;br&gt;Laryngeal papilloma&lt;br&gt;Buschke-Löwenstein tumors</td>
</tr>
<tr>
<td>7</td>
<td>Cutaneous</td>
<td>Butchers' wart</td>
</tr>
<tr>
<td>8</td>
<td>Cutaneous</td>
<td>EV (benign)&lt;br&gt;EV (squamous cell carcinoma)</td>
</tr>
<tr>
<td>9</td>
<td>Cutaneous</td>
<td>EV (benign)</td>
</tr>
<tr>
<td>10</td>
<td>Cutaneous</td>
<td>Verruca plana</td>
</tr>
<tr>
<td>11</td>
<td>Genital mucosa</td>
<td>CIN&lt;br&gt;Laryngeal papilloma&lt;br&gt;EV (benign)</td>
</tr>
<tr>
<td>12</td>
<td>Cutaneous</td>
<td>EV (benign)</td>
</tr>
<tr>
<td>13</td>
<td>Oral mucosa</td>
<td>Focal epithelial hyperplasia</td>
</tr>
<tr>
<td>14</td>
<td>Cutaneous</td>
<td>EV (benign)&lt;br&gt;EV (squamous cell carcinoma)</td>
</tr>
<tr>
<td>15</td>
<td>Cutaneous</td>
<td>EV (benign)</td>
</tr>
<tr>
<td>16</td>
<td>Genital mucosa</td>
<td>CIN; cervical carcinoma</td>
</tr>
<tr>
<td>17</td>
<td>Cutaneous</td>
<td>EV (benign)&lt;br&gt;EV (squamous cell carcinoma)</td>
</tr>
<tr>
<td>18</td>
<td>Genital mucosa</td>
<td>CIN; cervical carcinoma</td>
</tr>
<tr>
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<td>Cutaneous</td>
<td>EV (benign)</td>
</tr>
<tr>
<td>20</td>
<td>Cutaneous</td>
<td>EV (benign)&lt;br&gt;EV (squamous cell carcinoma)</td>
</tr>
<tr>
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<td>Cutaneous</td>
<td>EV (benign)</td>
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<tr>
<td>22</td>
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<td>EV (benign)</td>
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<tr>
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<td>Cutaneous</td>
<td>EV (benign)</td>
</tr>
<tr>
<td>26</td>
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<td>Verruca (immunosuppressed patient)&lt;br&gt;Verruca plana</td>
</tr>
<tr>
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<td>Cutaneous</td>
<td>Verruca (immunosuppressed patient)&lt;br&gt;Verruca plana</td>
</tr>
<tr>
<td>28</td>
<td>Cutaneous</td>
<td>Verruca plana</td>
</tr>
<tr>
<td>29</td>
<td>Cutaneous</td>
<td>Verruca vulgaris</td>
</tr>
<tr>
<td>30</td>
<td>Genital and oral mucosa</td>
<td>Laryngeal carcinoma&lt;br&gt;CIN</td>
</tr>
<tr>
<td>31</td>
<td>Genital mucosa</td>
<td>CIN; cervical carcinoma</td>
</tr>
<tr>
<td>Page</td>
<td>Location</td>
<td>Condition</td>
</tr>
<tr>
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<td>--------------------------------</td>
</tr>
<tr>
<td>32</td>
<td>Oral mucosa</td>
<td>Focal epithelial hyperplasia</td>
</tr>
<tr>
<td>33</td>
<td>Genital mucosa</td>
<td>CIN; cervical carcinoma</td>
</tr>
<tr>
<td>34</td>
<td>Genital mucosa</td>
<td>CIN (genital)</td>
</tr>
<tr>
<td></td>
<td>(cutaneous)</td>
<td>Bowen's disease (cutaneous)</td>
</tr>
<tr>
<td>35</td>
<td>Genital mucosa</td>
<td>CIN; cervical carcinoma</td>
</tr>
<tr>
<td>36</td>
<td>Cutaneous</td>
<td>EV (benign)</td>
</tr>
<tr>
<td>37</td>
<td>Cutaneous</td>
<td>Keratoacanthoma</td>
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<tr>
<td>38</td>
<td>Cutaneous</td>
<td>Malignant melanoma</td>
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<tr>
<td>39</td>
<td>Genital mucosa</td>
<td>CIN; cervical carcinoma</td>
</tr>
<tr>
<td>40</td>
<td>Genital mucosa</td>
<td>CIN</td>
</tr>
<tr>
<td>41</td>
<td>Cutaneous</td>
<td>Cutaneous squamous cell carcinoma</td>
</tr>
<tr>
<td>42</td>
<td>Genital mucosa</td>
<td>CIN</td>
</tr>
<tr>
<td>43</td>
<td>Genital mucosa</td>
<td>CIN (normal cervical mucosa)</td>
</tr>
<tr>
<td>44</td>
<td>Genital mucosa</td>
<td>CIN (normal cervical mucosa)</td>
</tr>
<tr>
<td>45</td>
<td>Genital mucosa</td>
<td>CIN; cervical carcinoma</td>
</tr>
<tr>
<td>46</td>
<td>Cutaneous</td>
<td>EV (benign)</td>
</tr>
<tr>
<td>47</td>
<td>Cutaneous</td>
<td>EV (benign)</td>
</tr>
<tr>
<td>48</td>
<td>Cutaneous</td>
<td>Cutaneous squamous cell carcinoma (transplant patient)</td>
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<td>49</td>
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<td>Verruca (immunosuppressed patient)</td>
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<td>EV (benign)</td>
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<tr>
<td>51</td>
<td>Genital mucosa</td>
<td>CIN; cervical carcinoma</td>
</tr>
<tr>
<td>52</td>
<td>Genital mucosa</td>
<td>CIN; cervical carcinoma</td>
</tr>
<tr>
<td>53</td>
<td>Genital mucosa</td>
<td>Normal cervical carcinoma</td>
</tr>
<tr>
<td>54</td>
<td>Genital mucosa</td>
<td>Condyloma acuminatum</td>
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<tr>
<td>55</td>
<td>Genital mucosa</td>
<td>Bowenoid papulosis</td>
</tr>
<tr>
<td>56</td>
<td>Genital mucosa</td>
<td>CIN</td>
</tr>
<tr>
<td>57</td>
<td>Oral and genital</td>
<td>CIN</td>
</tr>
<tr>
<td></td>
<td>mucosa (cutaneous)</td>
<td>Verruca vulgaris</td>
</tr>
<tr>
<td>58</td>
<td>Genital mucosa</td>
<td>CIN</td>
</tr>
<tr>
<td>59</td>
<td>Genital mucosa</td>
<td>VIN</td>
</tr>
<tr>
<td>60</td>
<td>Cutaneous</td>
<td>Epidermoid cyst</td>
</tr>
</tbody>
</table>
regulatory region (about 1 kb) that contains the origin of replication and many of the control elements for transcription and replication. This region has been referred to by several terms, including the noncoding region (NCR), the upstream regulatory region (URR), and the long control region (LCR). The term LCR will be used throughout.

DNA sequences of 15 human and 7 animal papillomaviruses are currently available (Table 2). The sequence homology between the different PV's varies throughout the genome, ORF's E1, E2 and L1 are highly conserved whereas the long control region (LCR), ORF's E4, E5 and L2 have been shown to be more type specific (Pfister and Fuchs, 1987).

The sequence homology between HPV 6b and HPV 11 within a papilloma virus group is close to 90% in the E1 region and about 75% in E5 region, (Dartmann et al., 1986), however, the cross hybridization between these HPV types was only 25% (Pfister and Fuchs 1987). Between HPV 11 and 16 belonging to different HPV groups the sequence homology in ORF E1 is close to 60% and in ORF E5 50%, (Dartmann et al., 1986).

The functions of the eight designated ORFs in the early region (E1-E8) and of the two ORFs in the late region (L1, L2) are shown in table 3.

The E1 ORF is the longest of the papillomavirus ORFs and is also one of the best conserved (Baker, 1987). Its function and protein expression is unknown for HPV,
<table>
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<th>Virus</th>
<th>Host</th>
<th>Associated with</th>
<th>Reference</th>
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<td>BPV 1</td>
<td>Cattle</td>
<td>Cutaneous fibropapillomas</td>
<td>Chen et al., 1982</td>
</tr>
<tr>
<td>BPV 2</td>
<td>-</td>
<td>&quot;</td>
<td>a</td>
</tr>
<tr>
<td>BPV 4</td>
<td>-</td>
<td>Alimentary tract papillomas</td>
<td>Patel et al., 1987</td>
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<tr>
<td>CRPV</td>
<td>Rabbit</td>
<td>Cutaneous papillomas</td>
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<td>DPV</td>
<td>Deer</td>
<td>Fibropapillomas</td>
<td>Groff and Lancaster, 1985</td>
</tr>
<tr>
<td>EEPV</td>
<td>Elk</td>
<td>&quot;</td>
<td>b</td>
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<tr>
<td>RhPV</td>
<td>Rhesus monkey</td>
<td>Genital intraepithelial neoplasia</td>
<td>Ostrow et al., 1991</td>
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<td>HPV 1</td>
<td>Human</td>
<td>See Table 1</td>
<td>Danos et al., 1982</td>
</tr>
<tr>
<td>HPV 2</td>
<td>-</td>
<td>&quot;</td>
<td>Hirsch-Behnam et al., 1990</td>
</tr>
<tr>
<td>HPV 5</td>
<td>-</td>
<td>&quot;</td>
<td>Zachow et al., 1987</td>
</tr>
<tr>
<td>HPV 6</td>
<td>-</td>
<td>&quot;</td>
<td>Schwarz et al., 1983</td>
</tr>
<tr>
<td>HPV 8</td>
<td>-</td>
<td>&quot;</td>
<td>Fuchs et al., 1986</td>
</tr>
<tr>
<td>HPV 11</td>
<td>-</td>
<td>&quot;</td>
<td>Dartmann et al., 1986</td>
</tr>
<tr>
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<td>-</td>
<td>&quot;</td>
<td>Seedorf et al., 1985</td>
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<td>-</td>
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<td>Goldsborough et al., 1989</td>
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<td>HPV 33</td>
<td>-</td>
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<td>Cole and Streeck, 1986</td>
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<td>Volpers and Streeck, 1991</td>
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<td>HPV 57</td>
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<td>Hirsch-Behnam et al., 1990</td>
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</tbody>
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\[\text{a} \text{ Unpublished, but sequence is available through GenBank by Groff D.E., Mitra R, and W.D. Lancaster.}
\[\text{b} \text{ A partial sequence has been published, but the complete sequence is available through GenBank by Ahola H., Bergman P., Ström A.C., Moreno-Lopez J., and U. Pettersson.} \]
<table>
<thead>
<tr>
<th>Function</th>
<th>ORF</th>
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<td>E1</td>
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<td>L1, L2</td>
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but for BPV the full-length E1 gene product has been shown to be a positive replication factor (Groff and Lancaster, 1986; Rabson et al., 1986; Ustav and Stenlund, 1991). The full-length E1 gene product has been detected in BPV 1 transformed rodent cells and has an apparent molecular size of 68 to 72 kDa (Santucci et al., 1990; Sun et al., 1990). In addition, a 23-kDa E1 ORF gene product has been detected in BPV 1 transformed mouse cells (Thorner et al., 1988). To date no function has been ascribed to this 23-kDa E1 protein, but it is postulated to play a role in replication since it shares a domain with the full-length E1 replication protein (Lambert, 1991). The full-length E1 ORF has also been shown to encode a repressor of viral transcription and transformation (Schiller et al., 1989).

