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Herpesvirus RNA in Human Urogenital Tumors (41740)

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Abstract. In situ hybridization was used to examine human urogenital cancers for herpes simplex virus type 2 (HSV-2) and human cytomegalovirus RNAs. Neither of these herpesvirus RNAs was found in prostate tissues. However, HSV-2 RNA was detected in premalignant and malignant cervical tissues.

Naturally occurring and experimentally induced animal tumors can be caused by herpesviruses (1). In humans, Epstein–Barr virus is strongly associated with two human cancers: African Burkitt lymphoma and nasopharyngeal carcinoma. Analogous evidence for the involvement of herpes simplex virus (HSV) and human cytomegalovirus (HCMV) in human cancers is less complete. However, both HSV and HCMV oncogenically transform mammalian cells in culture (2–5).

The association of HSV and HCMV with the human genital tract is well documented. These viruses have been isolated from normal male and female urogenital organs as well as from cancer biopsies (6, 7). There have been sporadic reports that sera from cervical cancer patients react with a soluble antigen prepared from HSV type 2 (HSV-2)-infected cells (8, 9) and with antigens isolated from HSV-2-transformed cells (10). HSV-specific antigens have been detected in vulvar cancer cells (11, 12) and in cervical cancer cells (8, 13, 14), in which HCMV antigens have also been reported (13). Seroepidemiological and virological evidence have also implied an association between HCMV and prostate and bladder cancers (15–20) and Kaposi sarcoma (21–24).

In DNA renaturation studies with one human cervical tumor, only a portion of the HSV genome appeared to be present and transcribed (25). Within the past few years, reports of hybridization experiments suggest that HSV-specific RNA could be localized in cells from carcinomas of the cervix (26–29) and that HCMV-specific RNA could be localized in Kaposi sarcoma cells (23, 30). For HSV and cervical cancer, the percentage of positive specimens ranged from 40–70%, figures that are compatible with epidemiological evidence suggesting that there are (at least) two different and independent causes of the disease (9, 31). We undertook *in situ* hybridization experiments in an attempt to confirm and expand upon the data connecting prior HSV-infection with cervical cancer and prior HCMV-infection with prostatic cancer.

Materials and Methods. *Human tissues.* Frozen human male urogenital tissues were received from Dr. Theodore I. Malinin of the National Prostatic Cancer Project. The human female tissues, which were punch biopsies, were collected at The Milton S. Hershey Medical Center. When possible, a small sample of each of these tissues was cocultivated on Vero cells. The remainder was immediately frozen in precooled hexane (32) and stored at −70°C until embedding.

Tissues were embedded in Tissue-Tek O.C.T. (Ames Co.). Sections 12 μm thick were prepared with a cryostat, fixed on pretreated glass slides (33), dehydrated, and stored in 100% ethanol at −70°C until used in hybridization experiments.

Hybridization. For *in situ* hybridization, the sections were dried and then sequentially submersed in 0.2 *N* HCl for 20 min at room temperature, heated at 70°C for 30 min, treated with proteinase K (1 μg/ml) at 37°C for 15 min, and then dehydrated through an

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ethanol series according to the procedure of Brahic and Haase (33).

Three probes were used for *in situ* hybridization: HSV-2, HCMV, and λ DNAs. HSV-2 (strain 333) was grown on Vero cells. The virus and the virus DNA were prepared by standard procedures (34). HCMV (strain Ad169) was grown on whole human diploid fibroblast cells (Flow 5000, Flow Laboratories, Inc.) and HCMV and HCMV DNA were prepared by standard methods (35). For both HSV-2 and HCMV DNAs, the final purification was achieved by CsCl buoyant density centrifugation. The three DNAs were radiolabeled *in vitro* by nick translation (36). For most experiments, the radioactive substrates were [^{3}H]dCTP and [^{3}H]TTP (New England Nuclear Corp.), and the final specific activity was approximately 10^7 cpm/μg. In the specified experiments, the radioactive substrate was [^{125}I]dCTP (New England Nuclear Corp.), and the final specific activity was approximately 4×10^7 cpm/μg.

