American Journal of Pathology, Vol. 138, No. 6, June 1991

Abundant Expression of EBER1 Small Nuclear RNA in Nasopharyngeal Carcinoma

A Morphologically Distinctive Target for Detection of Epstein–Barr Virus in Formalin-fixed Paraffin-embedded Carcinoma Specimens

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The highly restricted expression of the Epstein–Barr virus (EBV) genome in malignancy has limited the use of EBV detection methods applicable to formalin-fixed paraffin-embedded carcinoma specimens. In EBV-transformed lymphocytes very short nonprotein coding EBV transcripts (EBERs) are expressed in much higher abundance (10^7 copies per cell) than other EBV latency transcripts. Using a ^3H riboprobe, the authors demonstrated EBER1 expression in NASOPHARYNGEAL CARCINOMAS (NPCs) as well as in parotid salivary gland. Recognition of EBER1 expression was facilitated by the intensity of hybridization and its characteristic morphology (nuclear with nucleolar sparing). EBER1 expression was not demonstrated in other epithelial malignancies arising from mucosal surfaces (oropharynx, uterine cervix) from which EBV shedding has been detected. Repeat study of the NPC specimens with digoxigenin-labeled probe yielded hybridization signal with subcellular morphologic detail and without background in a 12-hour procedure. Thus the EBER1 transcript is an appropriate target for* in situ *hybridization detection of EBV in formalin-fixed paraffin-embedded carcinoma specimens. (Am J Pathol 1991, 138:1461–1469)

Epstein–Barr virus (EBV) is a ubiquitous virus that replicates in the mucosal epithelium of the parotid glands, oropharynx, and uterine cervix.[1] Epstein–Barr virus is consistently detected in nasopharyngeal carcinoma by nucleic acid hybridization and antigen detection.[2–8] The application of EBV detection methods to carcinoma specimens has been limited, however, by the highly restricted expression of the EBV genome in malignancy. Only two viral proteins are known to be expressed in nasopharyngeal carcinomas (NPC), the EB nuclear antigen 1 (EBNA-1) and the latency membrane protein (LMP).[8] Methods to detect these antigens in carcinoma have been reliably applied only to fresh or snap-frozen tissues. *In situ* hybridization to detect EBV nucleic acid in carcinoma also has been applied, but is technically demanding because of the small number of viral genomes or commonly targeted transcripts per cell.

Study of EBV Burkitt's cell lines originally demonstrated the existence of very short RNA transcripts whose level of expression was orders of magnitude greater than the expression of other EBV latency transcripts (up to 10^7 copies per cell).[9–11] In contrast to the EBNA-1 and LMP genes, these EB early RNAs (EBERs) do not code for protein. The EBER1 and 2 genes also differ from other EBV genes in their small size, 165 and 169 nucleotides, respectively.[12] The function of the EBERs is unknown, but we recently have demonstrated their utility as targets for *in situ* hybridization in formalin-fixed tumor tissue of lym-

Supported by ACS PDT405 and NIH K11 AI00648-03 (to R. F. Ambinder), NIH RO1 CA42245 (to S. D. Hayward), Caswell Caplan, and the Hodson Trust.

Accepted for publication February 12, 1991.

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phohematopoietic origin (to detect EBV in Reed-Sternberg cells in some cases of Hodgkin's disease).[13]

We report here that the EBER1 is expressed in some EBV-infected epithelial tissues, including most, if not all, nasopharyngeal carcinomas, and is readily detected in routinely prepared formalin-fixed paraffin-embedded sections. We used the EBER1 antisense probe to investigate the presence of EBV in a sampling of other squamous cell or undifferentiated epithelial malignancies arising from mucosal surfaces (tongue, larynx, and uterine cervix) from which EBV shedding has been detected.

