JOURNAL OF VIROLOGY, Oct. 1987, p. 3295–3298
0022-538X/87/103295-04$02.00/0
Copyright © 1987, American Society for Microbiology
Vol. 61, No. 10

Integration of Human Papillomavirus Type 16 DNA Sequences: a Possible Early Event in the Progression of Genital Tumors

SYLVIE SCHNEIDER-MAUNOURY, ODILE CROISSANT, AND GÉRARD ORTH*

Unité des Papillomavirus, Unité de l'Institut National de la Santé et de la Recherche Médicale 190, Institut Pasteur, 75015 Paris, France

Received 27 January 1987/Accepted 9 June 1987

The keratinocyte line SK-v harbors only integrated human papillomavirus type 16 (HPV16) DNA sequences, although it originated from vulvar Bowenoid papules predominantly containing multiple copies of free HPV16 genomes. We have cloned a fragment of cell DNA that contains the integrated HPV16 DNA sequences and have shown that integration interrupts the HPV16 genome in open reading frames E2 and L2 and creates a deletion of 813 base pairs. This allows the expression of open reading frames E6 and E7, as actually substantiated by Northern (RNA) blot analysis of SK-v RNAs with subgenomic HPV16 RNA probes. Using a unique flanking cellular DNA sequence as the probe, we have shown that the integration of HPV16 sequences had already occurred in the premalignant lesions from which the SK-v cell line was derived.

The genomes of human papillomavirus types 16 and 18 (HPV16 and HPV18) have been associated with the majority of genital neoplasias (3, 4, 7, 12, 20), mainly as free DNA molecules in intraepithelial neoplasias (4, 8) and as DNA sequences integrated into the host cell genome in invasive cervical cancers (13, 16) and derived cell lines (23, 27). This finding suggests that integration of HPV DNA sequences plays a role in the progression of genital tumors. To analyze this problem, we have studied a keratinocyte line, designated SK-v, established from a specimen from the total vulvectomy of a patient (referred to as patient SK) with Bowenoid papulosis of the vulva. After subculture 20, SK-v cells harbored 10 to 20 HPV16 genome equivalents that resulted from the amplification of a region of the cell DNA containing a single copy of the viral genome plus flanking cellular sequences. When injected into nude mice, SK-v cells gave rise to slow-growing tumors with the histological features of a Bowen's carcinoma in situ (G. Orth, N. Jibard, F. Breitburd, O. Croissant, and S. Jablonska, manuscript in preparation).

The first aim of our study was to analyze the structure of the integrated HPV16 genome and flanking cellular DNA sequences. The DNA extracted (1) from the SK-v cell subculture 40 was treated with *Hin*dIII, a no-cut enzyme for HPV16 (8), and the cleavage products were fractionated by centrifugation in a preformed 5 to 40% sucrose gradient (1). DNA fragments of about 13.5 kilobases (kb) were introduced into bacteriophage λ L47-1 DNA (14) at the *Hin*dIII site and were cloned in *Escherichia coli* K12 C600 as previously described (1). A 13.5-kb insertion containing HPV16 DNA sequences was purified from recombinant phage DNA molecules and subcloned in plasmid pSP64 (17). The recombinant plasmid was designated pSK-v. The heteroduplex molecules, formed between the cloned SK-v cell DNA fragment and an HPV16 DNA recombinant plasmid, showed a single-stranded segment joining both tails of the cellular DNA (Fig. 1A). The segment corresponds to a deletion of about 800 nucleotides in the HPV16 genome at the integration site. At the 5′ virus-cell DNA junction, 449 nucleotides were sequenced (280 in viral sequences and 169 in cellular sequences), and 323 nucleotides were sequenced at the 3′ virus-cell DNA junction (170 in viral sequences and 153 in cellular sequences). Viral sequences showed no difference from the nucleotide sequence determined for HPV16 DNA (24). The 5′ integration site is located at position 4474 (Fig. 1B and Fig. 2), interrupting open reading frame (ORF) L2 and is 3 base pairs downstream from the only HPV16 DNA integration site determined so far (16). The 3′ integration site is located at position 3661, interrupting ORF E2 downstream from the 3′ end of ORF E4 (Fig. 1B and 2). This integration site is found between two inversely repeated sequences of 14 base pairs (Fig. 2). The deletion (813 base pairs) spans the 3′ end of ORF E2, the complete ORF E5, and the 5′ end of ORF L2 (Fig. 1B). It also includes the putative early polyadenylation signal at position 4213 and the consensus TATA box upstream from the late region at position 4289 (24). The distribution of stop codons at the virus-cell junctions (Fig. 2) indicates that integration does not generate large fusion ORFs. Such an integration pattern is compatible with the expression of ORFs E6 and E7, reported to be transcribed in a specimen from a patient with cervical cancer (25) and in cancer-derived cell lines (22, 25).