For each tissue section, the hybridization mixture (12 μl) consisted of 5×10^4 cpm of radiolabeled, denatured DNA in 0.01 *M* Tris, pH 7.4, 0.001 *M* EDTA, 6× SSC (1× SSC is 0.15 *M* NaCl, 0.015 *M* sodium citrate), and 50% deionized formamide, containing 100 μg/ml of denatured salmon sperm DNA. The section and hybridization mixture were covered with a siliconized coverslip, which was sealed with rubber cement. Hybridization was accomplished at 47°C for 48 hr. After hybridization, the slides were washed extensively in 2× SSC and 0.001 *M* EDTA, dehydrated through an alcohol series, and air dried. After dipping in Kodak NTB-2 emulsion, the samples were exposed in the dark at 4°C for 1 month (for the ^{3}H-labeled probes) or 24 hr (for the ^{125}I-labeled probes). After development, the samples were stained with Giemsa.

Results. *Human male urogenital tissue.* A total of 17 samples were examined: 7 normal prostates, 5 benign prostatic hyperplasias (BPH), and 5 carcinomas. A portion of each tissue was homogenized to a 10% (wet wt/vol) solution. Aliquots were inoculated onto Vero or Flow 5000 (whole human embryo) cells, which are permissive for HSV and HCMV, respectively. Neither virus was isolated from any of the tissues. This result is in agreement with the data of Baker *et al.* (37), who attempted to isolate HSV from urethral swabs and urine from 209 patients about to undergo transurethral resection of the prostate. All the cultures were negative for HSV; however, Baker *et al.* (37) did not attempt to isolate HCMV.

The probes for *in situ* hybridization (HSV-2, HCMV, and λ DNAs) were nick translated to high specific activity with [^{3}H]TTP and [^{3}H]dCTP as substrates. Each probe was tested by hybridization to uninfected and virus-infected cells. Table I shows the grain counts from a representative set of control experiments. Following the procedures of Brahic and Haase (33), we routinely achieved low background as well as high specificity (Table I).

There were no cells dramatically positive for virus RNA with either HSV or HCMV DNA probes in any of the 17 male tissues examined. Therefore, for each tissue section, silver grains were counted over 10 random

TABLE I. GRAIN COUNTS FOR CONTROL *IN SITU* HYBRIDIZATION EXPERIMENTS

Probes	HSV-2-infected Vero cells[a]	HCMV-infected Flow 5000 cells[b]	Uninfected cells
HSV-2 DNA	264 ± 38	N.D.[c]	6.4 ± 1.7
HCMV DNA	N.D.	234 ± 39	5.1 ± 1.5
λ DNA	7.1 ± 1.8	6.8 ± 1.3	N.D.

Note. Grains were counted over 10 random fields, each containing (approximately) 50 cells. The average value and standard deviation of grains/field were calculated. Note that the grain counts for ^{3}H-labeled λ DNA hybridized to virus-infected cells were virtually the same as for ^{3}H-labeled virus DNAs hybridized to uninfected cells. Exposure time was 3 months. The low average value of (approximately) 5 grains/cell reflected the low multiplicity of infection (MOI) (many cells were uninfected) and the fixation at an early time postinfection (pi).

[a] MOI = 0.2; cells fixed at 8 hr pi.

[b] MOI = 0.2; cells fixed at 40 hr pi.

[c] Not done.

fields, each containing (approximately) 50 epithelial cells. This procedure is entirely analogous to the one Eglin *et al.* (28) successfully employed in related experiments. Table II presents examples of the data expressed as average plus-or-minus standard deviation of grains per field. By statistical analyses, none of the 17 male tissues was positive for HSV-2 or HCMV RNA.

Human female cervical tissue. All of the female cervical samples were punch biopsies, which were inherently small. Most of the tissue was sent to the pathology laboratory for histological examination and diagnosis. Therefore, the remaining tissue samples for our experiments were extremely minute. Because of the limited amount of tissue in each biopsy, virus isolation by cocultivation could be attempted in about half of the cases. These were negative for HSV.

For the first six samples, there was sufficient tissue to use both HSV-2 and HCMV DNAs as probes, as well as to test the effect of prior RNase treatment upon hybridization. Two observations important to subsequent experiments were made. (i) HCMV DNA hybridization was uniformly negative. To save tissue, further hybridization with an HCMV DNA probe was not undertaken. (ii) No dramatically HSV-2 RNA-positive cells (which would appear as many silver grains/cell) were seen.