The E2 proteins are DNA-binding proteins (Boshart et al., 1984; Androphy et al., 1987a) and their ability to regulate viral gene expression appears to be dependent upon the ability to bind specific sequences in the DNA recognized by E2 (Hawley-Nelson et al., 1988; Hirochika et al., 1988; Spalholz et al., 1988). The full-length E2 protein E2TA stimulates transcription of viral genes through its interaction with conditional enhancers located in the LCR (Harrison et al., 1987; Haugen et al., 1987; Hirochika et al., 1988; Spalholz et al., 1988). In addition to the full-length E2 transcriptional trans-activator, the BPV 1 E2 ORF also encodes two N-terminally truncated E2 ORF gene products, the E2 transcriptional repressor E2TR and the E8/E2 transcriptional repressor E8/E2TR that can inhibit viral-mediated transformation and repress E2 transcriptional trans-activation (Lambert et al., 1987). This transcriptional repression is mediated through competitive DNA binding at the E2 DNA binding sites or by forming functionally attenuated heterodimers with the E2TA (Lambert, 1991). A direct role for E2TA in viral plasmid replication has recently been demonstrated (Ustav and Stenlund, 1991).

The E4 ORF does not contain an initiation codon in HPV 16, but spliced PV mRNAs have been described where an initiation codon and a short aminoterminal sequence of the protein is provided by upstream exons in the E1 (Rotenberg et al., 1989) or E6 ORFs (Pettersson et al., 1987). Although located in the early region, E4 ORF encodes a protein associated with the late gene expression during the development of papillomas (Doorbar et al., 1986). For HPV 1, the E4 protein is a very abundant cytoplasmic protein that is present predominantly in the terminally differentiated keratinocytes of the wart (Doorbar et al., 1986).
Little is known about the function and expression of the E5 region of human papillomaviruses. In BPV 1, the E5 gene has a strong transforming effect on mouse fibroblasts (Schiller et al., 1986; Bergman et al., 1988).

Of E3 and E8 ORF's practically no data are available whether they have a coding function or not. However, the N-terminal end of the E8/E2 transcriptional repressor E8/E2TR is encoded from the E8 ORF (Lambert, 1991).

The L region of PVs consists of two ORFs. The size of the major structural protein, encoded by the highly conserved L1 ORF, is 50-60 kd (Orth and Favre, 1985). The less conserved L2 ORF encodes the minor structural protein of 70-76 kd (Orth and Favre, 1985), which has been suggested to carry the type-specificity (Komly et al., 1986; Tomita et al., 1987).

The single long control region (LCR) is located between the stop-codon of L1 and the first ATG codon of E6 (Smith and Campo, 1985). The size of LCR varies from 454 bp (HPV 8) to 979 bp (HPV 1). The LCR region exhibits the greatest variability amongst the sequenced HPVs. Gene expression and genome replication are controlled from this regulatory region. It contains enhancer elements that are responsive to cellular factors as well as to viral-encoded transcriptional regulatory factors. It was shown for HPV 16 (Cripe et al., 1987) and HPV 18 (Thierry and Yaniv, 1987) that specific regulatory elements are located in the LCR. Putative nuclear factor 1 (NF1) binding sites have been demonstrated in the LCR of HPV 16 (Gloss et al., 1989). NF1 is involved in replication and transcription of adenovirus (Cleat and Hay, 1989). It has also been reported that the LCR of HPV 16 contains elements that mediate response to both glucocorticoids and progesterone with increase in E6 and E7 transcription. Interaction between HPV 16, ras and fos oncogenes and progestins was also shown to effect neoplastic transformation in experimental models (Crook et al., 1988; Chan et al., 1989; Pater et al., 1990).

The E7 genes of HPV 16 and HPV 18 have been shown to encode transforming proteins. The most extensive studies have been carried out with the E7 gene of HPV 16, which has been shown to encode a multifunctional protein possessing both transcriptional modulatory and transformation properties similar to that of adenovirus E1A (Phelps et al., 1988). E7 is able to trans-activate the adenovirus E2 promotor, and it cooperates with an activated ras oncogene to transform primary baby rat kidney cells (Phelps et al., 1988; Storey et al., 1988). The E7 protein is 98 amino acids in size. The N-terminal 37 amino acid part of the E7 protein contains regions of striking
similarity to portions of conserved domains 1 and 2 of adenovirus E1A, which have been shown to be necessary for transformation (Phelps et al., 1988). These regions in Ad E1A contain the amino acid sequences necessary for the association of the product of the retinoblastoma tumor suppressor gene (p105-RB) (Whyte et al., 1988). Regions of amino acid similarity in SV40 large T antigen, that are also important in cellular transformation, are also involved in binding to p105-RB (De Caprio et al., 1988). Like Ad E1A and SV40 large T antigen, the human papillomavirus E7 protein can also complex with p105-RB (Dyson et al., 1989). Thus, all three groups of viruses may employ similar mechanisms of transformation. Interestingly, the E7 proteins of the nononcogenic human papillomaviruses HPV 6 and HPV 11 also bind p105-RB, but with lower affinity than the oncogenic HPV 16 and HPV 18 (HPV 11, 4- to 6 fold weaker and HPV 6, 20-fold weaker) (Münger et al., 1989). The aminoterminal half of E7 was shown to determine the affinity for binding to p105-RB and the transformation properties (Münger et al., 1991). The mechanism by which E7 modulates transcription has not yet been identified. The carboxy-termini of the genital HPV E7 gene products all contain the repeated motif Cys-X-X-Cys, which is also characteristic of domain 3 of adenovirus E1A, that has been implicated in transcriptional trans-activation (Phelps et al., 1988). The E7 proteins of the genital papillomaviruses are zinc-binding, and it seems likely that these motifs are involved in zinc binding (Barbosa et al., 1989).

The E6 protein is a 158 amino acid protein. Like the large T antigen of SV40 (Lane and Crawford, 1979; Linzer and Levine, 1979) and the E1B 55-kD protein of adenovirus 5 (Sarnow et al., 1982), the E6 protein of HPV 16 can form a complex with the tumor suppressor protein p53 (Werness et al., 1990). The E6 protein of HPV 18 can also form a complex with the p53 protein, but with lower affinity (50%) than HPV 16. The HPV 6 and HPV 11 E6 proteins showed no binding to p53 (Werness et al., 1990). The E6 proteins of HPV 16 and HPV 18 have been shown to promote the degradation of p53 (Scheffner et al., 1990).

3.5. PROPAGATION AND ASSAY IN CELL CULTURE

None of the papillomaviruses have yet been succesfully propagated in cell culture to yield virus particles. The result of a large number of early attempts to propagate papillomaviruses in a variety of cell cultures gave negative or equivocal results (Butel, 1972).
When virions derived from plantar warts are inoculated onto cultured keratinocytes, the viral DNA replicates in an extrachromosomal form and some viral transcripts are made (Christian et al., 1987; Taichman and La Porta, 1987). However, viral capsid proteins and viral particles are not synthesized, viral cytopathic effect is not seen, and the viral DNA is lost after a few passages. Cell culture of wart-derived keratinocytes has also not yielded viral particles. The failure to propagate the virus in keratinocytes may be the result of incomplete cellular differentiation. Kreider et al. (1987) reported that fragments of HPV infected human foreskin epithelium could be grafted beneath the kidney capsule of athymic mice. The epithelium had been infected with HPV 11 extracted from condylomata acuminata, and the infected tissue would differentiate to form a condylomatous cyst, containing HPV particles. This system has also been developed for HPV 1 (Kreider et al., 1990), but is not likely to be useful for studies of HPV 16 and HPV 18, the viruses most strongly associated with cervical carcinoma, because there probably is only minute amounts of virus particles with which to initiate the infection. An alternative approach has been to induce differentiation of a human keratinocyte cell line latently infected with HPV 16, by grafting onto nude mice (Sterling et al., 1990). Terminally differentiated cells then contained amplified levels of HPV 16 DNA, virus capsid antigen, and virus particles.

3.6. BIOLOGY OF PAPILLOMAVIRUS INFECTION

Most papillomaviruses are epitheliotropic. Only few (BPV 1, 2 and 5) infect fibroblasts, (Pfister, 1984; Smith and Campo, 1985). The incubation time of infection by HPV probably varies from a few months to over one year (Pfister, 1984). It has been proposed that the targets for PV infection are basal cells of the squamous epithelium. Basal cells are non-permissive for PV's but the cells become more and more permissive with increasing differentiation. Viral capsid production and virus maturation only occurs in terminally differentiated keratinocytes of the superficial cell layer (Pfister, 1984; Giri and Danos, 1986). Papillomaviruses are strictly host and tissue specific. The tissues most susceptible to PV infections include the genital mucous membranes and the skin; tracheobronchial esophageal squamous epithelium are also sensitive to HPV infections (Syijänen et al., 1982; Orth and Favre, 1985). Certain HPV types seem to be associated with lesions on the mucous membranes whereas others infect the skin. Thus, HPV types 6, 11, and 13 infect only mucosa although they have different preferential sites, and HPV types 1 to 5 cause exclusively cutaneous infections. Distinct clinical syndromes are associated with certain HPV types, e.g. HPV 5 and HPV 8 are associated with skin lesions in epidermodyplasia verruciformis (EV) (Pfister, 1984; Syijänen, 1987).
The latency seems to be an established feature in papillomavirus infections. HPV DNA has been detected in biopsies from normal epithelium adjacent to treated lesions (Ferency et al., 1985), in women without signs of gynecological neoplasia (Toon et al., 1986), as well as in histologically normal epithelium of the patients with genital cancer (Macnab et al., 1986).