To substantiate the transcription of ORFs E6 and E7 in SK-v cells, poly(A)$^+$ RNAs were prepared from cells at subculture 43, were fractionated on denaturing agarose gels, and were transferred to nitrocellulose. The membranes were hybridized in 50% formamide at 55°C with RNA probes in the conditions described by Melton et al. (17). The probes were specific for the complete HPV16 genome and for *Pst*I HPV16 DNA fragments (24) containing ORFs E1, E2, and E4 (Fig. 1B, fragment A), the noncoding region and ORFs E6 and E7 (Fig. 1B, fragment B), and a part of ORF L2 (Fig. 1B, fragment E). Two major RNA species of 1.0 and 1.2 kb hybridized with the probes for the complete HPV16 genome (Fig. 3, lane a), ORFs E6 and E7 (Fig. 3, lane c), and ORFs E1, E2, and E4 (Fig. 3, lane d). No hybridization was detected with the probe for ORF L2 (Fig. 3, lane b). Because of their sizes and the relative intensity of the signals obtained with the different probes, it is most likely that these transcripts correspond to sequences of the E6-E7 region spliced to sequences of the E2-E4 region, as previously demonstrated for the HPV16 transcripts in the specimen from the patient with cervical cancer and in a cancer-derived cell line (25).

* Corresponding author.

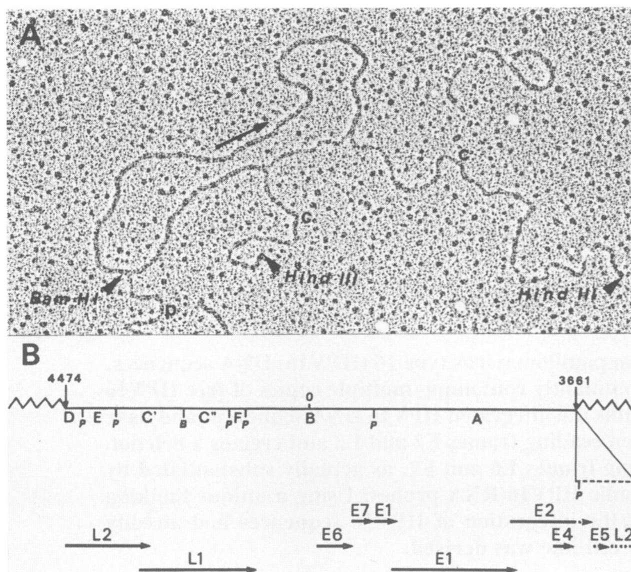

FIG. 1. (A) Heteroduplex analysis of HPV16 sequences integrated in the SK-v cell genome. *Hin*dIII-excised SK-v DNA and pSP62/HPV16 plasmid linearized by *Sma*I were annealed and spread in the presence of 50% formamide at 20°C (5). Inspection of the heteroduplex molecules under an electron microscope permitted the identification of plasmid (p) and cellular (c) sequences. The different regions of the heteroduplex molecules were measured with a digital length calculator (Numonics Corp.) connected to a Nova 3D computer (Data General Corp.) (1). The ends of the cloned SK-v cell DNA fragment (*Hin*dIII) and the insertion site of the HPV16 genome in pSP62 (*Bam*HI) are indicated. The orientation 5'→3' of the HPV16 genome, deduced from the map of the pSP62/HPV16 recombinant plasmid (1), is indicated (→). (B) Map of the HPV16 DNA sequences integrated into the SK-v cell genome. The map of the HPV16 insertion was deduced by heteroduplex mapping and nucleotide sequencing (see legend to figure 2). The origin of the HPV16 DNA cleavage map *Bam*HI (B) (8) and nucleotide sequence (0) (24), the positions of ORFs and of *Pst*I sites (P) (16, 24), and the locations of the integration sites are indicated. Symbols: ⁓, cellular sequences; ———, HPV16 sequences; – – –, HPV16 DNA sequences deleted in SK-v cells.