TABLE II. EXAMPLE OF GRAIN COUNTS FOR EACH TYPE OF MALE TISSUE

Probe	Tissue		
	Normal	BPH[a]	Carcinoma
HSV-2 DNA	1.0 ± 0.5	1.4 ± 1.1	0.4 ± 0.7
RNase/HSV-2 DNA[b]	0.8 ± 0.8	1.1 ± 1.0	1.0 ± 0.8
HCMV DNA	0.5 ± 0.5	0.7 ± 0.7	1.0 ± 1.0
RNase/HCMV DNA[b]	0.5 ± 0.7	0.5 ± 0.7	0.5 ± 0.5
λ DNA	0.4 ± 0.5	1.1 ± 1.0	1.0 ± 0.9
Reaction mix[c]	0.6 ± 1.0	1.3 ± 1.0	1.0 ± 1.1

Note. Each column in the "Tissue" category represents an example of one biopsy. Sequential cryostat sections of that one biopsy were hybridized to the individual probes listed on the left in the column category "Probe." The probes were ^{3}H-labeled. Grains were counted over 10 random fields, each containing (approximately) 50 epithelial cells. The average value and standard deviation of grains/field were calculated. Exposure time was 3 months.

[a] Benign prostatic hyperplasia.

[b] Tissue section treated with RNase before hybridization.

[c] No radioactive probe added to hybridization mixture.

Therefore, grains were counted in 10 random fields containing (approximately) 50 epithelial cells per field. To measure nonspecific binding, grains were counted in 10 random fields of adjacent glass slide, where the results were uniformly 1 ± 1 grain/field. As one negative control, grains were counted over 10 random fields containing (approximately) 50 normal cells in nonepithelial, connective tissue. A second negative control was to count grains in 10 random fields of epithelial cells from a subsequent section hybridized to λ DNA. Thus, there were always two negative controls: identical HSV-2 DNA probe hybridized to epithelial and connective cells in two different regions of the same tissue section, and λ DNA probe hybridized to epithelial cells in a subsequent tissue section. In addition, when a sufficient amount of tissue permitted, a section was treated with pancreatic RNase prior to hybridization, providing an abolished positive control. If the silver grains represented HSV RNA, then prior RNase treatment should have prevented hybridization and, therefore, silver grain formation.

Representative grain counts from the initial experiments are presented in Table III. For the HSV-2 DNA probe, RNase treatment prior to hybridization reduced the grain count to background level (equivalent to the λ DNA probe hybridization). These data demonstrate specificity. Statistical analysis by the Student's *t* test was used to compare the grain count achieved with the HSV-2 DNA probe with the grain counts for both negative controls. The epithelial layer was considered to contain HSV-2 RNA when the analysis of its grain count gave values of $P \leq 0.05$ against both negative controls. This criterion was chosen to avoid a false-positive result. The result of the statistical analysis was confirmed by comparison to the grain count for the abolished positive control (RNase pretreatment; Table III), when there was sufficient tissue to undertake this control.

To achieve a higher signal-to-background ratio, the HSV-2 DNA probe was radiolabeled *in vitro* by nick translation with [^{125}I]dCTP as the radioactive substrate. The rest of the *in situ* hybridization reaction was held constant. However, instead of the 4-week exposure time required for the ^{3}H-labeled probes, the ^{125}I-labeled probes required only a 24-hr exposure.

TABLE III. EXAMPLE OF GRAIN COUNTS FOR DIFFERENT TYPES OF CERVICAL TISSUE

Probe	Tissue		
	Dysplasia	Dysplasia	Carcinoma *in situ*
HSV-2 DNA	15.2 ± 7.0	14.1 ± 7.6	17.9 ± 7.8
RNase/HSV-2 DNA[a]	7.1 ± 2.6	5.1 ± 2.5	8.3 ± 1.6
HCMV DNA	6.1 ± 3.7	2.5 ± 1.3	7.1 ± 1.8
RNase/HCMV DNA[a]	5.7 ± 2.3	4.5 ± 1.5	N.D.[b]
λ DNA[c]	5.6 ± 2.6	3.6 ± 2.3	6.8 ± 2.9
HSV-2 DNA/normal cells[d]	7.8 ± 2.2	5.6 ± 1.5	5.7 ± 2.2

Note. Each column in the "Tissue" category represents one biopsy. Sequential cryostat sections of that biopsy were hybridized to the individual probes listed on the left in the column category "Probe." Grain counts represent the average value and standard deviation of grains/field for 10 random fields. The probes were ^{3}H-labeled. Exposure time was 3 months.

