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Detection of Epstein-Barr Virus DNA Sequences in Nasopharyngeal Carcinoma Cells by Enzymatic DNA Amplification

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The presence of Epstein-Barr virus (EBV) DNA sequences was examined by the polymerase chain reaction in 50 nasopharyngeal carcinoma (NPC) biopsy specimens and in two primary epithelial tumor cell cultures derived from patients with NPC. The detection limit was a single EBV genome equivalent by agarose gel electrophoresis followed by Southern blot analysis of the amplified products. EBV DNA sequences were detected in all 41 undifferentiated NPC cell specimens, in 2 of 4 moderately differentiated NPC cell specimens, and in 3 of 5 keratinized NPC cell specimens. Undifferentiated NPC cells were also found to contain higher copy numbers of EBV than cells of the other two types of NPC. Our data suggest that EBV replication may be closely associated with the differentiation of NPC tumor cells. The results also demonstrated a sensitive and specific method for the detection of EBV DNA sequences in NPC tumor cells.

Epstein-Barr virus (EBV), a ubiquitous human herpesvirus, is the causative agent of infectious mononucleosis (5) and has been suggested to be closely related to at least three human cancers, Burkitt's lymphoma (6, 7), nasopharyngeal carcinoma (NPC) (7, 8), and lymphoma in immunocompromised individuals. NPC is one of the most common cancers of males in southeastern Asia, including southern China and Taiwan. EBV DNA has been detected in endemic Burkitt's lymphoma (12), in NPC tissues (10), and in cell lines derived from NPC by Southern blot hybridization (2). Fahraeus et al. reported the presence of EBV DNA in 90% of NPC tissues examined (4). However, it is technically difficult to analyze the EBV in NPC tissues because of specimen size, and there has been no report correlating the presence and the content of EBV DNA in different stages of NPC.

Recently, a powerful technique known as the polymerase chain reaction (PCR), which is capable of amplifying specific DNA by making thousands of copies in a matter of hours in vitro, was described. PCR is based on repeated cycles of template denaturation coupled with highly specific oligonucleotide primers to extend the DNA chain with the assistance of a thermostable enzyme, *Taq* polymerase (15). The primers are positioned so that the products of each cycle can serve as a template for the next cycle of DNA extension, which can result in exponential accumulation of the DNA bracketed by the two primers. Amplification of specific target DNA by PCR allows the use of very small amounts of target DNA and permits easy identification of product DNA after amplification (15).

In the following text we describe the use of PCR to detect the presence and determine the content of EBV DNA in various types of NPC cells.

MATERIALS AND METHODS

NPC biopsies, primary epithelial tumor cells, and cultured cells. Biopsy tissues of NPC were obtained from the Department of Otorhinolaryngology of Chang-Gung Memorial Hospital, Taoyuan, Taiwan. The stages of differentiation of NPC cells were determined in accordance with the guidelines of the World Health Organization (WHO). Three subtypes have been derived from the WHO classification scheme: keratinizing squamous-cell carcinoma (WHO type 1), nonkeratinizing carcinoma (WHO type 2), and undifferentiated carcinoma (WHO type 3). Keratinizing carcinoma (type 1) is defined as a tumor having squamous differentiation in the form of intercellular bridges or keratinization over most of its extent. Sometimes the keratinized pearls are visible by light microscopy. Nonkeratinizing carcinoma (type 2) has cells at differing levels of maturation but lacks light-microscopic evidence of squamous differentiation. Undifferentiated tumors (type 3) consist of cytologically uniform cells with ovoid vesicular nuclei, prominent nuclei, and indistinct cell borders resulting in a syncytial pattern (14). A total of 50 NPC biopsy specimens were examined in this study, including 41 cases of undifferentiated (WHO type 3), 4 cases of moderately differentiated (WHO type 2), and 5 cases of keratinized (WHO type 1) NPC tissues. Also analyzed in this study were one metastatic lymph node from the left side of the neck of a patient with NPC, two primary NPC cell lines derived from 2 of 41 undifferentiated NPC biopsy specimens and cultured in vitro as described previously (2), and six normal nasopharyngeal tissue specimens. Non-NPC tumors (Table 1) were surgically removed from patients. The malignant lymphoma analyzed was a Burkitt's-type tumor originating from an ovary. Lymphoid hyperplasia tissues were obtained from the nasopharynx region. The remaining tumors were diagnosed as keratinizing squamous-cell carcinomas. B-lymphocyte cells of the cell lines B95-8 (ATCC CRL 1612), Raji (ATCC CCL 86), Namalwa (ATCC CRL 143), and CA46 (ATCC CRL 1648) were obtained from the American Type Culture Collection and cultured according to the instructions of the supplier. Among these cell lines, B95-8, Raji, and Namalwa were EBV positive, whereas CA46 was EBV negative.