Methods

In Situ *Hybridization*

The *in situ* hybridization method has been described previously.[13,14] Five-micron–thick tissue sections were floated in a bath of distilled water onto acid-cleaned 3-aminopropyltriethoxysilane–coated slides and heated in a 65°C incubator. They were dewaxed in xylene and rehydrated in serial graded ethanol washes (100%, 95%, 70%), then digested with proteinase K (20 μg/ml) for 30 minutes at 37°C. Slides were treated with triethanolamine, acetic anhydride, and dehydrated again in serial graded ethanol washes (70%, 95%, 100%). The ^{3}H-labeled riboprobes (5×10^6 cpm) or digoxigenin-labeled riboprobes were applied in formamide solution (50% formamide, 0.1 mol/l [molar] Pipes, pH 7.8, and 0.01 ethylinediaminetetra-acetic acid [EDTA]) in a volume, usually 20 μl, sufficient to cover the section. An acid-washed, siliconized coverslip was placed over the section and sealed with rubber cement. The slide was hybridized for 6 hours at 50°C. After hybridization, the slides were submerged in 4 × SSC (0.6 mol/l sodium chloride, 0.06 mol/l sodium citrate) and the coverslips removed. The tissue sections were washed at room temperature (RT) with frequent changes of 1 × SSC for 30 minutes and 0.1 × SSC for 15 minutes. The slides were incubated in 10 μg/ml RNAse A in 2 × SSC at 37°C for 15 minutes and then dehydrated through graded ethanols. Air-dried slides hybridized with ^{3}H label were dipped in the dark in Kodak NTB-2 liquid emulsion diluted 1:1 with 0.6 mol/l ammonium acetate and exposed in a light-tight container at 4°C for 1 week. Slides were developed in Kodak D-19, fixed in 30% sodium thiosulfate, and stained with hematoxylin and eosin. Air-dried slides hybridized with digoxigenin label were incubated in buffer 1 (100 mmol/l [millimolar] TRIS HCl, 150 mmol/l NaCl, pH 7.5) for 1 minute at RT, washed in buffer 1 containing 2% normal sheep serum and 0.3% Triton X-100, incubated with anti-digoxigenin antibody-conjugate (1:500 with buffer 1 containing 1% normal sheep serum and 0.3% Triton X-100 for 3 hours at RT, washed twice in buffer 1 and equilibrated for 10 minutes with buffer 2 (100 mmol/l TRIS-HCl; 100 mmol/l NaCl; 50 mmol/l $MgCl_2$, pH 9.5). Color solution was prepared with 45 μl nitrobluetetrazolium salt (NBT) solution and 35 μl X-phosphate solution (both from the Boehringer digoxigenin kit) added to 10 ml of buffer 2. The reaction was completed after 1 hour and the reaction was stopped by washing the slides for 5 minutes with buffer 3 (10 mmol/l TRIS-HCl, pH 8.0, 1 mmol/l EDTA). Slides were counterstained with aqueous eosin solution.

Riboprobes

Recombinant plasmids with the B95–8 strain EBV large internal repeat (IR) subcloned into the Bluescribe M13 + vector (Stratagene) as *Bam*HI fragments in both sense (IR+) and antisense (IR−) orientations relative to the T7 polymerase were constructed. Recombinant plasmids with the EBER1 sequences subcloned into pGEM Blue (Promega) in both sense (EBER1+) or antisense (EBER1−) orientations relative to the T7 and or SP6 polymerase promoter were provided by G. Howe.[15] The EBER plasmids contained 89% of the EBER1 gene (148 nt). Plasmids were linearized and transcribed *in vitro* in the presence of ^{3}H-UTP and ^{3}H-ATP or digoxigenin-UTP (Boehringer). In those reactions where ^{3}H was used, only those reactions in which >75% incorporation was achieved were used for hybridizations. In those reactions using digoxigenin, 1% of the transcription products of 1 μg of EBER plasmid DNA and 0.2 ng of digoxigenin-labeled RNA (provided as a control by the manufacturer) were compared by dot-blot hybridization. In each case the signal associated with the EBER digoxigenin RNA was comparable to or exceeded the digoxigenin control RNA supplied by the manufacturer.

Analysis

Nasopharyngeal carcinoma was graded according to standard histopathologic criteria. Cases with the typical lymphoepithelioma histology were classified as undifferentiated. Those showing squamous differentiation were classified according to level of differentiation. Cells were considered to show specific hybridization if the deposition of grains over an individual neoplastic cell was predominantly nuclear and showed nucleolar sparing.