[a] RNase treatment prior to hybridization.

[b] Not done, insufficient tissue.

[c] Counted over epithelial cells in subsequent section.

[d] Same tissue section as "HSV-2 DNA" but counted over cells in normal, connective tissue.

Representative grain counts are presented in Table IV. There was an improvement in the signal-to-background ratio with the ^{125}I-labeled probe compared with the ^{3}H-labeled probe; however, the background increased (compare Tables III and IV).

The results for the female tissues are compiled in Table V. The category of "dysplasia" includes mild, moderate, and severe dysplasia. Five of six samples were positive for the presence of HSV RNA. The category "carcinoma" includes carcinoma *in situ* and invasive carcinoma. Four of six samples were positive for the presence of HSV RNA. Overall, 9 of 12 (75%) premalignant and malignant human cervical tissues were positive for HSV RNA.

TABLE IV. EXAMPLES OF GRAIN COUNTS FOR THE ^{125}I-LABELED PROBE AND HUMAN CERVICAL TISSUE

Probe	Tissue	
	Dysplasia	Atypia
HSV-2 DNA	85.6 ± 12.1	94.7 ± 12.8
λ DNA[a]	49.3 ± 11.2	47.3 ± 8.5
HSV-2 DNA/normal cells[b]	55.5 ± 9.5	50.8 ± 13.5

Note. Each column in the "Tissue" category represents one biopsy, as an example. Sequential cryostat sections of that biopsy were hybridized to HSV-2 and λ DNAs. Grain counts are presented as the average value and standard deviation of grains/field for 10 random fields. Exposure time was 24 hr.

[a] Counted over epithelial cells in subsequent section.

[b] Same tissue section as "HSV-2 DNA" but counted over cells in normal, connective tissue.

Discussion. McDougall *et al.* (26, 27) reported finding dramatically positive, multigrain cells in tissues containing cells undergoing premalignant changes and in tissues containing malignant cells. In sharp contrast, Eglin *et al.* (28) found no dramatically positive cells, with the exception of three cases where HSV was isolated. By grain counts and statistical analyses, Eglin *et al.* (28) detected HSV RNA in premalignant and malignant cells. The *in situ* hybridization data of Maitland *et al.* (29) are difficult to interpret because the authors acknowledge problems with nonspecific binding of probe DNA. In addition,

TABLE V. SUMMARY OF *IN SITU* HYBRIDIZATION RESULTS FOR HUMAN CERVICAL TISSUE

Tissue	Probe	
	HSV-2 DNA	HCMV DNA
Dysplasia	5/6[a]	0/2
Carcinoma	4/6	0/4
Total	9/12[b]	0/6

[a] Number positive, by statistical analysis of grain count, divided by number of samples. Combined data for both ^{3}H-labeled and ^{125}I-labeled probes.

[b] In addition, one sample of cervical ectropion koilocytotic atypia was HSV grain-positive; one sample of hyperkeratosis was negative; and one sample of normal cervix was negative.

Maitland *et al.* (29) reported binding of adenovirus type 2 DNA to tissues. These data were directly contradicted by the results of Eglin *et al.* (28). Most curious was the observation that all the tissues examined by Maitland *et al.* (29) that were positive for adenovirus DNA binding were also positive for HSV DNA binding; not one tissue positive for adenovirus DNA was negative for HSV DNA. Our data (Tables III and IV) are in excellent twofold agreement with the results of Eglin *et al.* (28): (i) no dramatically positive hybridization of HSV-2 DNA was detected; but (ii) by statistical analysis, hybridization of HSV-2 DNA was detected in 75% of premalignant and malignant tissues. Thus, four independent groups detected selective hybridization of HSV-2 DNA to the RNA of dysplastic and malignant cells compared with normal cervical cells ((26–29); this work).

A fundamental question is whether the detected hybridization of HSV-2 DNA was to HSV RNA or normal cell RNA. Maitland *et al.* (29) reported that RNA from human placenta hybridized to limited regions (principally the "joint region" and physical ends) of HSV-1 and HSV-2 DNAs. Blot hybridization experiments by Peden *et al.* (38) and Puga *et al.* (39) suggest that there is homology between the repeated sequences of HSV DNA and normal human cell DNA. All these data suggest, but do not prove, that a limited portion of the HSV genome may share base sequences with normal human cell DNA. Thus, it could be argued that the HSV-2 DNA probe hybridized to HSV RNA or to normal cell RNA or to both. For the two latter possibilities, it would have to be argued further that the HSV-cross-hybridizing normal cell RNA was expressed in greater abundance in the epithelial cells than in the connective tissue cells (Tables III and IV). The technique of *in situ* hybridization cannot distinguish these different possibilities easily, if at all.