Sample DNA preparation. Biopsy tissues, primary tumor cells (approximately 10^3 cells), normal nasopharyngeal tissues, and B lymphocytes were treated with proteinase K (1 mg/ml) freshly prepared in 10 mM Tris hydrochloride (pH 8.0)–10 mM EDTA–0.5% sodium dodecyl sulfate at 56°C

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TABLE 1. Prevalence and amount of EBV DNA in NPC biopsy and other tissues

Tissue source[a]	No. of EBV genome equivalents/ μg of DNA[b]	No. of samples
Type 1 NPC	<1	2
	50	2
	500	1
Type 2 NPC	<1	2
	23	2
Type 3 NPC	10–100	7
	100–500	4
	500–1,000	15
	1,000–5,000	10
	7,000	4
	38,500	1
NPC lymph node	6,300	1
Malignant lymphoma	25,000	1
Lymphoid hyperplasia	50–500	3
Hypopharyngeal carcinoma	<1	3
	500	1
Epithelial carcinoma of palate	500	1
Epithelial carcinoma of sinus maxilla	500	1
Tongue carcinoma	<1	1
Normal tissue	<1	5
	50	1

[a] Non-NPC tumors: malignant lymphoma was of Burkitt's type; hypopharyngeal carcinoma, epithelial carcinomas of the palate and sinus maxilla, and tongue carcinoma were keratinizing squamous-cell carcinomas.

[b] The EBV content in all the biopsy tissues was estimated as described in the text. <1 indicates that the EBV content was below the detection limit of the method used.

overnight. DNA was then purified by repeated phenol-chloroform-isoamyl alcohol (49:49:2) extraction followed by ethanol precipitation.

PCR assays and primers. Purified sample DNA was subjected to PCR amplification in a thermal cycler (Coy Laboratory Products, Inc., Ann Arbor, Mich.). Three sets of EBV-specific oligonucleotide primers corresponding to the *Bam*HI Nhet, Y, and E fragments of strain B95-8 EBV (19) were prepared and used in this study. The sequences of these primers and the locations and distance bracketed by each set of primers are shown in Fig. 1. A typical 100-μl amplification reaction mixture contained 10 mM Tris (pH 7.5), 50 mM NaCl, 10 mM $NgCl_2$, the four deoxyribonucleoside triphosphates (1.5 mM each), the two primers (1 μM each), and 2.5 U of *Taq* DNA polymerase. The amplification procedure included 3 min of denaturation at 95°C in the first cycle (1 min thereafter) followed by 2 min of annealing at 55°C and 2 min of DNA extension at 72°C. This amplification was repeated for 32 cycles. After the reaction, 1/10 of the reaction mixture was fractionated electrophoretically in a 2% agarose gel and visually inspected under UV light for the presence of DNA bands of appropriate sizes after ethidium bromide staining. If the initial result was negative for EBV DNA, a portion, usually one-fifth of the first reaction product, was subjected to another 32 cycles of amplification with freshly supplemented primers and *Taq* DNA polymerase. Positive (B95-8, Raji, or Namalwa), negative (CA46), and component (*Taq* polymerase buffer, primers, and deoxyribonucleoside triphosphates) controls were always included with each PCR amplification assay. To prevent unexpected contamination, each DNA sample was checked with all three sets of PCR primers. Only those samples showing EBV-specific bands with all primers were then considered to be positive for EBV.