Results

Nine specimens from six patients with primary NPC were studied with the EBER1 sense and antisense probes (Ta-

ble 1). In each specimen of primary NPC, the EBER1 antisense probe produced hybridization signal associated with the malignant epithelial cells (Figure 1A, B). In some specimens the signal was quite intense and in others relatively sparse. There was considerable variation in hybridization intensity among the malignant cells of a given specimen. In one case, the majority of cells were hybridization negative. In each case, hybridization signal was predominantly nuclear with relative nucleolar sparing (Figure 1B). With the EBER sense probes there was no hybridization signal, thus indicating that the EBER antisense probes were detecting RNA (not shown).

Areas of metaplastic or normal respiratory epithelium in NPC specimens did not hybridize with the EBER1 antisense probe. Similarly tumor-infiltrating lymphocytes did not hybridize with the EBER1 antisense probe. In specimens with tumor metastatic to lymph nodes, hybridization was limited to the malignant cells and not associated with lymphocytes.

Fifteen epithelial tumors from the aerodigestive tract, including five metastatic tumors to lymph nodes, also were studied. Included in this series were squamous cell carcinomas of the tongue, oropharynx, larynx, and lung as well as undifferentiated carcinoma of the larynx (laryngeal lymphoepithelioma). Similarly, 10 cervical carcinomas including one cervical lymphoepithelioma were studied. Hybridization was not seen in either the malignant epithelial cells or normal tissues in these specimens. Hybridization was seen in morphologically normal parotid salivary gland tissue from a patient with Hodgkin's disease (Figure 2).

For comparison with the results of using the EBER1 antisense probe, four specimens were studied with the large internal repeat antisense probe (Figure 3). This probe was much more inconsistent in its results. In the best case, there was diffuse cytoplasmic hybridization with malignant epithelial cells. The intensity of signal with comparably labeled probe was less than that associated with the EBER1 antisense probe and the differentiation of signal from background was more difficult.

Nasopharyngeal carcinomas specimens previously studied with ^{3}H-labeled probe were subsequently studied with digoxigenin-labeled EBER1 antisense probes (Figure 4). The results were similar to those obtained with the ^{3}H label, but the background disappeared entirely. Whereas in the ^{3}H-labeled hybridization, grains were predominantly nuclear with nucleolar sparing, in the digoxigenin-labeled specimens, signal was exclusively nuclear with nucleolar sparing (Figure 4C, D). The signal appeared clumped and marginated against the nuclear membrane and nucleolus. The nucleolus itself and areas of nucleoplasm were devoid of signal. Some of the malignant cells hybridized intensely with the EBER1 probe, whereas other, immediately adjacent, carcinoma cells were entirely negative (Figure 4C). The absence of background, and the distinctive morphologic signal made it possible to recognize a positive specific signal in one metastatic NPC specimen, which had been interpreted initially as showing only background hybridization with ^{3}H-labeled probe (Table 1). In addition, the hybridization and development procedures were carried out in a day.

Discussion

Epstein–Barr virus has been detected previously by *in situ* hybridization in epithelial tissues, including desquamated oral epithelium from patients with infectious mononucleosis,[16,17] in the stratified squamous epithelium in the skin,[18] in the stratified squamous epithelium of the cervix,[19] as well as in nasopharyngeal carcinoma and in

Table 1.

Case	Location	(P)rimary, (D)irect Extension or (M)etastatic	Pathology	Hybridization
1	Nasopharynx	P	Undifferentiated carcinoma	Positive
2	Nasopharynx	P	Undifferentiated carcinoma	Positive
3	Nasopharynx	P	Poorly differentiated squamous carcinoma	Positive
3	Cervical node	M	Poorly differentiated squamous carcinoma	Positive
3	Nasopharynx	D	Poorly differentiated squamous carcinoma	Positive
4	Cervical node	M	Undifferentiated	Positive*
5	Chest wall	M	Poorly differentiated squamous carcinoma	Positive
5	Cervical node	M	Poorly differentiated squamous carcinoma	Positive
6	Maxillary sinus	D	Moderately differentiated squamous carcinoma	Positive

* Positivity only recognized with digoxigenin-labeled probe.