In benign squamous cell lesions the PV genome exists exclusively as an extrachromosomal circular molecule with a copy number of about 30-100 copies per diploid cell (Smith and Campo, 1985). These molecules can exist as oligomeric circles of concatenated structures (Wettstein and Stevens, 1982).

Integrated HPV genomes have often been identified in biopsy specimens of cervical cancers (Durst et al., 1985; Lehn et al., 1985; Di Luca et al., 1986; Cullen et al., 1991), in cell lines isolated from cervical neoplasms (Yee et al., 1985; El Awady et al., 1987), and in immortalized keratinocytes (Barbosa and Schlegel, 1989). Two groups (Di Luca et al., 1986; Lehn et al., 1988) have reported the detection of integrated HPV sequences in over 50% of CIN, suggesting integration as a prognostic indicator of the subset of preinvasive lesions likely to develop into cancer. Another group, however, detected integrated HPV DNA in only 5% of the CINs compared to 81% of the cancers (Cullen et al., 1991). Of these, HPV 16 was present in integrated form in 72% (29/40) and in episomal form in 28% (11/40) of carcinomas. HPV 18, on the other hand, was found to be integrated in all HPV 18 containing carcinomas investigated (23/23), suggesting that this might be related to its greater transforming activities in vitro (Barbosa et al., 1989) and its reported clinical association with more aggressive cervical cancers (Barnes et al., 1988; Kurman et al., 1988; Walker et al., 1989). So far, HPV 16, 18, 31, 35, 39, and 45 have been shown to appear in an integrated form (Boshart et al., 1984; Durst et al., 1985; Beaudenon et al., 1987a; Cullen et al., 1991).

Integration seems to occur as head to tail tandem repeats in multiple sites of the host genome, (Boshart et al., 1984; Durst et al., 1985), and it seems to happen randomly, or as a preferential selection of these constructs (Lehn et al., 1985). The integrational pattern reveals a remarkable specificity for the site of disruption within the circular viral DNA or selection of a specific integrational pattern. This regularly occurs within the 3' end of the E1 ORF, and the 5' end of the adjacent E2 ORF (Pater and Pater, 1985; Schwarz et al., 1985; Baker et al., 1987), and obviously disrupts an intragenomic viral regulation exerted by E2 functions on other early gene expressions.
as originally demonstrated for bovine papillomaviruses. (Cripe et al., 1987; Lambert et al., 1987; Thierry and Yaniv, 1987).

Immunologic impairment of the host may enhance HPV infections. The frequency of HPV infections in renal transplant recipients is greater than in an immunocompetent population. Thus, genital HPV infections among the immunosuppressed renal transplant recipients were reported to be nine times more frequent, by the use of cytology and histopathology, than in the general population (Halpert et al., 1986). It is unclear whether HPV infection is more frequent during pregnancy (Fife et al., 1987; Schneider et al., 1987a; Peng et al., 1989; Rando et al., 1989; Smith et al., 1989; Soares et al., 1990).

3.7. TRANSFORMATION BY PAPILLOMAVIRUSES

In BPV 1, the papillomavirus that has been studied most completely, the transforming activity has been localized to a fragment representing 69% of the genome and, within that fragment, to the E5 and E6 genes (Yang et al., 1985; Groff and Lancaster, 1986; Schiller et al., 1986). Each transformed cell contains many copies of the viral genome, which remains extrachromosomal and is necessary for the maintenance of the transformed state (Law et al., 1981).

HPVs are inefficient at transforming established rodent cell lines and can transform primary rodent cells only in cooperation with an activated ras oncogene (Matlashewski et al., 1987; Phelps et al., 1988). In tests with human keratinocytes, the oncogenic HPV types 16 and 18 display much greater transforming activity than those of the nononcogenic HPV types 6 and 11 (Schlegel et al., 1988).

In carcinoma cell lines containing HPV DNA, integration of the viral genome generally results in disruption of the E1, E2 and E5 ORF's, (Schwarz et al., 1985; Shirasawa et al., 1987). E6 and E7 are retained and expressed as mRNA in carcinoma cell lines containing HPV 16 and HPV 18 (Schwarz et al., 1985; Takebe et al., 1987). They remain intact also in some cancer tissues containing integrated HPV DNA, (Smotkin and Wettstein, 1986; Androphy et al., 1987b; Seedorf et al., 1987; Takebe et al., 1987). This suggests that E6 and E7 play a role in the maintenance of tumorigenic state of the cell lines.

The major transforming activity of HPV 16 and 18 is localized to the E7 ORF (Phelps et al., 1988). Human keratinocytes transfected with HPV 16 or HPV 18 DNAs
become immortal and carry the viral genome in an integrated state (Durst et al., 1987; Schlegel et al., 1988). The immortalized cells are not tumorigenic in nude mice. HPV 16 and HPV 18 transfected keratinocytes, if allowed to stratify in the "raft" system, display the morphology of intraepithelial neoplasia (McCance et al., 1988). In a quantitative assay of transformation of human keratinocytes by HPV DNAs both oncogenic (HPV 16 and HPV 18) and nononcogenic (HPV 6 and HPV 11) HPVs induce transient cell proliferation, but only the oncogenic HPVs give rise to immortalized cells (Schlegel et al., 1988).

It is widely accepted that PVs alone are insufficient to cause malignant transformation; (zur Hausen, 1977; Gissmann, 1984a) chemical and/or physical carcinogens are likely required to act synergistically as cofactors. Ultraviolet light seems to be the cofactor in EV syndrome, where malignant growth preferentially arises from pityriasislike lesions at sun-exposed skin areas (Gissmann, 1984a). Some laryngeal papillomas treated by radiation therapy have developed into squamous cell carcinoma after 5-40 years, thus X-radiation is assumed to be a physical risk factor (Gissmann, 1984a). Smoking cigarettes has been shown to increase the risk of cervical cancer two-fold compared to non-smoking (zur Hausen, 1982).

3.8. GENITAL INFECTIONS

Papillomavirus infection of the genital tract is a common sexually transmitted disease. Genital warts are the most clearly recognized clinical lesions of these infections. Condylomata occur predominantly in young adults and in sexually promiscuous populations (Oriel, 1971). The age distribution of condyloma patients is very similar to that of patients with gonorrhea (or chlamydia in Sweden). About two-thirds of the sexual partners of condyloma patients develop genital warts after an incubation period ranging from three weeks to 8 months, with an average of 2.8 months (Oriel, 1971).

Condylomata may be florid and exophytic (condyloma acuminatum) or flat (condyloma planum) (Fig. 3). In males, the exophytic condylomata occur on the penis, in the urethra, around the anus, in the anal canal, on the perineum, and more rarely on the scrotum. In the female, they involve the vaginal wall, the vulva, the perineum, the anus, the urethra and the cervix. In the infected individual, condylomata are often found at more than one site in the genital tract (Meisels et al., 1982). Many of the condylomatas regress spontaneously or respond to treatment. They may increase in numbers and size in pregnancy and regress after delivery (Rudlinger et al., 1986). Recurrence of disease is correlated to the presence of the virus in the normal epithelium adjacent to the lesion (Ferenczy et al., 1985).
In the late 1970s, flat condylomata of the cervix was recognized as one of the most common manifestations of papillomavirus infection of the female genital tract (Meisels et al., 1977; Reid et al., 1980). Exophytic condylomatas are less common on the cervix. The flat lesion on the cervix is generally seen only by colposcopic examination (Purola and Savia, 1977; Rylander et al., 1985) after acetic acid application and is often indistinguishable from low-grade cervical intraepithelial neoplasia (CIN I). In contrast to the finger-like papillary projections of the exophytic condylomatas, the flat lesion may have irregularities of surface contour. It usually involves the transformation zone but may extend beyond it. The lesion can be identified cytologically and histologically as a condylomata by the presence of koilocytotic cells and other features of warts, such as dyskeratosis and parakeratosis.

Several papillomavirus types have been associated with condylomatas. HPV 6 and the closely related HPV 11 are responsible for the large majority of exophytic condylomas at all locations. The flat condylomata of the cervix is etiologically more heterogeneous than exophytic condylomas and may be associated with any of the HPV types (most often HPV 16) found in the cervix. Bowenoid papulosis is associated with HPV 16 infection.

### 3.9. INFECTIONS AT OTHER SITES

Skin warts are a common disease, mostly affecting children and young adults. Specific HPV types are characteristic for the skin lesions which are almost exclusively
benign. Only rare malignancies have been reported, (zur Hausen, 1977). HPV types 2, 4 and 7 are present in common warts and HPV 3, 10 and 28 in plane warts (table 1) (Jablonska et al., 1985). HPV 7 induces warts in butchers and in people handling meat. It was originally suggested to represent an unknown animal PV-type (Ostrow et al., 1981; Orth and Favre, 1985). However, in a recent study, DNA of 37 bovine tumors was negative when hybridized with HPV 7 (Oltersdorf et al., 1986). Furthermore, two cases were reported revealing HPV 7 in warts of non-butchers, (deVilliers et al., 1986). Thus, HPV 7 is likely to be of a human origin.

Epidermodysplasia verruciformis (EV) is a rare autosomal recessive disease with HPV involvement in etiology. Skin lesions are flat or pityriasis-like covering in severe cases the whole body (Lutzner, 1978). Up to 30% of the patients eventually develop in situ carcinomas, usually in sun-exposed areas (Smith and Campo, 1985). Eighteen distinct HPV types have been detected in EV lesions (table 1). HPV 5 and 8 are the risk types for malignant transformation of the skin lesions (Grussendorf-Conen, 1987).