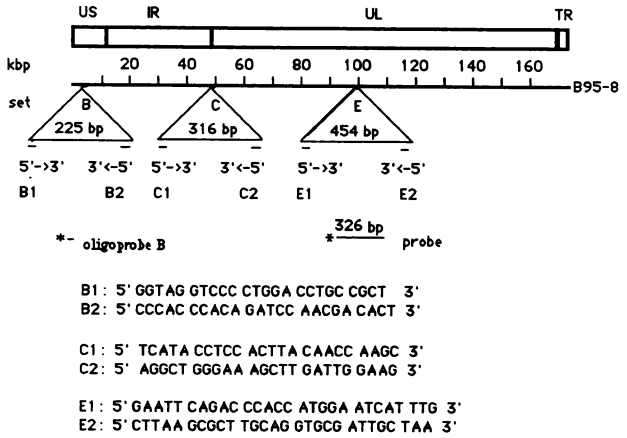

FIG. 1. Sequences of PCR primers and their locations on the genome of EBV strain B95-8. The PCR primer sets B, C, and E are located between nt 3676 and 3900, nt 47738 and 48053, and nt 98360 and 98814 on the B95-8 map, respectively. The locations of the probes for the regions bracketed by primer sets B and E also are shown on the map. US and UL represent the short and long unique regions on the genome. IR and TR indicate the regions of the major internal and terminal repeats, respectively.

Southern blot hybridization analysis. DNA was transferred to a Zeta-Probe blotting membrane (Bio-Rad Laboratories, Richmond, Calif.) from the agarose gel after electrophoresis. The DNA fragments on the membrane were hybridized with radioactively labeled EBV genome DNA fragments or synthetic oligonucleotide probes. A 326-bp DNA fragment obtained from the digestion of strain B95-8 EBV *Bam*HI-E with restriction endonucleases *Xba*I and *Bgl*I (positioned between nucleotides [nt] 98396 and 98723) was labeled with [α-^{32}P]dCTP by the nick translation method (13) and used as a probe for PCR products amplified with primer set E. The PCR products amplified with primer set B were detected with a 24-base synthetic oligonucleotide, 5' TGGGA TC GAA TGACA GAGAA GCTG 3' (positioned between nt 3776 and 3800) which was end labeled with [γ-^{32}P]ATP by polynucleotide kinase reaction (3). The hybridization conditions followed the procedure recommended by Maniatis et al. (13).

Estimation of detection limit and EBV copy number. The detection limit of EBV DNA by PCR was determined by positive identification of EBV DNA in serial dilutions of DNA prepared from p*Bam*HI-E and Namalwa cells. Plasmid p*Bam*HI-E contained a 7.9-kb *Bam*HI E fragment of EBV strain B95-8 in the *Bam*HI site of pBR322 (19). Namalwa cells contained two genome equivalents of EBV per cell (11).

Estimation of EBV DNA content in NPC biopsy specimens. One microgram of biopsy DNA and 10-fold dilutions thereof were subjected to PCR analysis. The DNA sample of the largest dilution demonstrating visible PCR products by ethidium bromide staining was chosen to compare with the detection limit established above. DNA samples showing negative results were subjected to an additional 32 cycles of amplification. The EBV-specific bands were finally determined by Southern blot hybridization analysis.

