Epstein–Barr Virus-specific Transcription in Normal and Malignant Nasopharyngeal Biopsies and in Lymphocytes from Healthy Donors and Infectious Mononucleosis Patients

By JONATHAN D. TUGWOOD,1 WAI-HON LAU,2 SAI-KI O,3 SHIU-YING TSAO,3 W. M. CRAIG MARTIN,3 WESLEY SHIU,3 CLAUDE DESGRANGES,4 PHILIP H. JONES5 AND JOHN R. ARRAND1.*

1 Paterson Institute for Cancer Research, Christie Hospital and Holt Radium Institute, Manchester M20 9BX, U.K., 2 Institute of Radiology and Oncology, Queen Elizabeth Hospital, Kowloon, Hong Kong, 3 Department of Clinical Oncology, Prince of Wales Hospital, Shatin, Hong Kong, 4 Laboratory of Epidemiology and Immunovirology of Tumours, Faculty of Medicine A. Carrel, Lyon, France and 5 Department of Surgery, Christie Hospital and Holt Radium Institute, Manchester M20 9BX, U.K.

(Accepted 8 December 1986)

SUMMARY

Cytoplasmic RNA was prepared from biopsy material obtained from the nasopharynx of normal individuals and nasopharyngeal carcinoma (NPC) patients. Similar RNA preparations were prepared from lymphocytes of healthy donors and infectious mononucleosis patients. RNA was radioactively labelled in vitro and hybridized to cloned fragments of B95-8 Epstein–Barr virus (EBV). In all seropositive cases the minimum pattern of EBV-specific RNA expression was like that observed previously in latently infected, EBV-positive lymphoblastoid cell lines or Burkitt's lymphoma biopsies. A novel observation was that all of the control biopsies from normal nasopharynxes expressed EBV-specific RNA, some of which appeared to be associated with a more active state of EBV infection. The pattern of expression in NPC patients was similar to that of the normal donors but showed some variations in complexity. In general the virus-specified small RNAs were not present in normal nasopharyngeal tissue but were expressed in the NPC biopsies.

INTRODUCTION

Epstein-Barr virus (EBV) is associated with a variety of human diseases. It is responsible for the onset of the self-limiting lymphoproliferative disease infectious mononucleosis (IM) and is also associated with several human cancers including Burkitt's lymphoma (BL), a B cell lymphoma which is found predominantly in children in parts of Africa (de The et al., 1978), polyclonal B-cell lymphomas in immunosuppressed patients (Hanto et al., 1982; Miller, 1984) and nasopharyngeal carcinoma (NPC), an epithelial cell malignancy commonest in southern Chinese adult males (Simons & Shanmugaratnam, 1982). It is well established that EBV is the aetiological agent of IM (Henle et al., 1968) but although the association of EBV with NPC and African BL appears unequivocal, in that patients have elevated serum antibody titres to viral antigens (Henle et al., 1969; Henle & Henle, 1976) and EBV genomes can be found in malignant cells (Klein et al., 1966; Wolf et al., 1973), the precise role of the virus in the development of these neoplasms is not clear.

Analysis of EBV DNA isolated from patients suffering from IM, BL or NPC has failed to reveal any disease-specific virus subtypes (Bornkamm et al., 1984). It is therefore conceivable that differences in virus gene expression may correlate with different EBV-associated diseases, particularly in the case of NPC which seems to be the only pathological instance in which EBV is found in a cell type other than B-lymphoid. Although NPC tissue is heavily infiltrated with
lymphocytes, these are predominantly T cells which are resistant to infection by EBV (Lemon et al., 1977; Thomas et al., 1984) and viral DNA is present only in the epithelial cells (Wolf et al., 1973).

EBV transcription in virus genome-positive lymphoblastoid cell lines and BL biopsies has been well characterized (Rymo, 1979; Thomas-Powell et al., 1979; Dambaugh et al., 1979; King et al., 1980; Arrand & Rymo, 1982; Van Santen et al., 1981, 1983; Arrand et al., 1983; Weigel & Miller, 1983) and it is clear that only a few regions of the EBV genome are expressed in this 'latent-type' infection. In contrast, EBV transcription in the B cells from IM patients has not previously been examined and similar information from the epithelial cells of NPC is scanty. One study (Raab-Traub et al., 1983) suggests an enhanced EBV expression in the malignant cells of NPC. However, in that work nude mouse-passaged tumours were predominantly used and it is not completely clear whether such cells are entirely representative of the initial tumour material.

In this study we have examined the complexity of EBV-specific RNA in NPC biopsies and in the lymphocytes of IM patients and compared it with the well characterized pattern in BL-related cells. As controls, normal nasopharyngeal tissue (on which no previous data exist) and lymphocytes from healthy EBV seropositive and seronegative individuals were studied. Anti-EBV antibody titres from the patients were also determined and related to the complexity of virus expression.