Laryngeal papillomas can be divided into two subgroups, juvenile papillomas are benign and occur mainly in children under 5 years (Gissmann, 1984a) and also, on the other hand, adult-onset laryngeal papillomas are thought to represent premalignant lesions (zur Hausen, 1977).

Furthermore, there is evidence to suggest that HPV also can infect several other sites in humans. HPV DNA has been demonstrated in lower respiratory tract papillomas (Syrjänen and Syrjänen, 1987), in digestive tract carcinomas (Hille et al., 1985; Kulski et al., 1986), conjunctival papillomas and head and neck tumours of oral cavity and paranasal sinuses (Beaudenon et al., 1987b; Syrjänen, 1987).

3.10. IMMUNE RESPONSE

Many clinical and pathological observations have pointed to the importance of immune response, especially T-cell response, in the course of papillomavirus infections.

The prevalence of serum immunoglobulin G (IgG) antibodies to E2, E7, L1, and L2 recombinant proteins encoded by HPV 6, HPV 16, and HPV 18 in women attending a sexually transmitted diseases (STD) clinic, and in hospitalized children has been reported (Jenison et al., 1990). Cervical secretions and serum of patients with condylomas or cervical intraepithelial neoplasia were reported to contain IgA
antibodies reactive with disrupted BPV 1 virions (Dillner L. et al., 1989, 1990). It was also suggested that the presence of an IgA antibody in human serum against an HPV 16 E2 peptide may serve as a method to screen for HPV 16 infection (Dillner J., 1989).

Characterization of the humoral immune response to HPV infection is still in its infancy. Nothing is known about the pattern of antibody response following initial infection, or reactivation of presumably latent infections. Furthermore, it is unknown whether antibody status correlate with clinical outcome. However, one report is correlating serum antibodies to an HPV 16 E7 fusion protein with cervical cancer (Jochmus-Kudielka et al., 1989).

3.11. EPIDEMIOLOGY

The data obtained from the large number of virological investigations are difficult to compare because the characterization of the patient populations and of samples obtained is sketchy. Furthermore, the techniques for viral identification have varied greatly. Nevertheless, a broad outline of the acute and long-term pathogenic potential of HPVs has emerged.

3.11.1. PREVALENCE OF HPV DNA IN NORMAL POPULATIONS

In large cancer-screening programs in which Papanicolaou (Pap) smears from essentially asymptomatic women are examined, about 2-3% of the smears show abnormal cytology (Meisels et al., 1982). Nearly all squamous cell abnormalities in the Pap smears (koilocytosis, CIN 1, higher-grade lesions) appear to be associated with HPV infections (Stanley et al., 1990). Higher prevalences of abnormal cytology are found in those attending sexually transmitted disease (STD) clinics.

The presence of HPV in exfoliated cervical cells has been studied with several hybridization techniques. Southern blot, dot-blot, or filter in situ hybridization (FISH) tests, using HPV 6, 11, 16, and 18 probes, could identify viral DNA in 5-11% of cytologically negative women compared to 35-92% of women with abnormal cytology (Lörincz et al., 1986; de Villiers et al., 1987; Martinez et al., 1988; Kiviat et al., 1989). However, the populations studied varied considerably. The mean age varied from teenagers to 50 years. The subjects in several studies were young, inner-city women, and in other studies women from areas with low cervical cancer incidence.
Characteristically, the female genital tract is infected at many sites (Bergeron et al., 1987). HPV DNA prevalence rates increase when more than one genital-tract site is sampled. Women recipients of renal allografts have a higher prevalence of genital-tract neoplasia, as compared with normal women (Schneider et al., 1983; Halpert et al., 1986; Alloub et al., 1989).

Koutsky et al. (1988) have estimated that genital-tract HPV infections are prevalent in approximately 10% of the men and women in the 15- to 49-year age group in the United States and that a large majority of these infections are subclinical. They further suggest that the true prevalence may be significantly higher because of the insensitivity of the commonly used HPV detection method (a single test of cells from one genital-tract site, using a small panel of HPV probes).

During the last few years, studies have been made based on the polymerase chain reaction (PCR) technology (Saiki et al., 1985; Evander et al., 1988; Shibata et al., 1988). The technique greatly improves the sensitivity of previous hybridization strategies.

3.11.2. CERVICAL INTRAEPITHELIAL NEOPLASIA (CIN) AND CANCER OF THE CERVIX.

Worldwide, about 500,000 new cases of invasive cancer of the cervix are diagnosed annually (Peto, 1986). In developing countries, cancer of the cervix is the most frequent female malignancy and is responsible for about 24% of all cancers in women. In developed countries, it ranks behind cancers of the breast, lung, uterus, and ovaries and accounts for 7% of all female cancers. In the United States, there are about 4,800 deaths annually from cervical cancer (Shah and Howley, 1990). In Sweden, 500 women were diagnosed as having cancer in the cervix 1988 (2.6% of all female cancers). The lifetime risk of dying from cervical cancer may vary as much as 10-fold between different countries. Squamous cell carcinoma of the cervix, which accounts for over 85% of cervical cancers, has all the characteristics of a sexually transmitted infectious disease. It is almost never seen in virgins and is most frequent in women with multiple sexual partners. Monogamous women whose sexual partners are promiscuous have a higher risk of cervical cancer than monogamous women whose partners are not (Buckley et al., 1981).
Invasive cancer is preceded by a progressive spectrum of abnormalities of the cervical epithelium which is considered as cancer precursors and is classified as CIN grade 1 (CIN 1) (mild dysplasia), CIN 2 (moderate dysplasia), and CIN 3 (severe dysplasia and carcinoma in situ) (Ferenczy and Winkler, 1987a, 1987b). Flat cervical condylomas are considered as part of the spectrum of CIN 1. The time interval between CIN 1 and invasive cancer may span several decades.

The evidence linking HPVs with squamous cell carcinomas of the cervix and of other lower-genital-tract sites has been derived from clinical and epidemiological investigations, from studies of the pathogenesis of cervical neoplasia, and from molecular studies examining the presence and expression of HPV genomes in cervical cancers.

HPV genomes have been recovered from a large proportion of invasive cervical cancers as well as from the precursor lesions of CIN 1, CIN 2, and CIN 3 and from cervical condylomas. Almost all HPV types of the genital tract are represented in the mild lesions and in subclinical infections. In contrast, HPV 16 and HPV 18 predominate in invasive cancers. Estimates based on dot blot, Southern blot and filter in situ hybridization studies from all over the world [North America (Lörincz et al., 1987; Reid et al., 1987), Central and South America (Lancaster et al., 1986; McCance et al., 1986; Reeves et al., 1987, 1989), Europe (Scholl et al., 1985; Di Luca et al., 1986; MacNab et al., 1986; Millan et al., 1986; de Villiers et al., 1987; Ikenberg et al., 1987; Meanwell et al., 1987; Fuchs et al., 1988), Asia (Yoshikawa et al., 1985; Tsunokawa et al., 1986; Choo et al., 1987) Africa (Gissman and Schneider, 1986), and Australia (Kulski et al., 1987)] indicate that HPV 16 is recovered from about 50-60% and HPV 18 from about 10-20% of invasive cervical cancers. In contrast, HPV 6 and HPV 11, which are seen frequently in subclinical infections and which account for a large majority of exophytic condylomas, are virtually absent in higher grade neoplasias and in cervical cancers.

The PCR technique (Saiki et al., 1985; Mullis and Faloona, 1986) has also, during the last years, been used for detection of HPV in CIN and cancer.

The predominance of HPV 16 and HPV 18 in high grade anogenital lesions and invasive cancers is evident not only for the cervix but also for the vulva, the penis, and the anus (Koutsky et al., 1988). The association of HPV 16 and HPV 18 with invasive carcinoma of the cervix is true not only for squamous cell carcinomas but
also for adenocarcinomas (Smotkin et al., 1986; Tase et al., 1988) and for undifferentiated small cell carcinomas (Stoler et al., 1991).

Women who have cervical cytological abnormalities consistent with HPV infection have a much higher incidence of cervical cancer as compared to women in the general population (Mitchell et al., 1986). In recent prospective studies, an attempt was made to determine whether progression of disease from low-grade to high-grade CIN is correlated with infecting virus type. In a study in the United Kingdom, progression of disease during a 19- to 24-month period of observation was noted in 9 of 46 (19.6%) HPV 6 associated lesions as compared to in 22 of 39 (56.4%) HPV 16 associated lesions (Campion et al., 1986). In an ongoing prospective investigation of over 500 women in Finland, progression of disease during a 2-year follow-up was seen in 25% of HPV 6/11-associated lesions and in 33% of HPV 16-associated lesions (Syrjänen et al., 1987). In Germany, progression was recorded in none of 12 women with HPV 6/11 lesions and in 5 of 20 women with HPV 16 or HPV 18 lesions during a mean observation period of 6 months (Schneider et al., 1987b). The significant progression rates of HPV 6/11-associated lesions in two of these three studies were surprising because HPV 6 and HPV 11 are virtually absent from invasive cervical cancers. However, double infection with other HPV types was not excluded by use of the sensitive PCR technique.
4. PURPOSE OF THIS STUDY

* To evaluate existing methods for detection of genital HPV infection.

* To develop new sensitive techniques for detection of genital HPV infection in general and to type recognized HPVs.

* To describe the prevalence of HPV in populations with normal cytology versus populations with different clinical stagings.
5. MATERIAL AND METHODS

5.1. STUDY POPULATION AND SPECIMEN COLLECTION

To study the prevalence of genital HPV infection in the female population women with cervical cancer, cervical intraepithelial neoplasia (CIN), HPV signs and normal cytology have been included. The groups with severe lesions were referred to the respective Department of Gynecology. The women with normal cytology either took part in a general population screening, attended the gynecological Department for some gynecological disorder or were part of a population-based study. The groups also had different age profiles. The HPV DNA results were related to the histopathological and cytological findings and the HPV type was determined.

In paper I, all women (n=168) referred to the Department of Obstetrics and Gynecology in Umeå during one year because of an abnormal PAP smear - and in 5 cases because of a suspected CIN diagnosis - were included in the study. Colposcopically directed biopsies were obtained from 155 of the women and cervical swabs from 105. Their mean age was 28 years (range 16-56).