RESULTS AND DISCUSSION

Detection limit of EBV DNA by PCR. We used DNA of Namalwa cells as a standard for the determination of the detection limit of EBV DNA in cells. With 32 cycles of PCR

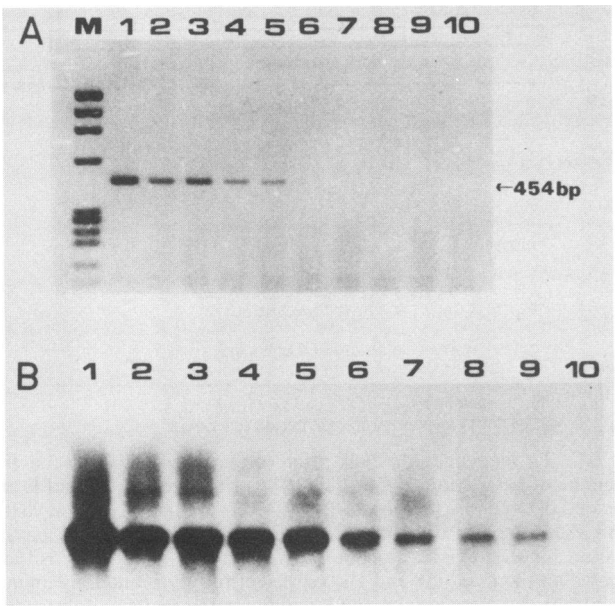

FIG. 2. Determination of detection limit of EBV DNA by the PCR technique. (A) Agarose gel electrophoresis of PCR products. A 454-bp DNA fragment indicated the presence of EBV DNA when primer set E was used in the PCR. Template DNAs used for the reaction were B95-8 DNA (lane 1, 0.5 μg) and Namalwa DNA (lane 2, 1 μg; lane 3, 0.5 μg; lane 4, 0.1 μg; lane 5, 10 ng; lane 6, 5 ng; lane 7, 1 ng; lane 8, 10 pg; lane 9, 1 pg; lane 10, 0.1 pg). *Hae*III-digested bacteriophage φX174 DNA (lane M) was used for DNA size standards. The sizes of these fragments were 1,353, 1,078, 872, 603, 310, 281, 271, 234, 194, 118, and 72 bp. (B) Autoradiogram of Southern blot (lanes are in the same order as in panel A) with the 326-bp DNA fragment generated from the digestion of EBV strain B95-8 *Bam*HI-E with restriction endonucleases *Xba*I and *Bgl*I (nt 98396 to 98723).

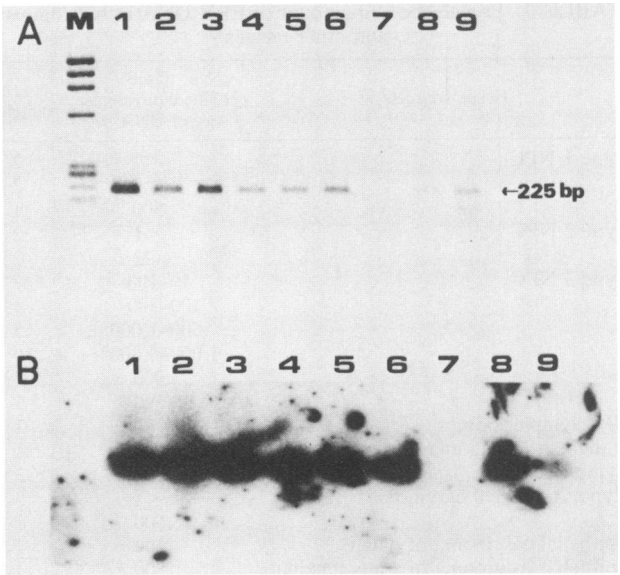

FIG. 3. PCR products of NPC biopsy specimens with primer set B. (A) agarose gel electrophoresis of PCR mixture. One microgram of total cellular DNA was subjected to PCR amplification. B95-8 (lane 1), Raji (lane 2), and Namalwa (lane 3) are all EBV-containing cell lines. NPC41 (lane 4), NPC667 (lane 5), and NPC67 (lane 6) are three different NPC biopsy specimens. CA46 (lane 7) is an EBV-free cell line. 122P (lane 8) and 155P (lane 9) are two primary cultures derived from NPC biopsy specimens NPC122 and NPC155, respectively. *Hae*III-digested φX174 DNA was included in lane M for DNA size standards. (B) Autoradiogram of the gel after Southern blot hybridization. The end-labeled oligonucleotide probe used in this experiment is described in the text.