METHODS

**Cloned restriction endonuclease fragments of EBV DNA.** Plasmids containing cloned EcoRI fragments of EBV DNA were described previously (Arrand et al., 1981). Plasmids pJJ1 and pJJ2 which respectively contain the genes specifying J RNA I and J RNA II (EBER I and II) were described by Jat & Arrand (1982). Plasmid pJF which contains both the J-RNA genes within an approximately 1 kb SacI/EcoRI fragment (Arrand & Rymo, 1982) was constructed by cleaving the EcoRI-J fragment with SacI and inserting into EcoRI + SacI-cleaved cosmid Homer II (Chia et al., 1982). All plasmids were propagated in *Escherichia coli* and DNA was prepared as before (Arrand et al., 1981).

**Preparation of cytoplasmic RNA**

**Solid tissues.** NPC biopsies were obtained from Hong Kong, Tunisia and England and similar samples of normal nasopharynx tissue were from Hong Kong and England. Typically the samples weighed 0.02 to 0.05 g. They were immediately snap-frozen in liquid nitrogen and stored at −70 °C. The frozen biopsies were disrupted in a Mikro-dismembrator (F.T. Scientific Instruments, Gloucester, U.K.) to produce a fine powder (Kreig et al., 1983). Cytoplasmic RNA and nuclear DNA were simultaneously prepared from the cells by the method of Favaloro et al. (1980). RNA and DNA were stored as ethanol precipitates at −70 °C. Typical yields were 30 to 50 μg of RNA and 50 to 100 μg of DNA.

**B lymphocytes.** Approximately 10 ml of heparinized blood was layered onto an equal volume of Ficoll/Hypaque (density 1.077 g/ml) in a sterile universal tube and centrifuged at approximately 1900 g for 15 min at 20 °C. White cells at the interface were removed and washed twice in sterile phosphate-buffered saline (PBS). The upper serum fraction was retained for subsequent analysis. Cells were resuspended in 3 ml RPMI 1640 containing 10% foetal calf serum, and plated out for 1 h at 37 °C in a plastic culture flask to which monocytes adhered. The non-adherent cells were then mixed with an equal volume of sheep erythrocytes treated with 2-aminoethylisothiouronium bromide hydrobromide (Kaplan & Clark, 1974) in a flat-bottomed glass bottle, centrifuged at approximately 300 g for 10 min at 20 °C and left to stand for at least 2 h at room temperature. The cells were gently resuspended, and rosetted T lymphocytes removed on a Ficoll/Hypaque gradient as before. The cells at the interface, predominantly B lymphocytes, were resuspended in 2 to 3 ml sterile PBS, washed twice and counted on a haemocytometer. Typically, $10^8$ to $1.3 \times 10^8$ cells per 10 ml of blood were obtained from IM patients. $10^6$ to $5 \times 10^6$ cells per 10 ml of blood were obtained from healthy donors. RNA and DNA were prepared from the B lymphocytes as described above.

**Analysis of RNA.** Southern blots of cloned EBV EcoRI fragments (Arrand et al., 1981) were used to detect EBV-specific transcripts. Total cytoplasmic RNA was degraded with alkali and end-labelled with $[^32P]ATP$ as described by Arrand & Rymo (1982). To increase specific activity, three 5 unit aliquots of polynucleotide kinase were used during the 30 min labelling reaction at 0, 10 and 20 min. Specific activities of $1 \times 10^8$ c.p.m./μg or higher were generally obtained. Hybridization of labelled RNA to Southern blots of cloned EBV DNA was performed by the 'BLOTTO' method described by Johnson et al. (1984). The blots were washed finally at 68 °C in 0.1 × SSC, 0.1% SDS for several hours.
EBV-specific RNA expression

Fluorescent antibody staining. Patients' anti-viral capsid antigen (VCA) IgG and IgA antibody titres were determined by indirect immunofluorescence as described by Henle & Henle (1966).

RESULTS

Identification of EBV-homologous transcripts in diseased and healthy individuals

Cytoplasmic RNA was prepared from NPC biopsy material, normal nasopharyngeal tissue and the B lymphocytes of IM patients and EBV seropositive or seronegative donors. The RNA was labelled in vitro and hybridized to nitrocellulose membranes containing individual cloned fragments of EBV DNA. Fig. 1 shows a restriction map of EBV and summarizes graphically the patterns of hybridization obtained using RNA from the various sources. Fig. 2 shows the results of the hybridizations. Data from individual nasopharyngeal biopsies are listed in Table 1.

Normal tissue

As has been observed in similar earlier studies (Rymo, 1979; Arrand & Rymo, 1982; Arrand et al., 1983), non-EBV-infected human tissues contain cytoplasmic RNA which is homologous to certain regions of the EBV genome. This cellular cross-hybridization has been examined in detail (Arrand et al., 1983) and appears to be due to short repetitive sequences which are conserved in higher eukaryotes. These cross-hybridizing transcripts were detectable in the RNA of seronegative donors (Fig. 2, panel 4) and were consistently observed in all blots. Cross-hybridization was observed to the EcoRI-D and -G1 and the BamHI-F, -Q and -Z fragments. The experimental protocol used, although having the disadvantage of detecting cross-hybridizing RNAs, was well suited to analysing transcription in biopsy material in which the RNA may be of poor quality. In addition to the cross-homology, EBV-specific RNA was detected in lymphocytes from normal seropositive donors and, somewhat unexpectedly, also in all 18 samples of normal nasopharyngeal tissue.