The design and evaluation of general and type-specific primers for HPV DNA amplification by using the PCR technique is described in paper II and III. The clinical specimens in these papers were collected from women with cervical cancer, CIN, HPV signs and normal cytology. The specimens consisted of biopsies, cervical smears and sections of formalin-fixed tissues.

In paper IV, 13 biopsies from Kenyan patients with histologically confirmed squamous cell carcinoma of the cervix were analysed for presence of HPV DNA by PCR. The mean age of the patients was 40.5 years.

All patients with cervical adenocarcinoma seen between 1980 and 1988 in the Umeå and Sundsvall Hospitals were selected, as described in paper V. Their formalin-fixed, paraffin-embedded biopsy specimens were re-examined and in 26 women the diagnose was confirmed. The biopsies were analysed for the presence of HPV DNA by PCR. The age of the women ranged between 32-85 years (mean=54.8).

In paper VI, 102 women with normal PAP smear on a routine visit to a family planning clinic in Nairobi, Kenya, were included in the study. Seventy-seven cervical scrapes were found to be accessible to HPV DNA amplification after β-globin PCR. The mean age of these women was 26.5 years.
In paper VII, 29 colposcopically directed biopsies from women attending a gynecological clinic in Debrecen, Hungary were analysed for presence of HPV DNA by PCR. Their mean age was 38.6 years. Cervical exfoliated cells with normal cytology were also obtained from 30 age-matched control women.

In paper VIII, cervical exfoliated cells were collected from 99 Swedish women with no cytological abnormality, attending the out-patient clinic of the Department of Obstetrics and Gynecology, Umeå. The median age of the women was 40 years.

A population-based study was performed in paper IX. All women aged 19, 21, 23 and 25, being inhabitants of a primary health care area in Umeå, were invited to participate in the study. Cervical scrapes were taken for HPV DNA analysis by PCR from 590 women.

5.2. NUCLEIC ACID HYBRIDIZATION METHODS

The fundamental finding for the development of hybridization technology is the observation that two complementary strands of nucleic acids, when separated, can reassociate to form a double stranded molecule (Marmur and Doty, 1962).

The stability of the formed hybrid depends on its melting temperature ($T_m =$ temperature, where half of the hybrids are dissociated). The latter, in turn, is affected by ionic strength of the hybridization buffer, content of G+C bases, length of the reacting molecules and some organic destabilizing agents, such as formamide. To characterize the hybridization reaction, the following empirical equation has been derived:

$$T_m = 81.5°C + 16.6 \log M + 0.41(\%G+C) - 500/n - 0.61(\%\text{formamide})$$

(Bolton and McCarthy, 1962), where $M$ is the ionic strength (mol/l), (\%G+C) is the composition of bases, and $n$ is the shortest chain in the duplex. This equation pertains to probes longer than approximately 50 nucleotides. For hybrids between oligonucleotides (14-20 bp) and immobilized DNA the stability decreases. The temperature at which 50% of these short duplexes dissociate ($T_d$) when the hybridization is performed under standard conditions (e.g., 0.9 M) is:

$$T_d = 4(G+C) + 2(A+T)$$
The number of mismatches in the bases between the two strands has an influence on the hybrid stability. For hybrids longer than 150 bp, $T_m$ of the DNA duplex decreases by 1°C for every 1% of mismatched base pairs (Bonner et al., 1973). For hybrids shorter than 20 bp, the $T_m$ decreases by approximately 5°C for every mismatched base pair (Wallace, 1983). The stringency of the hybridization depends on how many mismatches in the new duplex are allowed, i.e. what are the requirements of specificity. The stringency can be adjusted by altering (a) the salt concentration, (b) temperature, (c) formamide concentration in the hybridization solution or (d) conditions in the posthybridization washes (Meinkoth and Wahl, 1984).

Nucleic acid hybridization has several applications. Among the first were the reassociation kinetics techniques, where hybridization of the single stranded molecules occur in solution (Wetmur and Davidson, 1968).

Other, and more common, applications are based on mixed phase systems; one component, usually the sample DNA, is bound to the solid support, and the second, the labeled probe, is in the liquid phase. The major advantages compared to the liquid phase systems is that separation of hybrid from the probe is simple and that self annealing of sample DNA is also prevented.

5.2.1. Dot blot hybridization.

Dot blot hybridization is a modification of the two phase hybridization (Fig. 4). Unfractionated denatured DNA is immobilized onto the filter and fixed (Kafatos et al., 1979). The hybridization results are semiquantitative. Protein traces (Meinkoth and Wahl, 1984) and vector homology due to bacterial contamination of the samples may cause background problems in dot blot hybridization (Ambinder et al., 1986). However, the background problems can be significantly reduced by blocking with cold vector (Tabrizi et al., 1991). The sensitivity varies from 1 pg to 10 pg depending on the probe used (Anderson and Young, 1985; Schuster et al., 1986a; Seto and Yen, 1987). For demonstration of HPV DNA the dot blot hybridization is capable of revealing whether the specimen contains HPV sequences or not, and it also serves as a
crude quantitation of the viral DNA. Determination of the HPV type is performed under high stringency conditions. The detection limit of the dot blot test has proved to be $10^5 - 10^6$ HPV DNA molecules (Tomita et al., 1986).

5.2.2. Southern blot hybridization.

In Southern blot hybridization (Southern, 1975), the sample DNA is cleaved by appropriate restriction enzymes and fractionated electrophoretically in an agarose gel (Fig. 5). After chemical depurination, neutralization, and denaturation, the DNA fragments are transferred onto the filters and fixed. Several modifications of the transfer conditions and fixation exist depending on the filter material (Reed and Mann, 1985; Khandjian, 1987; Rigaud et al., 1987). The HPV type can be determined by restriction enzyme pattern using any HPV as a probe under low stringency conditions. Information about the physical state and possible rearrangements in the HPV genome during the integration can be obtained by Southern blot hybridization using an appropriate panel of restriction enzymes (Durst et al., 1983, 1985; Gissman et al., 1984b; Millan et al., 1986). With two dimensional electrophoresis the different virus conformations may be separated more efficiently (Wettstein and Stevens, 1982; Boshart et al., 1984). The sensitivity equals the dot blot method, $10^5 - 10^6$ genome equivalents of HPV can be detected by Southern blot hybridization (Gissman et al., 1982b), but the specificity is higher.
5.2.3. In situ hybridization.

Although not used in this study, the in situ hybridization, can be used for demonstration of nucleic acid sequences directly (in situ) in the cells (Gall and Pardue, 1969). Cells or tissue sections are fixed on a slide and hybridization is carried out basically as in the mixed phase system. Microscopic examination enables the assessment of DNA (RNA) localization related to morphological aspects of the lesions. The in situ hybridization technique has been used for demonstration of HPV DNA in tissues (Beckman et al., 1985; Milde and Lönig, 1986; Burns et al., 1987). (Fig. 6). Radioactive ssRNA probes labeled with $^{3}$H or $^{35}$S are capable of detecting 20-50 genomes of HPV per cell (Stoler and Broker, 1986; Schneider et al., 1987b). Biotin labeled probes have been reported to detect 10 copies of HPV DNA per cell (Wells et al., 1987).

Figure 5. Southern blot hybridization under non-stringent conditions. Autoradiography reveals the characteristic fragment pattern of the different HPV types, after digestion with Pst I.

Figure 6. A biopsy from a cervical acetowhite lesion. In situ hybridization revealed HPV-16 positive nuclei in the koilocytotic cells of the upper layer of the moderately neoplastic tissue.

Photo: Evy Hagelqvist
5.2.4. Filter in situ hybridization (FISH).

Hybridization can also be performed in situ on cells spotted on a filter. The method is known as filter in situ hybridization (FISH) (Brandsma and Miller, 1980; Wagner et al., 1984; Schneider et al., 1985) (Fig. 7). Whole cells are lysed on the filter and denatured. Hybridization and detection of the hybrids are carried out as in the other two-phase systems. The sensitivity of FISH is reported to be approximately 100 copies of BPV per cell using $^{32}$P-labeled probes (Wagner et al., 1984).

![Figure 7. Filter in situ hybridization (FISH) under stringent conditions. The filter was divided in two parts. One half was hybridized to HPV 6/11 and the other half to HPV 16/18.](image)

5.2.5. Polymerase chain reaction.

The polymerase chain reaction (PCR) is a technique by which one can amplify DNA (RNA) by enzymatic synthesis (Mullis and Faloona, 1986; Saiki et al., 1985, 1988) (Fig. 8). The target DNA is denatured by heating (92-95°C). Two sequence specific oligonucleotides with opposite direction, flanking a predetermined stretch, are allowed
to anneal to the single-stranded DNA at 30-70°C. A heat-stable DNA polymerase starts to synthesize a new DNA strand (72°C), by using the annealed oligonucleotides as primers. The size of the resulting amplification product is determined by the distance between the oligonucleotide primers. The three steps in the cycle: denaturation, annealing, and extension typically goes through 30-40 cycles and the amplification product increases exponentially, since the product itself functions as a target for annealing and subsequent extension. The detection limit is, theoretically, one genome copy, a sensitivity at least $10^4$ greater than Southern blotting (Young et al., 1989). The amplification products can be visualized by gel electrophoresis followed by ethidium bromide staining or by hybridization as in the other systems.
Principles of PCR

Cycle 1
Step 1. Denaturation of target DNA

Step 2. Annealing of primers

Step 3. Elongation of DNA

Cycle 2
Step 1. Denaturation of DNA

Step 2. Annealing of primers

Step 3. Elongation of DNA

Figure 8. Polymerase chain reaction: Principles of the method.
6. RESULTS AND DISCUSSION

The golden standard for HPV detection has for a long time been the Southern blot hybridization (Southern, 1975). Other methods that have been used for HPV detection includes the dot-blot hybridization (Kafatos et al., 1979), sandwich hybridization (Ranki et al., 1983), filter in situ hybridization (FISH) (Brandsmaa and Miller, 1980; Wagner et al., 1984; Schneider et al., 1985) and in situ hybridization (Beckmann et al., 1985; Stoler and Broker, 1986). The different methods have their "pros" and "cons". Southern blot is the most specific method but time-consuming, dot-blot hybridization, sandwich hybridization, and FISH are relatively fast methods, but with lower specificity. In situ hybridization can detect HPV DNA and HPV mRNA in specific cells, but needs a relatively high number of HPV genomes or mRNAs.