amplification using primer set E, we were able to detect EBV DNA sequences in 0.5 ng of Namalwa cell DNA. On the basis of the facts that Namalwa cells contain two copies of the EBV genome in each cell (11), that the genome size of EBV is 172,282 bp, and that the size of the human genome is about 3×10^9 bp, the PCR detection limit is approximately 250 genome equivalents of EBV per cell (Fig. 2A, lane 6). An additional 32 cycles of PCR with the same primers (data not shown) or Southern blot hybridization of the products from the first 32 cycles of amplification can increase the sensitivity of detection of EBV DNA by PCR by at least 500-fold (Fig. 2B, lane 9), which brings the detection limit down to approximately a single EBV genome equivalent. When the cloned EBV fragment was used as a template, the sensitivity increased to 10^{-5} pg (data not shown). This result is similar to the limit established for the detection of hepatitis B virus DNA sequences in serum (9). This method is by far the most sensitive method currently available for EBV detection. Therefore, this method is very useful for the detection of specific DNA or RNA sequences in very small samples. Recently, Sixbey et al. also used the PCR technique to detect EBV DNA in throat washings, which usually contain few cells (17).

EBV DNA in NPC tissues and in cultured cells. Three sets of primers and PCR methods were used to examine the presence of EBV DNA in NPC biopsy specimens and in primary cell cultures. A 225-bp band appeared after PCR (primer set B), indicating the presence of EBV DNA in all three NPC biopsy tissue specimens and in two primary cell cultures previously shown to contain EBV (Fig. 3A and B, lanes 4 to 6, 8, and 9) as well as in B95-8, Raji, and Namalwa cells (Fig. 3A and B, lanes 1 to 3). The hybridization band of specimen 155P (Fig. 3B, lane 9), which was derived from an EBV-positive WHO type 3 tumor, was very light. It was probably due to poor transfer of DNA from the agarose gel. This 225-bp DNA fragment was cloned into pUC18 and was demonstrated to be EBV sequence (data not shown). All NPC biopsy tissues and cultured cells derived from NPC were also analyzed with primer sets C and E by PCR. All the EBV-positive samples shared positive results and gave 316-bp (primer set C) and 454-bp (primer set E) bands. The PCR products of DNA samples positive for all three sets of primers were considered EBV positive (Table 1). The data suggested that EBV DNA was found in three of five keratinized (WHO type 1) NPC cells and in two of four moderately differentiated (WHO type 2) NPC cells (Table 1). On the other hand, EBV DNA was detected in all 41 undifferentiated (WHO type 3) NPC cells (Table 1). EBV DNA was also found in lymph node tissue of a patient with NPC as well as in other head and neck carcinoma tissues and lymphoid hyperplasia tissues (Table 1). However, EBV DNA was found in one of six normal nasopharyngeal tissue specimens and in one of four biopsy specimens of hypopharyngeal carcinoma tissues. All these findings were consistent with the recent suggestion that the permissive cell types for EBV replication also include epithelial cells (1).