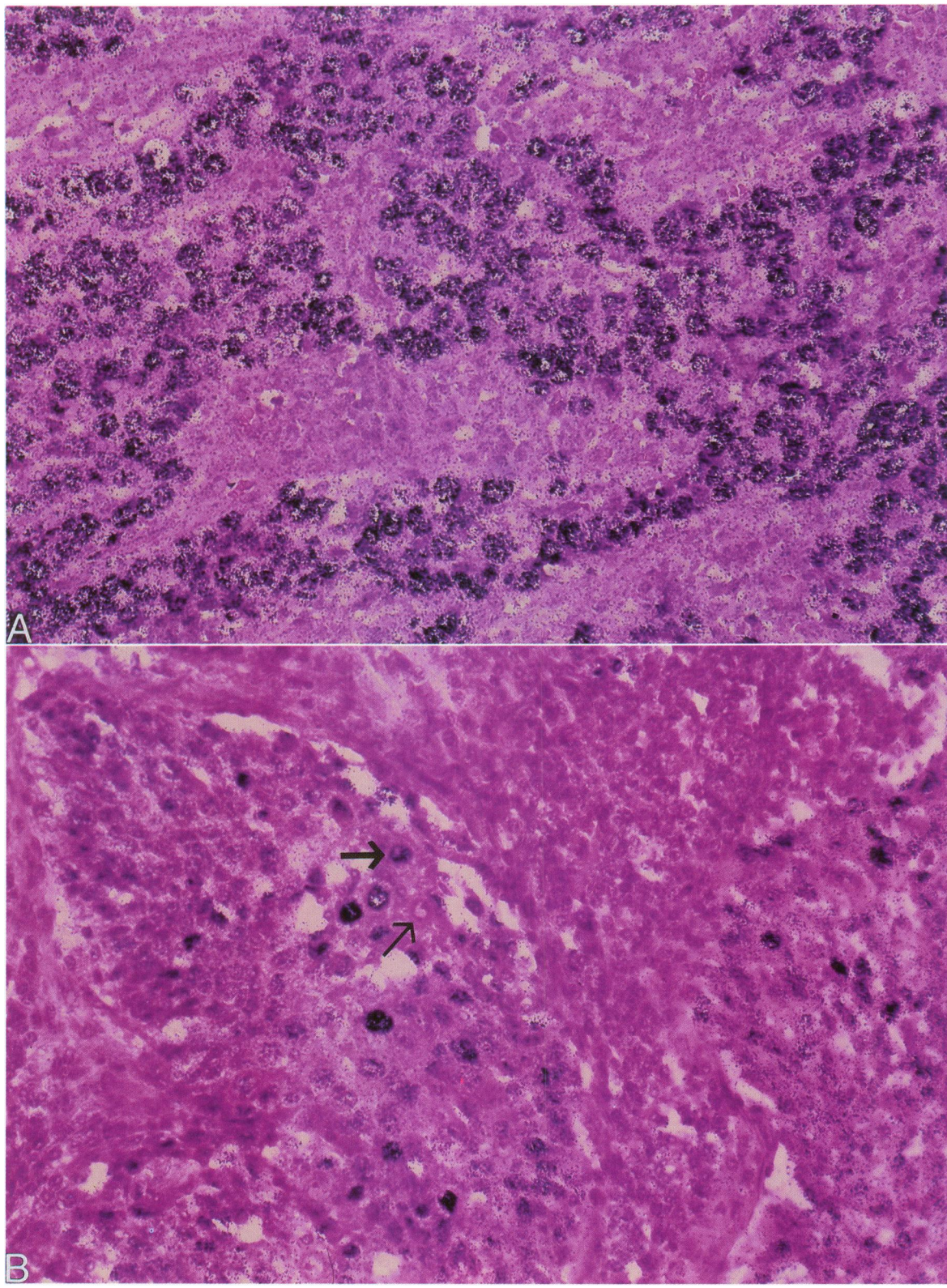
A
B

Figure 1. In situ *hybridization of NPC specimens with ^{3}H-labeled EBER1 antisense probe.* **A:** *Primary NPC showing intense hybridization with most tumor cells but weak or absent hybridization with others.* **B:** *NPC metastatic to lymph node. Many tumor cells show characteristic nuclear localization. In some tumor cells, nuclear sparing can beappreciated (thick arrow). Other tumor cells were not associated with EBER1 signal (thin arrow). Lymphocytes in the middle of the field were entirely negative.*