Our second aim was to find out whether the integration of HPV16 sequences had taken place in vivo or had resulted from the establishment of the cells in vitro. Such a study was possible because parts of the vulvectomy specimen had been kept frozen. A DNA preparation from the lesions, digested by *Hin*dIII (Fig. 4B, lane g), yielded mainly forms I and II monomeric HPV16 DNA molecules, with a labeling intensity corresponding to 500 to 1,000 copies of HPV16 DNA molecules per diploid cell. The data indicate that the majority of the viral genomes were present as episomes. Faint bands with a lower mobility were also detected, which could correspond to oligomeric HPV16 DNA molecules or integrated viral sequences. To characterize these bands, we prepared a probe from a unique SK-v cell DNA sequence adjacent to the integrated HPV16 genome. After digestion of pSK-v with a mixture of *Bam*HI and *Hin*dII (Fig. 4A) and blot hybridization of the products with ^{32}P-labeled human placental DNA (15, 26), only a 1-kb fragment (referred to as BH5) showed no labeling, indicating that it consisted of unique DNA sequences. This fragment was isolated by electroelution (15). Blot hybridization experiments done with BH5 DNA as the probe and with *Hin*dIII-digested DNA preparations obtained from human placenta (Fig. 4B, lane a) or from normal fibroblasts grown from a biopsy from patient SK (Fig. 4B, lane b) resulted in a single 10-kb band corresponding to the normal allele of the integration locus. An additional 13.5-kb band was detected with the BH5 probe in SK-v cells from subculture 34, which corresponded to the single band detected after rehybridization with an HPV16 DNA probe (Fig. 4B, lanes c and d).

SK-v cells from an earlier passage (passage 13) also harbored integrated head-to-tail tandem repeats of the HPV16 genome (Orth et al., in preparation). This integration pattern resulted in the detection of two 19.5- and 24-kb bands, in addition to the 13.5-kb band, after hybridization of SK-v cell DNA with an HPV16 DNA probe (Fig. 4B, lane e). After rehybridization with a BH5 DNA probe (Fig. 4B, lane f), the three HPV16 DNA-containing bands were detected, indicating that all of the viral sequences were integrated at the same cellular site. When the DNA extracted from the lesions of patient SK was hybridized with ^{32}P-labeled BH5 DNA, three bands were detected with the same mobility (13.5, 19.5, and 24 kb) as the bands observed in SK-v cells at early passages. A 10-kb band corresponding to the unoccupied allele was also detected (Fig. 4B, lane h). In addition, a band migrating like form II HPV16 DNA molecules was found, resulting probably from some contamination of the BH5 probe by viral sequences. The similar labeling intensity of the 10- and 13.5-kb bands indicates that an important fraction of the cells in the lesions had integrated HPV16 DNA sequences into their genomes, as the result of a clonal expansion.

Finally, we wished to determine whether the integration of HPV16 DNA sequences occurred at specific sites in the cell genome. We used BH5 DNA as the probe to look for rearrangements of this locus in cellular DNA extracted from genital tumors. DNA preparations from 16 specimens of invasive carcinoma of the cervix, previously shown to contain integrated HPV16 genomes, and a DNA preparation from a vulvar carcinoma in situ (Bowen's disease), containing only integrated HPV33 sequences (1), were analyzed by blot hybridization after *Hin*dIII treatment. Only the 10-kb band was detected (data not shown), indicating that in these tumors, integration did not occur at the same cellular site as in the Bowenoid papules of patient SK.

Integration of HPV DNA sequences is observed in the majority of cervical cancers (8, 13, 16), but it is not clear whether this event plays a role in the progression of genital tumors or whether it is only a consequence of DNA rear-

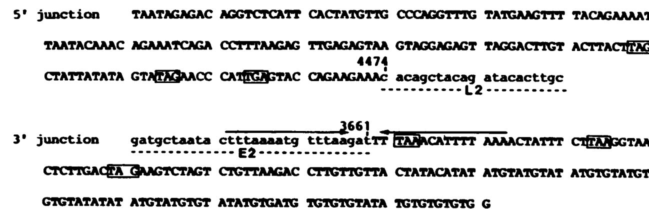

FIG. 2. Nucleotide sequence of the virus-cell junctions. Restriction fragments spanning the junctions were isolated and inserted in M13 mp18 and M13 mp19 vectors (18). DNA sequencing was done by the dideoxy chain termination method (21), as modified by Biggin et al. (2). The nucleotide sequence of the 5' and 3' virus-cell junctions on the sense strand of HPV16 DNA is represented. Upper- and lowercase letters represent cellular and HPV16 sequences, respectively. The interrupted E2 and L2 ORFs are indicated (– – –), and the inverted repeat at the 3' junction is indicated (→←). The stop codons closest to the integration sites in each frame of the cell DNA are boxed.