EBV DNA content in NPC tissues. The amount of EBV DNA in NPC specimens that contain EBV was estimated by comparison with that in Namalwa cells, which were shown to contain two copies of EBV per cell (11), and with EBV

*Bam*HI-E DNA. The results are shown in Table 1. The amount of EBV DNA varied among NPC biopsy specimens. This variation may be due to the numbers of NPC cells in each biopsy specimen, which are different, or the actual EBV content per NPC cell. However, the fact that WHO type 3 NPC cells had the highest EBV DNA content (500 to 38,500 EBV genome equivalents per μg of DNA in 34 of 41 samples) of the three types of NPC cells coincides with the observation that the tumor cells of this type were mostly undifferentiated and mitotically active. Therefore, the EBV replication may be closely associated with the stages of cellular differentiation. Previous observations suggested that EBV gene expression in mitotically active epithelial cells was restricted to EBV-encoded nuclear antigen (EBNA1), which is required for the viral replication and partitioning of the EBV genome to daughter cells (18). It has also been reported that only EBNA1 protein can be consistently detected in NPC tissues (4). The presence of this protein in the tumor tissues presumably permits the replication of the viral genome in cells. Our data suggest that EBV replication is regulated with cell differentiation, as evidenced by the presence of significantly higher EBV DNA contents in type 3 NPCs and lower EBV DNA contents in type 1 and type 2 NPCs. These data seem to imply that EBV DNA replication was favorable in undifferentiated epithelial cells. This is in agreement with the earlier reports that expression of productive-cycle viral antigens (early antigens and viral capsid antigen) and virions were restricted to terminally differentiated cells such as desquamated epithelial cells in pharyngeal washing (16) and that EBV replication occured in undifferentiated epithelial cells (1).

The positivity of EBV DNA in hypopharyngeal carcinoma is probably not statistically different from that in WHO type 1 and 2 NPCs. Other non-NPC tumors (palate carcinoma, maxillary carcinoma, and tongue carcinoma) were classified as keratinizing squamous-cell carcinomas and were also associated with lower contents of EBV DNA in their tissues.

The amount of DNA obtained from two primary cultures (122P and 155P) was limited. Therefore, no serial dilutions of DNA were performed to determine the EBV DNA content quantitatively. Ideally, establishing an EBV-containing NPC cell line will allow us to circumvent this problem.

In summary, by using PCR we were able to demonstrate the direct detection of wide ranges of EBV DNA at low levels in three types of NPC cells. Our data also suggest that EBV replication in nasopharyngeal epithelial cells seems to be related to the differentiation stages of the NPC cells. In light of this, understanding and assessing the correlation of these points become important for understanding EBV pathogenesis. It has been suggested that uncontrolled EBV replication in the pharyngeal epithelium may be central to the evolution of some (or all) EBV-associated malignancies (1). To further examine this correlation, it seems reasonable to look at the replication-associated antigens as well as the latent proteins of EBV in different types of NPCs. Additionally, other factors (cellular or viral) in differentiated cells that may suppress EBV replication are worth investigating. Identification of these genes may be essential for understanding the role of EBV in NPC tumorigenesis. With the availability of PCR, it will be feasible to perform reverse transcription on and amplify RNA from small numbers of tumor cells.