certain other lymphoepitheliomalike epithelial neoplasms arising in salivary glands, thymus, and lung.[20–24]

In general, *in situ* investigations of EBV in epithelial malignancies have used the large internal repeat as probe and, with a single exception,[20] have used fresh or snap-frozen,[21,22] but not formalin-fixed paraffin-embedded tissue. The large internal repeat probe has been applied to the study of formalin-fixed paraffin-embedded lymphohematopoietic malignancies by several investigators.[25–28] Very sensitive single-copy nonradiometric detection of EBV in lymphoblastoid cell lines using the large internal repeat probe also has been reported.[29,30]

Sensitive *in situ* hybridization detection of viral cellular genes may be facilitated by large hybridization targets.[29] The EBV large internal repeat probe targets DNA sequences between 10 and 40 thousand nucleotides in length.[30] The EBER genes, which are less than 170 nucleotides in length, thus are orders of magnitude smaller than the conventional targets of EBV *in situ* hybridization.

We investigated EBER transcripts as appropriate targets for *in situ* hybridization, despite their small size, for two reasons. First they exist as ribonucleoproteins complexed with the cellular protein La with extensive intramolecular base pairing and stable secondary structure.[9,11] As such, they may be more resistant to nuclease degradation than other transcripts and thus be especially useful in the investigation of routinely prepared formalin-fixed paraffin-embedded clinical specimens. Second their abundance in latently infected lymphoid cells suggested that any loss of sensitivity because of their small size might be compensated for by their high copy number.

Howe and Steitz[15] first used EBER antisense transcripts for *in situ* hybridization in a study aimed at determining the subcellular distribution of EBER transcripts in lymphocytes.[15] They demonstrated that the EBER1 and 2 transcripts are localized to the nucleus sparing the nucleolus.

We have previously used the EBER1 antisense probe for *in situ* hybridization of Hodgkin's disease.[13] In that study, the probe was sensitive and specific, detecting EBV in Reed–Sternberg cells and their variants but not in the polymorphous benign infiltrate.

The function of the EBERs and the significance of nuclear localization with nucleolar sparing remains unknown. Because this subcellular distribution is not a characteristic of background or nonspecific hybridization, however, its recognition facilitates diagnostic interpretation. Background is absent and the distinctive subcellular distribution of signal is most readily appreciated with digoxigenin-labeled probes.

EBERs have primary sequence homology with the adenovirus VA1 and VA2 small RNAs and the cellular U6 small RNA. Under the hybridization conditions used, this homology does not lead to cross-hybridization in epithelial, lymphohematopoietic,[13] and neural tissues (MacMahon et al, manuscript submitted for publication) or in human EBV-negative lymphoid or epithelial cell lines. Howe and Steitz similarly described an absence of cross-hybridization to cellular RNAs.[15]