rangements occurring in tumor cells (8). The available data on the structure and transcription of integrated HPV DNA sequences in genital tumors (1, 13, 16, 25) and derived cell lines (22, 23, 25), including the SK-v cell line, indicate specific viral mechanisms. Integration usually interrupts ORFs E1 or E2 of the viral genome, and deletions in viral sequences involving the early polyadenylation signal are usually found at the integration site. This integration pattern allows the transcription of ORFs E6 and E7 as spliced messengers containing the 3′ end of the early region and cellular sequences (22, 25). The E7 protein of HPV16 has already been identified in a cervical cancer-derived cell line (25).

Bowenoid papules are intraepithelial neoplasms with a rather benign clinical course (10, 19), although there are isolated reports of conversion into typical Bowen's disease (6) or squamous cell carcinomas (11). The lesions of patient SK were widespread on the vulva, involving the perineal and inguinal regions, and showed features of Bowen's atypia (patient 2 in reference 19). This supported the indication for a complete vulvectomy. Furthermore, it must be stressed that the tumors induced in nude mice by SK-v cells have the histological features of Bowen's carcinoma in situ (Orth et al., in preparation). Integrated HPV DNA sequences have been characterized in only two cases of carcinoma in situ (Bowen's disease) of the external genitalia (1, 8), but the presence of free viral genomes at a high copy number could preclude the detection of integrated viral sequences in the majority of intraepithelial neoplastic lesions. Our study indicates that integration of HPV sequences can be an early event in the progression of genital tumors and is not sufficient by itself to confer a fully malignant phenotype.

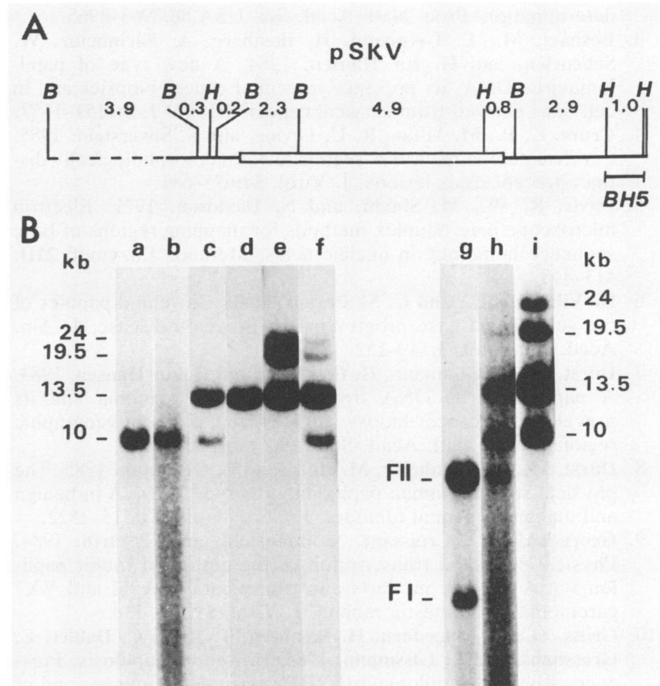

FIG. 4. Evidence for the presence of integrated HPV16 DNA sequences in Bowenoid papules of patient SK. (A) Localization of unique sequences (BH5) in recombinant plasmid pSK-v. Symbols: ▭, HPV16 sequences; ———, cellular sequences; - - -, pSP64 sequences. The positions of the *Bam*HI (B) and *Hin*dII (H) sites and the position of the cell DNA fragment consisting of unique sequences (BH5) are indicated. The sizes of the fragments are given in kilobases. (B) Blot hybridization experiments. Total cellular DNA was prepared from human placenta (lane a), fibroblasts grown from a biopsy from patient SK (lane b), SK-v subcultures 34 (lanes c and d) and 13 (lanes e, f, and i), Bowenoid papules from patient SK (lanes g and h), as previously described (1). After *Hin*dIII digestion, samples (10 μg) were electrophoresed in 0.5% agarose slab gels and were transferred to nitrocellulose membranes (15). The latter were hybridized with ^{32}P-labeled HPV16 DNA (lanes d, e, and g), dehybridized, and then rehybridized with ^{32}P-labeled BH5 DNA (lanes a, b, c, f, h, and i). The specific activity of the probes was 1×10^8 to 2×10^8 cpm/μg. The hybridizations were done in the presence of 50% formamide and 10% dextran sulfate, at 42°C (26). The dehybridization was done twice in distilled water for 15 min each at 68°C. The hybrids were detected by autoradiography after 5 h (lanes e, and g), 3 days (lanes a, c, f, and i), or 7 days (lanes b and h) of exposure. kb, Kilobases; FI, HPV16 supercoiled circular DNA molecules; FII, HPV16 relaxed circular DNA molecules.