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LITERATURE CITED

1. **Allday, M. J., and D. H. Crawford.** 1988. Role of epithelium in EBV persistence and pathogenesis of B-cell tumors. Lancet **i**:855–857.
2. **Chang, Y. S., S. Y. Lin, P. F. Lee, T. Durff, H. C. Chung, and M. S. Tsai.** 1989. Establishment and characterization of a tumor cell line from human nasopharyngeal carcinoma tissue. Cancer Res. **49**:6752–6757.
3. **Devlin, P. E., K. L. Ramachandran, and R. L. Cate.** 1988. Southern analysis of genomic DNA with unique and degenerate oligonucleotide probes: a method for reducing probe degeneracy. DNA **7**:499–507.
4. **Fahraeus, R., H. L. Fu, I. Ernberg, J. Finke, M. Rowe, G. Klein, K. Falk, E. Nilsson, M. Yadav, P. Busson, T. Tursz, and B. Kallin.** 1988. Expression of Epstein-Barr virus-encoded proteins in nasopharyngeal carcinoma. Int. J. Cancer **42**:329–338.
5. **Henle, G., W. Henle, and V. Diehl.** 1968. Relation of Burkitt's tumor associated herpes-type virus to infectious mononucleosis. Proc. Natl. Acad. Sci. USA **59**:94–101.
6. **Henle, G., W. Henle, G. Klein, P. Gunren, P. Clifford, R. Morrow, and J. Ziegler.** 1971. Antibodies to early Epstein-Barr virus induced antigens in Burkitt's lymphoma. J. Natl. Cancer Inst. **46**:861–871.
7. **Henle, W.** 1971. Evidence for a relation of the Epstein-Barr virus to Burkitt's lymphoma and nasopharyngeal carcinoma, p. 361–425. *In* W. Nakahara, K. Nishioka, T. Hirayama, and Y. Ito (ed.), Recent advances in human tumor virology and immunology. Proceedings of the 1st International Symposium of the Princess Takamatsu Cancer Research Fund. University of Tokyo Press, Tokyo.
8. **Henle, W., G. Henle, P. Burtin, Y. Cachin, P. Clifford, A. de Schryver, G. de Thé, V. Diehl, J. H. C. Ho, and G. Klein.** 1970. Antibodies to Epstein-Barr virus in nasopharyngeal carcinoma, other head and neck neoplasms and control groups. J. Natl. Cancer. Inst. **44**:225–231.
9. **Kaneko, S., S. M. Feinstone, and R. H. Miller.** 1989. Rapid and sensitive method for the detection of serum hepatitis B virus DNA using the polymerase chain reaction technique. J. Clin. Microbiol. **27**:1930–1933.
10. **Klein, G., B. C. Giovanella, T. Lindahl, R. J. Fialkow, S. Singh, and J. S. Stehlin.** 1974. Direct evidence for the presence of Epstein-Barr virus DNA and nuclear antigen in malignant epithelial cells from patients with poorly differentiated carcinoma of the nasopharynx. Proc. Natl. Acad. Sci. USA **71**:4737–4741.
11. **Lawrence, J. B., C. A. Villnave, and R. H. Singer.** 1988. Sensitive, high-resolution chromatin and chromosome mapping in situ: presence and orientation of two closely integrated copies of EBV in a lymphoma line. Cell **52**:51–61.
12. **Lindahl, T., G. Klein, B. M. Reedman, B. Johansson, and S. Singh.** 1974. Relationship between Epstein-Barr virus (EBV) DNA and the EBV-determined nuclear antigen (EBNA) in Burkitt lymphoma biopsies and other lymphoproliferative disorders. Int. J. Cancer **13**:764–772.
13. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
14. **Mills, S. E., and R. E. Fechner.** 1989. The nose, paranasal sinuses, and nasopharynx, p. 657–794. *In* S. S. Sternberg (ed.), Diagnostic surgical pathology. Raven Press, Publishers, New York.
15. **Saiki, R. K., S. Scharf, F. Faloona, K. B. Mullis, G. T. Horn, H. A. Erlich, and N. Arnheim.** 1985. Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science **230**:1350–1354.

16. **Sixbey, J. W., J. G. Nedrud, N. Raab-Traub, R. H. Hanes, and J. S. Pagano.** 1984. Epstein-Barr virus replication in oropharyngeal epithelial cells. N. Engl. J. Med. **310:**1225–1230.
17. **Sixbey, J. W., P. Shirley, P. J. Chesney, and D. M. Buntin.** 1989. Detection of a second widespread strain of Epstein-Barr virus. Lancet **ii:**761–765.
18. **Sixbey, J. W., E. H. Vesterinen, J. G. Nedrud, N. Raab-Traub, L. A. Walton, and J. S. Pagano.** 1983. Replication of Epstein-Barr virus in human epithelial cells infected *in vitro*. Nature (London) **306:**480–483.
19. **Skare, J., and J. L. Strominger.** 1980. Cloning and mapping of BamHI endonuclease fragments of DNA from the transforming B95-8 strain of Epstein-Barr virus. Proc. Natl. Acad. Sci. USA **77:**3860–3864.