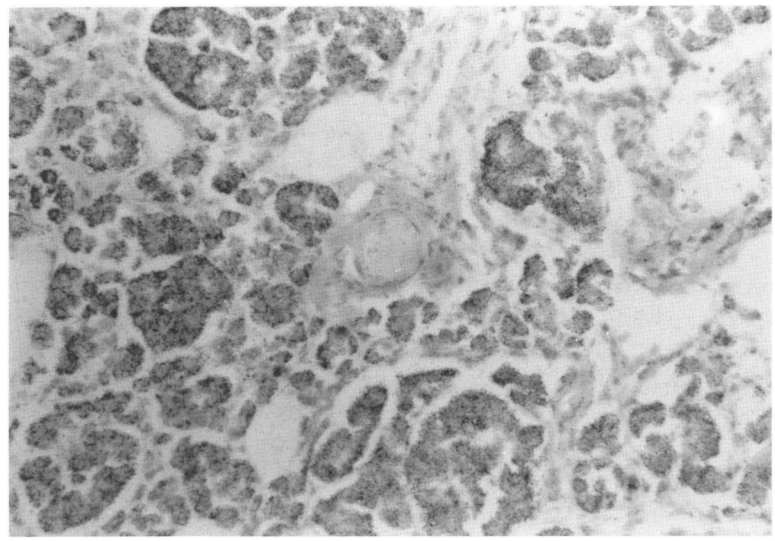

Figure 2. In situ *hybridization with ^{3}H-labeled EBER1 antisense probe of parotid salivary gland from a patient with Hodgkin's disease without neoplastic involvement. Hybridization is seen in the acini of the gland.*

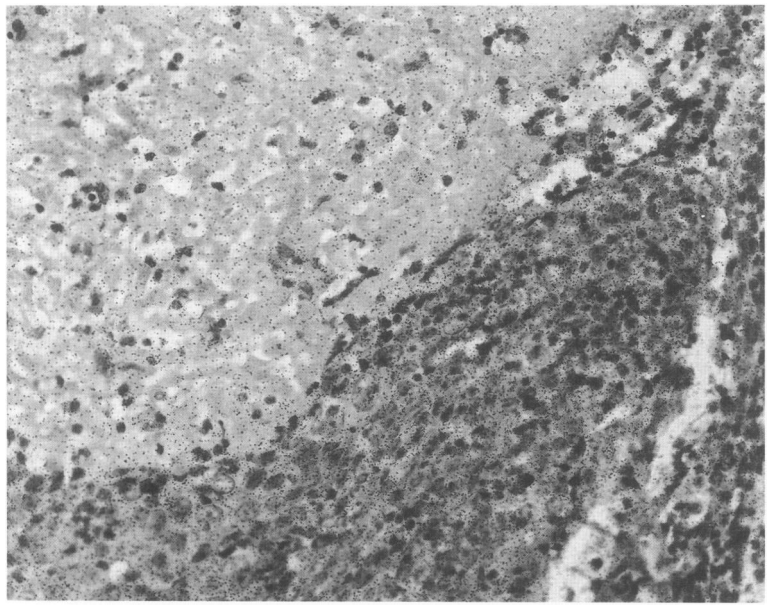

Figure 3. In situ *hybridization of nasopharyngeal carcinoma with ^{3}H-labeled EBV large internal repeat probe. Hybridization is diffuse and it is difficult to distinguish signal from background.*

Our present results demonstrate that the EBER genes are expressed in some EBV-infected epithelial tissues. We have shown EBER expression in morphologically normal salivary gland epithelium as well as in nasopharyngeal carcinoma. It is of note that in some NPC specimens EBER1 expression varies considerably between adjacent tumor cells. This suggests that tumor cells may be heterogeneous with regard to EBV gene expression. In contrast, DNA studies using the internal repeat probe showed a homogeneous pattern of positivity among tumor cells.[20]

Epstein–Barr virus also has been shown to replicate in the uterine cervix *in vitro* and *in vivo*, raising the possibility that EBV may play a role in cervical carcinoma.[31] In this study, we sampled 10 cases of cervical carcinoma, including one lymphoepithelioma, to determine whether there was EBER1 expression. In none of the cases did we find evidence of EBV infection. This does not rule out a role for EBV in cervical cancer, but suggests that either EBV is only occasionally associated with cervical cancer, or that the biology of that association is substantially different from the biology of the EBV association with nasopharyngeal carcinoma.