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1. Rapp F. Oncogenic Herpesviruses. Boca Raton, CRC Press, Vols I and II, 1980.
2. Duff R, Rapp F. Oncogenic transformation of hamster cells after exposure to herpes simplex virus type 2. Nature New Biol **233**:48–50, 1971.
3. Duff R, Rapp F. Oncogenic transformation of hamster embryo cells after exposure to herpes simplex virus type 1. J Virol **12**:209–217, 1973.
4. Albrecht T, Rapp F. Malignant transformation of hamster embryo fibroblasts following exposure to ultraviolet-irradiated human cytomegalovirus. Virology **55**:53–61, 1973.
5. Geder L, Lausch R, O'Neill F, Rapp F. Oncogenic transformation of human embryo lung cells by human cytomegalovirus. Science **192**:1134–1137, 1976.
6. Amstey M. Genital herpesvirus infection. Clin Obstet Gynecol **18**:89–100, 1975.
7. Melnick J, Lewis R, Wimberly I, Kaufman R, Adam E. Association of cytomegalovirus (CMV) infection with cervical cancer: Isolation of CMV from cell cultures derived from cervical biopsy. Intervirology **10**:115–119, 1978.
8. Aurelian L, Davis H, Julian C. Herpesvirus type 2-induced, tumor-specific antigen in cervical carcinoma. Amer J Epidemiol **89**:1–9, 1973.
9. Gilman S, Docherty J, Clarke A, Rawls W. Reaction patterns of herpes simplex virus type 1 and type 2 proteins with sera of patients with uterine cervical carcinoma and matched controls. Cancer Res **40**:4640–4647, 1980.
10. Notter M, Docherty J. Reactions of antigens from herpes simplex virus transformed cells with sera from squamous cell carcinoma patients. Cancer Res **36**:4394–4401, 1976.
11. Kaufman R, Dreesman G, Burek J, Korhonen M, Matson D, Melnick J, Powell K, Purifoy D, Courtney R, Adam E. Herpesvirus-induced antigens in squamous-cell carcinoma in situ of the vulva. N Engl J Med **305**:483–488, 1981.
12. Cabral G, Marciano-Cabral F, Fry D, Lumpkin C, Mercer L, Goplerud D. Expression of herpes simplex virus type 2 antigens in premalignant and malignant human vulvar cells. Amer J Obstet Gynecol **143**:611–619, 1982.
13. Pacsa A, Kummerländer L, Pejtsik B, Krommer K, Pali K. Herpes simplex virus-specific antigens in exfoliated cervical cells from women with and without cervical anaplasia. Cancer Res **36**:2130–2132, 1976.
14. Dreesman G, Burek J, Adam E, Kaufman R, Melnick J, Powell K, Purifoy D. Expression of herpesvirus-induced antigens in human cervical cancer. Nature (London) **283**:591–593, 1980.
15. Geder L, Sanford E, Rohner T, Rapp F. Cytomegalovirus and cancer of the prostate: *In vitro* transformation of human cells. Cancer Treat Rep **61**:139–146, 1977.
16. Sanford E, Dagen J, Geder L, Rohner T, Rapp F.

Lymphocyte reactivity against virally transformed cells in patients with urologic cancer. J Urol **118**:809–810, 1977.