6.1. PREVALENCE OF HPV INFECTION IN WOMEN REFERRED FOR ABNORMAL PAP SMEARS, USING SOUTHERN BLOT AND FILTER IN SITU HYBRIDIZATION (FISH) (I).

The Southern blot and FISH techniques were used to determine the prevalence of HPV infection in women referred for abnormal Papanicolaou (PAP) smears to the Department of Obstetrics and Gynecology, Umeå University Hospital. All women (n=168) referred to the Department during one year (1986-1987), because of abnormal smears, were included in the study. Their mean age was 28 years (range 16-56). Biopsies (n=196) obtained from 155 of the women were histopathologically examined and analysed for the presence of HPV DNA by the Southern blot technique. A second Pap smear was collected from 105 women and analysed for the presence of HPV DNA by the FISH technique. Altogether, with both Southern blot and FISH, 46% of the women were HPV DNA positive. Of the women analysed by Southern blot, 39% (61/155) harboured HPV DNA and 25% (38/155) proved HPV 16 positive. Of the samples analysed by FISH, 27% (28/105) with atypical cells in their first Pap smear contained HPV DNA (HPV 6, 11, 16, 18 or 31), compared to 11% (13/119) of samples from a group of reference women, comparable in age, with normal cytology. With the Southern blot technique, HPV DNA was detected in 66% (33/50) of women with CIN III lesions compared to 17% with benign lesions. Fifty-four percent (27/50) of the women with CIN III lesions, 21% of women with CIN I-II lesions and 4.8% of women with benign lesions carried HPV 16 DNA.

The Southern blot hybridization demonstrated a higher frequency of HPV positives than the FISH technique (39% vs 27%). The Southern blot was likely to detect all
HPV types independent of the HPV probe used, since the hybridization was performed under low stringency conditions. Every type has a specific restriction pattern that can be visualized after low-stringent hybridization with any HPV type. However, the FISH technique can only detect the HPV types used as probes (HPV 6, 11, 16, 18, 31), but was considered to be a screening method for HPV DNA in cytological specimens. About $10^5$-$10^6$ genome copies of HPV is needed for Southern blot detection and about $10^4$ copies in a local cell cluster for FISH detection.

A higher prevalence of HPV DNA was shown among women with CIN III lesions compared to women with HPV signs or normal cytology. It was also demonstrated that HPV 16 was more prevalent in CIN III lesions than in women with normal cytology or HPV signs. This was in concordance with earlier studies (reviewed by Koutsky et al., 1988) where a positive association was found between HPV 16 (or 18) DNA and grade of neoplasia.

6.2. DESIGN OF A PCR DETECTION SYSTEM FOR HPV DNA.

The copy number of HPV genomes in the cell can be expected to vary. To detect low copy numbers of HPV genomes in genital lesions, a more sensitive method than the commonly used methods had to be employed. The polymerase chain reaction technique was introduced as a method for amplifying minute amounts of DNA by the use of Klenow DNA polymerase (Saiki et al., 1985; Mullis and Faloona, 1986) and later by the use of the thermostable Taq DNA polymerase (Saiki et al., 1988). The sensitivity of HPV DNA detection was shown to be greatly improved by the use of the polymerase chain reaction technique (Evander et al., 1988; Shibata et al., 1988). Theoretically, one copy of the HPV genome could be detected by the PCR technique. However, in practice, 10-100 copies were usually shown to be detected. The PCR method for amplification of HPV DNA could either be designed to specifically detect HPV types or to amplify a broad spectrum of HPV types.

6.2.1. Strategy for design of oligonucleotide primers for PCR detection of HPV DNA.

* Select the target for amplification.
  - A specific HPV type (e.g. HPV 6, 16, 18, 33, etc).
  - A specific HPV group (e.g. HPV 6 and 11).
  - HPVs with a specific tropism (e.g. mucosal, cutaneous)
  - All HPVs.
* Select the genome region to be amplified.
  - Early (E), Late (L) or Long Control Region (LCR).
  - E1-E8, L1-L2.
* Compare the DNA sequence of the selected region to different HPV types.
  - For type-specific primers find regions of low similarity.
  - For general primers find regions of high similarity.
* Check if the selected primer sequence can be found in any other HPV sequence.
* Check the primer construct concerning:
  - GC/AT content.
  - 3'end.
  - Internal homologies.
  - Dissociation temperature.
* Combine the two primers to a primer pair, considering:
  - Homologies between the primers.
  - A balanced dissociation temperature.
  - The distance between the primers, which gives the size of the amplification product.
* Perform a PCR with the primer pair, using cloned HPVs.
  - Optimize the PCR conditions concerning buffer, Mg²⁺- concentration, temperatures, number of cycles.
  - Check the specificity of the primer pair.
* Perform a PCR under routine conditions with a pilot panel of clinical specimens.

6.2.2. Design of general primers for amplification of HPV DNA (II, III).

Integration of the genome has been correlated to a transformed cellular phenotype (Durst et al., 1985; Lehn et al., 1985; DiLuca et al., 1986). The L1 and L2 region respectively, might be deleted during HPV infection when the HPV genome integrates itself into the cellular chromosome. The E6 and E7 regions (and the 5'-part of the E1 region) have been found to be retained in all cervical cancer biopsies containing the HPV genome and in all HPV transformed cell lines (Pater and Pater, 1985; Schwarz et al., 1985; Baker et al., 1987). The integrated HPV 16 genome has been found to be transcriptionally active in invasive carcinoma and carcinoma cell lines. The major transcripts were transcribed from the E6 and E7 regions (Shirasawa et al., 1988). Six different genital HPV types, HPV 6b, 11, 16, 18, 31, and 33, were screened for the presence of consensus sequences within the E6, E7 and E1 regions (II). Two consensus sequences within the E7 and the 5'-part of the E1 region were identified. When searching for the consensus sequence in the entire HPV 1a, 5a, and 8 genomes,
we could not find any sequence with less than seven mismatches. One primer pair, GP60/GP124, was designed using the consensus sequences (II). The primer located in the E7 region (GP60) had degenerate bases at six positions. To reduce the degeneracy of the E7-primer, inosine was introduced in two of the degenerate base positions. The E1-primer (GP124) had three degenerate base positions. The genital HPVs 6b, 11, 16, 18, 31, and 33 were the only HPVs that generated an amplification product at an annealing temperature of 60°C. The general primer pair could amplify HPV 13 and 30 (isolated from the oral mucosa) as well as the cutaneous HPV types HPV 2, 3 and 7 at lower annealing temperatures but not HPV 1a, 4, 5a, or 8 (II). To check the function of the general primers in a clinical situation, cervical cancer biopsies and cervical scrapes from women with normal cytology, condyloma, or cervical intraepithelial neoplasia (CIN) were analysed for presence of HPV DNA. The general primer-based PCR demonstrated HPV DNA in 11/12 cervical cancer biopsies and in 26/57 cervical scrapes (15/21 CINs, 10/21 condylomas, and 1/15 with normal cytology) (II). By hybridizing the amplification products to type-specific probes we could demonstrate 8 different HPV types. HPV 6, 11, 13, 16, 18, 30, 31, 33 (II). Another primer pair based on a consensus sequence in the HPV genome, was designed within the E1 region (III). It was shared by HPV 6, 11, 16, and 31. This primer pair was shown to specifically amplify these four types, using cloned genomes as well as clinical specimens (III).

Other regions of the HPV genome have been utilized for general amplification of HPV DNA. Consensus sequences from the L1 region were chosen for design of primers capable of amplification of a broad spectrum of HPV types. (Manos et al., 1989; Snijders et al., 1990). Conserved primer pairs located in the E1 region (Gregoire et al., 1989) as well as in the E6 region (Resnick et al., 1990; Yoshikawa et al., 1990) have also been designed. A comparison of the consensus primer pairs is difficult to make. They are located in different regions of the genome and generate amplimers of differing sizes. Primer pairs selected from regions retained after integration are also likely to amplify DNA from cervical cancer and high grade CINs in contrast to primer pairs selected from other regions. Usually, a short amplimer gives a higher yield than a long amplimer but other factors, as discussed in paragraph 6.2.1 are also important.

6.2.3. Design of type-specific primers for amplification of HPV DNA (III).

To detect a specific HPV type by one PCR reaction, type-specific primers for HPV 6, 16, 18, and 33 were selected from the E1, E6, and E7 open reading frames (ORFs).
These early regions were chosen on account of their presence in all types of HPV lesions. One primer pair within the E1 ORF was specific for HPV 16. The eight primer pairs from the E6 and E7 ORFs specifically amplified HPV 6, HPV 16, HPV 18, and HPV 33 sequences. All primer sequences were compared with all other sequenced HPV genomes to discover possible homologies. The nucleotide homology between the primers and heterotypic DNA was usually 50-70%. The sizes of the amplimers within the E6 region were chosen to be specific for each analysed HPV type. Four different HPV types could in this way be determined directly. The primer pairs were evaluated using cloned HPV genomes and found to specifically amplify the four HPV types. The primer pairs were also evaluated and found to be useful for detection of HPV DNA in clinical material, i.e. biopsies, cytological smears and sections of formalin-fixed tissues. The presence of different regions from the HPV genome may depend on the staging of the clinical specimen (Matsukura et al., 1986; Smotkin and Wettstein, 1986; Shirasawa et al., 1988). Two regions of the HPV genome may be analysed in parallel to ascertain the presence of HPV DNA in clinical specimens from advanced neoplasia when rearrangement of the HPV genome could have occurred.

Many other HPV type-specific primers have been described (e.g. Shibata et al., 1988; Xiao et al., 1988; He et al., 1989; Melchers et al., 1989; van den Brule et al., 1989).