We did not detect EBER expression in normal respiratory epithelium in the nasopharynx (thought to be the locus from which NPC arises[32]) nor in squamous metaplasia even in tissue adjacent to EBV-associated NPC. Thus it appears that although the epithelial cells of the oropharynx and nasopharynx may be intermittently bathed in EBV throughout the lifetime of an individual, the vast majority of these cells are either not infected with EBV or are infected but not expressing EBERs. Similarly we did not detect EBER expression in normal cervical epithelium. Replication of EBV in oral and cervical epithelium is well documented. Our failure to detect EBER1 expression in these tissues may indicate that EBV-infected cells are rare, were missed by inadequate sampling, or, alternatively, that lytically infected cells do not express EBERs. The latter interpretation is supported by the finding that the EBERs are not expressed in the lytic EBV infection associated with hairy leukoplakia[33] and blot hybridization studies of RNA from control biopsies of normal nasopharyngeal tissue that demonstrated other EBV RNA species but not the EBERs.[34]

Down-regulation of EBER expression in lytic replication might explain the uneven EBER1 expression among NPC cells. It is possible that there is considerable heterogeneity in the state of the EBV genome in NPC, with some cells latently infected and others lytically replicating. Latent and replicating forms of EBV DNA have been detected by Southern blot hybridization in approximately 40% of lymphomas and lymphoproliferative diseases in the immunocompromised.[35] Lytic infection of some NPC cells might explain the high titers to EBV lytic antigens detected in NPC patients.

There are several potential clinical applications related to the detection of EBV in NPC. EBER *in situ* hybridization of nasopharyngeal exfoliative cytology might be used as a screening technique for the early detection of NPC. Epstein–Barr virus serology has been employed for screening populations at risk for NPC and in monitoring the clinical course of disease.[36,37] Exfoliative cytology to date, however, has been somewhat disappointing and has not been shown to have the same value in high-risk NPC populations as exfoliative cytology in cervical cancer. The sensitivity and specificity of such screening might be markedly improved by the ability to detect EBV in exfoliated cells. Similarly detection of EBV in carcino-