17. Sanford E, Geder L, Laychock A, Rohner T, Rapp F. Evidence for the association of cytomegalovirus with carcinoma of the prostate. J Urol **118**:789–792, 1977.
18. Sanford E, Geder L, Dagen J, Laychock A, Rohner T, Rapp F. Humoral and cellular immune response of prostatic cancer patients to cytomegalovirus-related antigens of cytomegalovirus-transformed human cells. J Surg Res **24**:404–408, 1978.
19. Dagen J, Sanford E, Rohner T, Geder L, Rapp F. Recognition of virally transformed cells by lymphocytes from patients with prostatic cancer. Urology **12**:532–536, 1978.
20. Laychock A, Geder L, Sanford E, Rapp F. Immune response of prostatic cancer patients to cytomegalovirus-infected and -transformed human cells. Cancer **42**:1766–1771, 1978.
21. Giraldo G, Beth E, Haguenau F. Herpes-type virus particles in tissue culture of Kaposi's sarcoma from different geographic regions. J Natl Cancer Inst **49**:1509–1526, 1972.
22. Giraldo G, Beth E, Kourilsky F, Henle W, Henle G, Mike V, Hurant J, Anderson H, Gharbi M, Kyalwazi S, Puissent A. Antibody patterns to herpesviruses in Kaposi's sarcoma: Serological association of European Kaposi's sarcoma with cytomegalovirus. Int J Cancer **15**:839–848, 1975.
23. Giraldo G, Beth E, Huang E-S. Kaposi's sarcoma and its relationship to cytomegalovirus (CMV). III. CMV DNA and CMV early antigens in Kaposi's sarcoma. Int J Cancer **26**:23–29, 1980.
24. Siegal F, Lopez C, Hammer G, Brown A, Kornfeld S, Gold J, Hassett J, Hirschman S, Cunningham-Rundles C, Adelsberg B, Parham D, Siegal M, Cunningham-Rundles S, Armstrong D. Severe acquired immunodeficiency in male homosexuals, manifested by chronic perianal ulcerative herpes simplex lesions. N Engl J Med **305**:1439–1444, 1981.
25. Frenkel N, Roizman B, Cassai E, Nahmias A. A DNA fragment of herpes simplex 2 and its transcription in human cervical cancer tissue. Proc Natl Acad Sci USA **69**:3784–3789, 1972.
26. McDougall J, Galloway D, Fenoglio C. Cervical carcinoma: Detection of herpes simplex virus RNA in cells undergoing neoplastic change. Int J Cancer **25**:1–8, 1980.
27. McDougall J, Crum C, Fenoglio C, Goldstein L, Galloway D. Herpesvirus-specific RNA and protein in carcinoma of the uterine cervix. Proc Natl Acad Sci USA **79**:3853–3857, 1982.
28. Eglin R, Sharp F, MacLean A, Macnab J, Clements J, Wilkie N. Detection of RNA complementary to herpes simplex virus DNA in human cervical squamous cell neoplasms. Cancer Res **41**:3597–3603, 1981.
29. Maitland N, Kinross J, Busuttil A, Ludgate S, Smart G, Jones K. The detection of DNA tumour virus-specific sequences in abnormal human cervical biopsies by *in situ* hybridization. J Gen Virol **55**:123–137, 1981.
30. Boldogh I, Beth E, Huang E-S, Kyalwazi S, Giraldo G. Kaposi's sarcoma. IV. Detection of CMV DNA, CMV RNA and CMNA in tumor biopsies. Int J Cancer **28**:469–474, 1981.
31. Ashley D. The biological status of carcinoma *in situ* of the uterine cervix. J Obstet Gynaecol Brit Common **73**:372–381, 1966.
32. Chayen J, Bitensky L, Butcher R. Practical Histochemistry. London, Wiley, pp1–8, 1973.
33. Brahic M, Haase A. Detection of viral sequences of low reiteration frequency by *in situ* hybridization. Proc Natl Acad Sci USA **75**:6125–6129, 1978.
34. Ecker JR, Hyman RW. Variables that may influence the alkaline sedimentation pattern of herpes simplex virus DNA. Arch Virol **68**:221–228, 1981.
35. Stinski M, Mocarski E, Thomsen D. The DNA of human cytomegalovirus: Size heterogeneity and defectiveness resulting from serial undiluted passage. J Virol **31**:231–239, 1979.
36. Rigby P, Dieckmann M, Rhodes C, Berg P. Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J Mol Biol **113**:237–251, 1977.
37. Baker L, Mebust W, Chin T, Chapman A, Hinthorn D, Towle D. The relationship of herpesvirus to carcinoma of the prostate. J Urol **125**:370–374, 1981.
38. Peden K, Mounts P, Hayward G. Homology between mammalian cell DNA sequences and human herpesvirus genomes detected by a hybridization procedure with high-complexity probe. Cell **31**:71–80, 1982.
39. Puga A, Cantin E, Notkins A. Homology between murine and human cellular DNA sequences and the terminal repetition of the S component of herpes simplex virus type 1 DNA. Cell **31**:81–87, 1982.

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