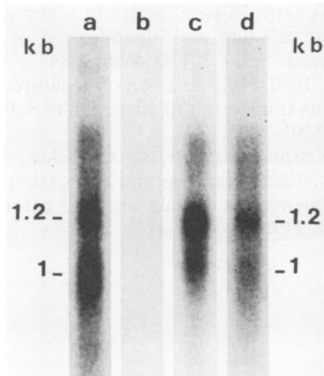

FIG. 3. Northern (RNA) blot analysis of the poly(A)$^+$ RNAs in SK-v cells. Cytoplasmic RNAs were prepared from cells at subculture 43 (15), and poly(A)$^+$ RNAs were isolated by oligo(dT)-cellulose chromatography, as previously described (9). Poly(A)$^+$ RNAs were fractionated on denaturing (6% formaldehyde) 1% agarose gels (9), transferred to nitrocellulose filters, and hybridized with ^{32}P-labeled RNA probes specific for the full-length HPV16 genome (13×10^4 cpm/cm^2) (lane a); the *Pst*I E fragment, contained in ORF L2 (0.8×10^4 cpm/cm^2) (lane b); the *Pst*I B fragment containing the noncoding region and ORFs E6 and E7 (3×10^4 cpm/cm^2) (lane c); and the *Pst*I A fragment containing ORFs E1, E2, and E4 (4.6×10^4 cpm/cm^2) (lane d). The RNA probes (specific activity, 10^9 cpm/μg) were obtained by in vitro transcription of the HPV16 DNA inserted in pSP62 at the *Bam*HI site and of the *Pst*I HPV16 DNA fragments A, B, and E inserted in pSP64 in the orientation allowing the transcription of antimessage RNAs by using bacteriophage SP6 RNA polymerase (17). The sizes of the transcripts are indicated in kilobases (kb). Lane a and lanes b, c, and d correspond to different experiments, respectively. The hybrids were detected by autoradiography after 16 h of exposure.

We are grateful to S. Jablonska for providing the biopsies of Bowenoid papules and for encouragement with this work. We thank O. Fichot for fruitful discussions, N. Jibard for assistance in some of the experiments, and B. Rubat du Mérac for help in preparation of the manuscript.

LITERATURE CITED

1. **Beaudenon, S., D. Kremsdorf, O. Croissant, S. Jablonska, S. Wain-Hobson, and G. Orth.** 1986. A novel type of human papillomavirus associated with genital neoplasias. Nature (London) **321**:246–249.
2. **Biggin, M. D., T. J. Gibson, and G. F. Hong.** 1983. Buffer gradient gels and ^{35}S label as an aid to rapid DNA sequence