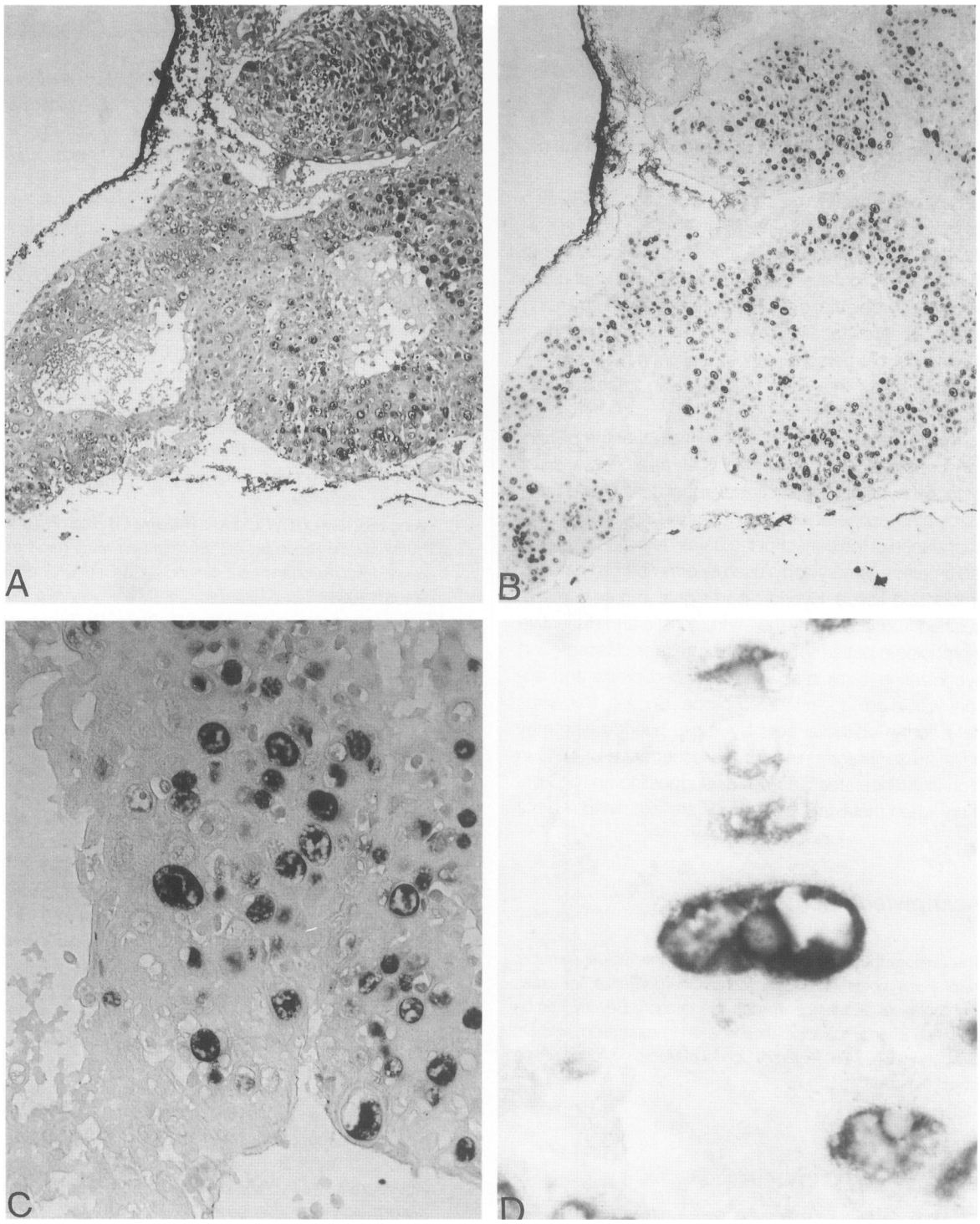

Figure 4. In situ *hybridization with digoxigenin-labeled EBER1-antisense probe of moderately differentiated NPC extending into the maxillary sinus.* **A**: *Hybridization with H&E counterstain.* **B**: *No counterstain. Shows absence of background.* **C**: *Higher power of* **A**. *Many malignant cells in the field do not show hybridization signal at all.* **D**: *High-power view of* **B**. *The hybridization signal is clumped and marginated against the nuclear membrane and nucleolus. The nucleolus and areas of nucleoplasm are devoid of signal.*

ma of unknown primary site may suggest a nasopharyngeal origin. Because the EBV association with NPC holds regardless of the geographic or ethnic origin of the afflicted patient and the sporadic or endemic pattern of incidence, the ability to perform reliable *in situ* hybridization on routinely prepared specimens thus might be diagnostically useful even in low-incidence areas. The results must be interpreted with caution, however, in light of

the findings of EBV in other epithelial tumors (eg, thymic carcinoma) on rare occasion.[20,38]

Digoxigenin-labeled EBER antisense probes are particularly well suited to clinical applications because their use avoids the long exposure times required with ^{3}H-labeled probes, provides superior resolution, and is not associated with background.

Polymerase chain reaction amplification (PCR) also can be applied to viral detection in formalin-fixed specimens, but the high sensitivity of PCR and the inability to identify the particular cells infected renders the technique somewhat problematic in its application to tumors arising from mucosal surfaces from which EBV is regularly shed, even in normal persons without malignancy. *In situ* hybridization has the virtue of identifying infected cells rather than free virus.

In summary, our results demonstrate that the EBER1 transcript, despite its small size, is an appropriate target for *in situ* hybridization of clinical specimens where EBV-associated epithelial cell malignancy is suspected. The abundance of the transcript facilitates detection with non-radiometric assay systems even in formalin-fixed paraffin-embedded tissue. Hybridization signal is readily distinguished from artifact by the characteristic and distinctive morphologic pattern of EBER1 expression. Background hybridization is minimal with ^{3}H-labeled probe and absent with digoxigenin-labeled probe. Epstein–Barr virus detection in epithelial cells by *in situ* hybridization may have a role in screening for the occurrence of NPC in high-incidence areas, as well as suggesting an NPC primary when metastatic specimens are examined.

Acknowledgments

The authors thank Dr. Keerti V. Shah for intellectual stimulus, helpful comments, and suggestions; George Pettus, for excellent technical assistance in slide preparation; Beverly Lambe and Jian Zhang, for assistance in probe preparation; and Dr. Gregory Howe, Yale University School of Medicine, for providing EBER plasmids.

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