6.2.4. Design of a two-step PCR, for amplification of HPV DNA using general primers (IX).

A nested general primer two-step PCR was assembled to amplify and detect, with high sensitivity, a broad spectrum of HPV types without the need for hybridization. Two general primer pairs, MY11/MY09 (Manos et al., 1989) and GP5/GP6 (Snijders et al., 1990) located within the L1 region were used. First, 20 cycles of amplification were performed with the outer primer pair (MY11/MY09). The inner primer pair (GP5/GP6) was added to the reaction mixture and the amplification continued for another 30 cycles. The two-step PCR system was used for detection of HPV DNA in a population-based study of 581 young Swedish women. A 3-fold increase in the detection rate compared to a one-step PCR, based on the outer primer pair (MY11/MY09), performed on the same study population was demonstrated.

Another two-step PCR system using general primers, has been described for detection of HPV DNA (Williamson and Rybicki, 1991). Two primer pairs arranged in a
nested fashion were located in the L1 region and could be used for amplification and
detection of HPV types found in genital lesions.

There are several successful concepts for designing functional PCR detection systems.
As mentioned in paragraph 6.2.1, the selection and design of the primers is of great
importance. The sensitivity of PCR is theoretically one copy of the target DNA.
Ordinarily, this is accomplished under conditions where the amplification reaction has
been optimized and the target DNA purified. However, during routine work in large-
scale studies or in clinical laboratories, PCR has to be applied directly on cervical
scrapes. The sensitivity of HPV DNA detection might then decrease, due to presence
of cellular components or other inhibitory substances.

6.3. PREVALENCE OF HPV INFECTION IN WOMEN WITH CERVICAL CANCER, CIN AND NORMAL CYTOLOGY, USING PCR.

The prevalence of HPV infection in the female population was investigated by using
the polymerase chain reaction technique. Both type-specific and general primers were
applied in PCRs to amplify HPV DNA. To study HPV infection in various lesions,
women with cervical cancer, cervical dysplasia, and normal cytology were analysed
as shown in papers IV-IX.

6.3.1. Prevalence of HPV infection in women with cervical squamous carcinoma
(IV).

Biopsy samples from 13 Kenyan women with squamous cell carcinoma of the cervix
were analysed for the presence of HPV 6, 16, 18, and 33 by type-specific PCR.
Infection with HPV 16 and/or HPV 18 was seen in 12/13 samples. HPV 16 was
confirmed in 11, and HPV 18 in 9 cases respectively, and both HPV 16 and 18 in 8
biopsies. HPV 33 was not seen in any of the samples, while HPV 6 was detected in
only 3 women. All women with HPV 6 infection were also positive for HPV 16
and/or HPV 18. The only biopsy negative for the four HPV types, was later found to
contain HPV 45 (data not shown).

All women in the study were shown to carry HPV DNA. This result should be
compared to the prevalence of genital HPVs in only 19% of Kenyan women without
cytological abnormalities (VI). In women with normal cytology from the same
geographical area, only 5% contained HPV 16 and 4% HPV 18 (VI). The most
common type carried by the women with cervical squamous carcinoma was HPV 16 (11/13), but HPV 18 DNA was also present in a majority of the biopsies. If one HPV genome at the time or several HPV genomes are expressed concomitantly in the same biopsy during multiple infections have not been reported. In other PCR studies of women with cervical squamous carcinoma, a high incidence of HPV infection (60-100%) and especially HPV 16 has been demonstrated (Xiao et al., 1988; He et al., 1989; Resnick et al., 1990). Other less well studied HPV types, HPV 31, 33, 35, 45, 51, 52, and, 56, together, have been found in an additional 10-15% of invasive cancers (Stanley, 1990).

6.3.2. Prevalence of HPV infection in women with cervical adenocarcinoma (V).

Paraffin-embedded biopsies from all patients diagnosed as having adenocarcinoma of the cervix, between 1980-1988, in Umeå and Sundsvall, were reexamined. The diagnose was confirmed in 26 women. By type-specific PCR, 11/26 (42%) were positive for HPV 16 or HPV 18, when analysed for these two types. HPV 16 was identified in 4/26 (15%) specimens and HPV 18 in 7/26 (26%). There were 13 pre- and 13 postmenopausal cases. Nine of eleven HPV positive specimens were from premenopausal women (median age 39 years) and only two were from postmenopausal patients (52 years and 80 years). The difference was statistically significant (p<0.015).

Presence of HPV DNA has been associated with cervical adenocarcinoma (Smotkin et al., 1986; Wilczynski et al., 1988). Also in Northern Sweden HPV 18 was the most common HPV type in cervical adenocarcinoma, but HPV 16 was also present at a high frequency (V). A comparison of the distribution of HPV 16 and HPV 18 in lesions of different pathology has suggested that the two types have different pathogenic potential. As compared to the distribution of HPV 16, HPV 18 genome is demonstrated less frequently in cancer precursors than in invasive cancers (Kurman et al., 1988). In one study of cervical cancers (Tase et al., 1988), HPV 18 accounted for nearly all virus-positive adenocarcinomas, whereas HPV 16 accounted for nearly all virus-positive squamous cell carcinomas. This sharp distinction has not been noted in all studies. It has also been suggested that cervical lesions associated with HPV 18 may progress at a more rapid rate than lesions associated with HPV 16. HPV 18 has been suggested to be associated with more aggressively invasive disease and to give severe lesions among very young women (Barnes et al., 1988, 1990; Walker et al., 1989). In paper V, all HPV 18 cases belonged to the younger group of women (pre-
or perimenopausal) and 5 out of 10 patients with a recent normal cytology were HPV 18 positive.

6.3.3. Prevalence of HPV infection in women with cervical intraepithelial neoplasia (CIN) (VII).

Twenty-nine biopsies from women living in Debrecen, Hungary, with histologically confirmed CIN were analysed for the presence of HPV 16 DNA by type-specific amplification within both the E6 and E7 ORFs. Altogether, 14/29 (48%) of the biopsies contained HPV 16 DNA. HPV 16 was present in only 2/30 (6.7%) of cytologically normal, matched control samples. All results were concordant both with the E6 and E7 ORFs.

In this study, HPV 16 could be detected at a high frequency among women with CIN compared to women with normal cytology. By Southern blot it was shown that 40% of women with CIN I-III, carried HPV 16 DNA (I). Others also demonstrated that HPV DNA (HPV 16 was the most frequent type) was present in a high share, 60-90%, of high grade CIN lesions (Stanley, 1990). HPV 16 was demonstrated in 11 out of 13 cervical squamous cell cancer patients (IV), compared to 48% of the women with CIN in this study. However, to evaluate the relevance of HPV infection in women with cervical cancer or CIN, a "base" level of HPV prevalence in cytologically normal women has to be established.

6.3.4. Prevalence of HPV infection in women from Kenya with normal cytology (VI).

Seventy-seven Kenyan women on routine visits to a family planning clinic, with normal cervical cytology were analysed for the presence of HPV DNA with the general primers from the E7 and E1 regions, presented in paper II. Their mean age was 26.5 years. HPV DNA was demonstrated in 19% (15/77) of the women. Four harboured HPV 16, three had HPV 18, one was positive for both HPV 16 and 33, and seven contained other HPVs. The mean age of the 15 HPV positive women was 25.7 years.

The prevalence of HPV 16 and HPV 18 (10%) in cytologically normal women from Kenya can be compared to the HPV prevalence (92%) in squamous cervical cancer biopsy specimens from the same geographic region (IV). HPV 16 was present in
11/13 (85%), compared to 5/77 (6.5%) of women with normal cytology and HPV 18 in 9/13 (69%) women with cervical squamous cell cancer compared to 3/77 (3.9%) with normal cytology. Other studies have suggested that HPV 18 was detected more frequently in cervical cancer specimens from Africa or the United States than from Europe (Gissmann and Schneider, 1986). However, it appears that the frequency of detecting HPV 16 or HPV 18 in cervical cancer specimens varies as much within a particular country and continent as it does between countries (Koutsky et al., 1988).

6.3.5. Prevalence of HPV infection in women from Umeå, Sweden with normal cytology (VIII).

Ninety-nine Swedish women, aged 17-72 (median 40), attending a gynecological clinic, were examined with three type-specific HPV 16 primer pairs from the LCR, E6 and E7 regions. All women had a normal PAP smear, a normal wet smear and no clinical signs of neither genital papillomavirus infection nor other genital infections. HPV 16 was detected in 21% (21/99) of the women. Sixty-nine of the women were without medication, except for hormonal replacement therapy. Only 10% (7/69) of these women carried HPV 16 DNA. Among the women with diseases and women with a relative progesterone dominance, 54% (14/26) were HPV 16 positive. This difference was statistically significant (p<0.01).

The high HPV 16 prevalence in women using contraceptive pills or attending for termination of pregnancy, might reflect a high sexual activity and thus increased risk of acquiring sexually transmitted agents. Genital HPV infections do fluctuate and latent and/or subclinical state might be activated during situations of immunosuppression (Schneider, 1990). Women that attend a gynecological clinic represent a heterogenous group although they have normal Pap smears. It is important to evaluate and describe the general health situation of the women included in a prevalence study. Women with diseases that require frequent hospital care and/or longterm use of antibiotics, might represent special groups in the HPV screening.

6.3.6. Prevalence of HPV infection in a population-based study of young Swedish women (IX).

The prevalence of HPV infection was investigated in cervical scrapes from a non-selected group of young Swedish women. All women aged 19, 21, 23 and 25, being inhabitants of a primary health care area in Umeå, Sweden, were asked to participate
in the study. Cervical scrapes from 590 women were analysed for the presence of HPV DNA by general primers. A comparison was also performed between a one-step PCR (40 cycles) and a two-step PCR (20 + 30 cycles), using general primers from the L1 region. By use of the one-step PCR, 5.9% (35/590) of the women were shown to carry HPV DNA, while with the two-step PCR HPV DNA was demonstrated among 20.3% (118/581) of the young women. The frequency of HPV positive women increased with age both with the one-step PCR, from 1.4% among women aged 19 to 9.2% among women aged 25, and with the two-step PCR, from 17.4% among women aged 19, to 31.9% among women aged 25. Among the women with normal Pap smears, 19.2% (102/530) carried HPV DNA with the two-step PCR, compared to 5.6% (30/539) with the one-step PCR. Of the women with atypical Pap smears, 26% (5/19) showed HPV DNA with the one-step PCR and 84% (16/19) with the use of the two-step PCR. More than 12 different HPV types were demonstrated by type-specific hybridization of the amplification products. The most prevalent types (by two-step PCR) were HPV 16 (2.7%) and HPV 6 (2.0%). The HPV 6 genome was detected in 10 out of 35 HPV positive samples, when using the one-step PCR, but only two additional HPV 6 containing scrapes were found with the two-step PCR. HPV 16 on the other hand was detected in 9/35 HPV positive scrapes, when using the one-step PCR, and seven additional HPV 16 positive scrapes were demonstrated with the two-step PCR. This could be a sign of variation of the copy number of certain HPV types in the lesions.