determination. Proc. Natl. Acad. Sci. USA **80**:3963–3965.
3. **Boshart, M., L. Gissmann, H. Ikenberg, A. Kleinheinz, W. Scheurlen, and H. zur Hausen.** 1984. A new type of papillomavirus DNA, its presence in genital cancer biopsies and in cell lines derived from cervical cancer. EMBO J. **3**:1151–1157.
4. **Crum, C. P., M. Mitao, R. U. Levine, and S. Silverstein.** 1985. Cervical papillomaviruses segregate within morphologically distinct precancerous lesions. J. Virol. **54**:675–681.
5. **Davis, R. W., M. Simon, and N. Davidson.** 1971. Electron microscope heteroduplex methods for mapping regions of base sequence homology in nucleic acids. Methods Enzymol. **21D**: 413–418.
6. **De Villez, R. L., and C. S. Stevens.** 1980. Bowenoid papules of the genitalia: a case progressing to Bowen's disease. J. Am. Acad. Dermatol. **3**:149–152.
7. **Dürst, M., L. Gissmann, H. Ikenberg, and H. zur Hausen.** 1983. A papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions. Proc. Natl. Acad. Sci. USA **80**:3812–3815.
8. **Dürst, M., A. Kleinheinz, M. Hotz, and L. Gissmann.** 1985. The physical state of human papillomavirus type 16 DNA in benign and malignant genital tumours. J. Gen. Virol. **66**:1515–1522.
9. **Georges, E., O. Croissant, N. Bonneaud, and G. Orth.** 1984. Physical state and transcription of the cottontail rabbit papillomavirus genome in warts and transplantable VX2 and VX7 carcinomas of domestic rabbits. J. Virol. **51**:530–538.
10. **Gross, G., M. Hagedorn, H. Ikenberg, T. Ruffi, C. Dahlet, E. Grosshans, and L. Gissmann.** 1985. Bowenoid papulosis. Presence of human papillomavirus (HPV) structural antigens and of HPV16-related DNA sequences. Arch. Dermatol. **121**:858–863.
11. **Hirai, A., N. Inamoto, and R. Harada.** 1979. So-called multicentric pigmented Bowen's disease progressing to squamous cell carcinoma. Jpn. J. Dermatol. (Tokyo) **89**:380–381.
12. **Ikenberg, H., L. Gissmann, G. Gross, E. I. Grussendorf-Conen, and H. zur Hausen.** 1983. Human papillomavirus type 16 related DNA in genital Bowen's disease and in Bowenoid papulosis. Int. J. Cancer **32**:563–565.
13. **Lehn, H., P. Krieg, and G. Sauer.** 1985. Papillomavirus genomes in human cervical tumors: analysis of their transcriptional activity. Proc. Natl. Acad. Sci. USA **82**:5540–5544.
14. **Loenen, W. A. M., and W. J. Brammar.** 1980. A bacteriophage lambda vector for cloning large DNA fragments made with several restriction enzymes. Gene **10**:249–259.
15. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
16. **Matsukura, T., T. Kanda, A. Furuno, H. Yoshikawa, T. Kawana, and K. Yoshiike.** 1986. Cloning of monomeric human papillomavirus type 16 DNA integrated within cell DNA from a cervical carcinoma. J. Virol. **58**:979–982.
17. **Melton, D. A., P. A. Krieg, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green.** 1984. Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Res. **12**:7035–7056.
18. **Norrander, J., T. Kempe, and J. Messing.** 1983. Construction of improved M13 vectors using oligodeoxynucleotide-directed mutagenesis. Gene **26**:101–106.
19. **Obalek, S., S. Jablonska, S. Beaudenon, L. Walczak, and G. Orth.** 1986. Bowenoid papulosis of the male and female genitalia. Risk of cervical neoplasia. J. Am. Acad. Dermatol. **14**:433–444.
20. **Riou, G. E., M. Barrois, V. Dutronquay, and G. Orth.** 1985. Presence of papillomavirus DNA sequences, amplification of c-myc and c-Ha-ras oncogenes and enhanced expression of c-myc in carcinomas of the uterine cervix. Papillomaviruses. Molecular and clinical aspects. UCLA Symp. Mol. Cell. Biol. **32**:47–56.
21. **Sanger, F., A. R. Coulson, B. G. Barrel, A. J. H. Smith, and B. A. Roe.** 1980. Cloning in single-stranded bacteriophage as an aid to rapid DNA sequencing. J. Mol. Biol. **143**:161–178.
22. **Schneider-Gädicke, A., and E. Schwarz.** 1986. Different human cervical carcinoma cell lines show similar transcription patterns of human papillomavirus type 18 early genes. EMBO J. **5**: 2285–2292.
23. **Schwarz, E., U. K. Freese, L. Gissmann, W. Mayer, B. Roggenbuck, A. Stremlau, and H. zur Hausen.** 1985. Structure and transcription of human papillomavirus sequences in cervical carcinoma cells. Nature (London) **314**:111–114.
24. **Seedorf, K., G. Krämmer, M. Dürst, S. Suhai, and W. G. Röwekamp.** 1985. Human papillomavirus type 16 DNA sequence. Virology **145**:181–185.
25. **Smotkin, D., and F. O. Wettstein.** 1986. Transcription of human papillomavirus type 16 early genes in a cervical cancer and a cancer-derived cell line and identification of the E7 protein. Proc. Natl. Acad. Sci. USA **83**:4680–4684.
26. **Thomas, P. S.** 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. USA **77**:5201–5205.
27. **Yee, C., I. Krishnan-Hewlett, C. C. Baker, R. Schlegel, and P. M. Howley.** 1985. Presence and expression of human papillomavirus sequences in human cervical carcinoma cell lines. Am. J. Pathol. **119**:361–366.