In this population-based study of young women (IX), 19.2% (102/530) of the women with normal cytology and 20.3% (118/581) of all women were shown to carry HPV DNA sequences by using general primers from the L1 region in a two-step PCR. In paper VI, general primers from the E7 and E1 regions (II) were used in a one-step PCR to demonstrate that 19% of Kenyan women on a routine visit to a family planning clinic carried HPV DNA. Their mean age was 26.5 years. In these two studies, general primers from different regions of the HPV genome were utilized and the HPV detection rate of 19.2% in the population based study was based on a two-step PCR. Furthermore, the number of women in paper VI was much lower than in the population-based investigation (77 vs 590). The prevalence of HPV 16 DNA was 2.7% among all women and 2.4% among cytologically normal women in the population based study (IX).
The prevalence of HPV in the female population has been studied extensively during the last decade. Several DNA hybridization methods have been used for detection of HPV infection. By Southern blot, dot-blot and FISH, HPV frequencies of 5-11% in cytologically normal women and 35-92% of women with abnormal cytology have been found (Lörincz et al., 1986; de Villiers et al., 1987; Martinez et al., 1988; Kiviat et al., 1989).

The PCR technique has been used in the recent years for detection of HPV DNA in CIN and cancer (Bevan et al., 1989; Claas et al., 1989; Ward et al., 1989; van den Brule et al., 1989, 1990a, b; Young et al., 1989; Manos et al., 1990; Beyer-Finkler et al., 1990; Nuovo, 1990). The PCR technique has consistently demonstrated a higher number of HPV infected individuals. Up to 100% of the cervical cancer biopsies were shown to contain HPV DNA sequences with the PCR technique; HPV 16 was the type most frequently present. A higher proportion of the women with CIN or HPV signs could also be shown to be infected with HPV, using PCR. HPV DNA sequences were present in 60-90% of high grade CIN; HPV 16 was the type most frequently present, but "unknown" HPVs were detected by PCR in a proportion (maximum 10-15%) of cases (Stanley, 1990).

The HPV prevalence in cytologically normal women from different geographic regions has been shown to vary considerably. By PCR, very high rates of cervical infection with HPV 16, 60-80%, in women with normal cervical cytology have been reported in the literature (Bevan et al., 1989; Tidy et al., 1989a,b; Ward et al., 1989; Young et al., 1989). In contrast lower and widely differing prevalence rates for HPV 16 have been published (Manos et al., 1990), 0% in Californians attending planned parenthood, 11% in women attending an annual routine gynaecological examination at the University of Mexico health clinic and 22% in women attending the University of Washington student health clinic. In a dutch study, 8% of normal women were found with HPV 16 in cervical swabs (van den Brule et al., 1990a).

There are several possible explanations for these wide disparities. The HPV prevalence may vary between different populations studied, as shown in the three papers concerning women with normal cytology presented herein (VI, VIII, IX) and by others (Manos et al., 1990). The women studied might also represent a heterogenous group although they have normal Pap smears (VIII). PCR is also
vulnerable to accidental contamination and high numbers of false positives are a hazard when using this technique (Kwok and Higuchi, 1989). Different primers and detection methods have also been used in the various reports. In paper IX, the detection rate increased about 3-fold when the more sensitive two-step PCR was used instead of the one-step PCR.

The prevalence of infection with HPV types may, of course, be genuinely different in different geographic locations, in differing social classes and in different age groups. For instance, in paper IX, the prevalence of HPV infection was shown to be substantially higher in women aged 25, then in the younger women. Furthermore, the female genital tract can be infected at many sites (Bergeron et al., 1987). In one report (Bauer et al., 1991), 33% of the women were infected at the cervix and 43% at the vulva. Altogether, 46% showed evidence of infection with HPV at one or both sites. Of these, 69% were positive at both sites. There might also be a fluctuation of the HPV prevalence. Subsequent swabs (10-12 at six weeks' interval) were taken from 15 cytologically normal women, 33-100% of their swabs were HPV 16 positive by type-specific PCR, giving a total of 13/15 HPV 16 positive women (Gissmann et al., 1989). Testing a single swab would have resulted in only 4-8 HPV 16 positive individuals.

The state of the HPV genome can be assumed to vary. At certain times it is likely to "rest" in a latent fashion and very few copies of the genome should be present. In the active state, the genome can be supposed to replicate to high copy numbers, thus being detected more easily. The technique used can be at it's limiting level of detection and with just a slight increase in the copy number, the specimen will be HPV positive.

The prevalence of HPV 16, by PCR, among cytologically normal women varies as different groups of women are studied (table 4). However, the prevalence of HPV 16 correlates with an increasing degree of dysplasia. In paper VII, 48% (14/29) of the women with CIN carried HPV 16 and in paper IV, HPV 16 was demonstrated in 85% (11/13) of the women with cervical squamous carcinoma. In the population-based study of young Swedish women, 16% (3/19) of the women with pathological signs were positive for HPV 16 compared to 2.4% (16/590) (table 4) of women with normal cytology (IX).
Table 4. HPV 16 prevalence among cytologically normal women.

<table>
<thead>
<tr>
<th>Paper</th>
<th>Country</th>
<th>Mean age years</th>
<th>HPV 16 prevalence % (no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VI</td>
<td>Kenya</td>
<td>26.5</td>
<td>6.5% (5/77)</td>
</tr>
<tr>
<td>VII</td>
<td>Hungary</td>
<td>38.6</td>
<td>6.7% (2/30)</td>
</tr>
<tr>
<td>VIII</td>
<td>Sweden</td>
<td>40</td>
<td>21%a (21/99)</td>
</tr>
<tr>
<td>IX</td>
<td>Sweden</td>
<td>22.5</td>
<td>2.4% (13/539)</td>
</tr>
</tbody>
</table>

a The group of women with normal cytology could be divided in two subgroups, one containing women without medication and the other women with diseases and a relative progesterone dominance. In the first group 10% carried HPV 16 DNA, in the second group, 54% were HPV 16 positive.

The prevalence of HPV 18 also showed a similar dependence. 69% (9/13) among women with cervical squamous carcinoma (IV) and 27% (7/26) among women with cervical adenocarcinoma (V) carried HPV 18 DNA. Six of thirteen (46%) premenopausal cases with cervical adenocarcinoma were positive for HPV 18 (V). Among women with normal cytology HPV 18 was found at a much lower frequency. In Kenya, 3.9% (3/77) of cytologically normal women were positive (VI) and in the population-based study in Sweden, 1.1% (6/539) of cytologically normal women carried HPV 18 DNA compared to 5.3% (1/19) of women with pathological signs (IX).

As described in paragraph 3.3, the anogenital papillomaviruses have been arranged in low- (HPV 6, 11, 42-44), intermediate- (HPV 31, 33, 35, 51, 52, 58) and high-risk (HPV 16, 18, 45) oncogenic viruses based on the results from both epidemiological studies and data from the ability to transform human keratinocytes in vitro (Lörincz et al., 1991; Schiffman et al., 1991; Schlegel, 1991). The prevalence of the various HPV types in different clinical stagings presented in this thesis, is in agreement with this pattern of risk categories.
7. GENERAL SUMMARY

* The prevalence of HPV infection, using Southern blot, was 39% among women with abnormal Pap smears.

* The prevalence of HPV infection, using FISH, was 27% among women with abnormal Pap smears.

* The prevalence of HPV infection, using Southern blot and filter in situ hybridization (FISH) together, was 46% among women with abnormal Pap smears.

* A system for type-specific detection of HPV by PCR was designed and found to be useful for demonstration of HPV DNA in clinical specimens.

* A system for detection of a broad spectrum of genital HPVs by PCR, using one degenerated primer pair, was designed and found to be useful for detection of HPV DNA in clinical specimens.

* A two-step PCR, using general primers, was shown to be more sensitive than a one-step PCR for detection of HPV DNA in clinical specimens.

* The prevalence of HPV infection among women with normal cytology was:

  19% among young Swedish women taking part in a population-based study, using general primers in a two-step PCR. 2.4% were HPV 16 positive.

  19% among Kenyan women on a routine visit to a family planning clinic, using general primers. 6.5% were HPV 16 positive.

  21% among women attending an gynecological clinic, using HPV 16 type-specific primers. This group could be divided into two subgroups where 10% of women without medication and 54% of women with diseases and women with a relative progesterone dominance were HPV 16 positive.
* The prevalence of HPV infection among women with cervical intraepithelial neoplasia (CIN) was:

48% among Hungarian women attending a gynecological clinic, using HPV 16 type-specific primers.

* The prevalence of HPV infection among women with cervical carcinoma was:

92% in Kenyan women with cervical squamous carcinoma, using HPV 6, 16, 18 and 33 type-specific primers. 85% were HPV 16 positive and 69% were HPV 18 positive.

42% in Swedish women with cervical adenocarcinoma, using HPV 16 and 18 type-specific primers. 15% were HPV 16 positive and 26% were HPV 18 positive.

* The prevalence of HPV 16 and HPV 18, infection was shown to increase with increased grade of neoplasia.
8. CONCLUDING REMARKS

* Southern blot is the generally accepted reference method for detection and typing of HPV DNA when present at high copy numbers.

* Filter in situ hybridization (FISH) has been used for screening of HPV DNA among cervical scrapes, but there is now no real need for this method.

* PCR can be used for sensitive and specific detection of HPV DNA at low copy numbers in a simple swab specimen. General primer pairs can be used for detection of most HPV types. Subsequent typing can be obtained by type-specific hybridization. Type-specific primer pairs can be used for direct detection of certain HPV types. A two-step PCR was found to be more robust in detecting HPV DNA in clinical specimens.
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10. LITERATURE CITED


keratinocytes obtained after transfection with human papillomavirus type 16 DNA. Oncogene 1:251-256.