In lymphocytes from seropositive donors (three samples), virus-specific transcription from the BamHI-H, -W and -Y regions was detected, and also from BamHI-K upon prolonged autoradiography. In two cases RNA homologous to EcoRI-J was also observed. In all cases the EBV-specific hybridization was very weak, presumably due to the low numbers of EBV-positive B lymphocytes in the circulation of healthy individuals. This pattern of expression is similar to that previously observed in immortalized lymphoblastoid cell lines infected in vitro (Van Santen...
Fig. 2. Hybridization of cellular cytoplasmic RNA to cloned EcoRI fragments of EBV DNA. Plasmid DNAs containing individual cloned EcoRI fragments of EBV DNA were cleaved with EcoRI plus BamHI (lanes A to C) or EcoRI (lanes D to J) and the products were separated by electrophoresis on 0.8% agarose gels. The letters above the lanes indicate the EcoRI fragment in the corresponding clone. Panel 1 shows the pattern of fragments after ethidium bromide staining and visualization under u.v. light. DNA was transferred to nitrocellulose membranes and hybridized with 32P-labelled, nick-translated EB virion DNA (panel 2) or 32P-labelled cytoplasmic RNA from: (panel 3), lymphocytes from an IM patient; (panel 4), lymphocytes from an EBV seronegative individual; (panel 5), a normal nasopharyngeal biopsy (no. 012); (panels 6, 7 and 8), NPC biopsies no. 3, 8 and 11, respectively. In separate experiments (not shown) no hybridization of labelled RNA from any of the cell types studied could be detected to the smaller EcoRI fragments K, L and M. In panel 7 the apparent hybridization to EcoRI-G2 is due to spillover of DNA from the adjacent EcoRI-G1 lane.

J. D. TUGWOOD AND OTHERS

et al., 1981; Arrand & Rymo, 1982) and is referred to as 'latent type'. This basic pattern of expression was also observed in all of the normal nasopharyngeal samples, with additional hybridization to the EcoRI-E region, and in some cases to BamHI-B (Fig. 2, panel 5). Hybridization to EcoRI-J was rarely observed in the normal nasopharyngeal biopsies (Table 1b). In order to distinguish between virus-specific RNA encoding the latent membrane protein (LMP) and the cellular RNA which cross-hybridizes with EcoRI-D, a probe specific for the
**EBV-specific RNA expression**

### Table 1. Analyses of NPC patients and normal individuals

(a) NPC biopsies

<table>
<thead>
<tr>
<th>Tumour no.</th>
<th>Origin*</th>
<th>WHO classification†</th>
<th>Tumour stage‡</th>
<th>Level of expression§</th>
<th>Anti-VCA titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>HK</td>
<td>III</td>
<td>$T_2N_2M_0$</td>
<td>Latent, $E^+, B^+, I^+, H^+$</td>
<td>1:1280 1:8190</td>
</tr>
<tr>
<td>4</td>
<td>T</td>
<td>NA</td>
<td>$T_2N_2M_0$</td>
<td>Latent</td>
<td>1:640 1:2560</td>
</tr>
<tr>
<td>5</td>
<td>HK</td>
<td>III</td>
<td>$T_2N_0M_0$</td>
<td>Latent, $E^+, B^+$</td>
<td>1:160 1:1024</td>
</tr>
<tr>
<td>6</td>
<td>HK</td>
<td>II</td>
<td>$T_2N_0M_0$</td>
<td>Latent, $E^+, B^+, I^+$</td>
<td>1:320 1:8190</td>
</tr>
<tr>
<td>7</td>
<td>T</td>
<td>NA</td>
<td>NA</td>
<td>Latent, $E^+$</td>
<td>1:20  1:320</td>
</tr>
<tr>
<td>8</td>
<td>HK</td>
<td>III</td>
<td>$T_1N_2M_0$</td>
<td>Latent, $E^+, B^+$</td>
<td>1:160  NA</td>
</tr>
<tr>
<td>9</td>
<td>HK</td>
<td>III</td>
<td>$T_3N_0M_0$</td>
<td>Latent, $E^+, B^+$</td>
<td>1:40  NA</td>
</tr>
<tr>
<td>10</td>
<td>HK</td>
<td>III</td>
<td>$T_3N_0M_0$</td>
<td>Latent, $E^+, B^+$</td>
<td>1:320  NA</td>
</tr>
<tr>
<td>11</td>
<td>T</td>
<td>NA</td>
<td>$T_3N_0M_0$</td>
<td>Latent, ($J^-$), $B^+$, $E^+$</td>
<td>1:320 1:2560</td>
</tr>
<tr>
<td>12</td>
<td>HK</td>
<td>III</td>
<td>$T_3N_0M_0$</td>
<td>Latent, $E^+, B^+$</td>
<td>1:640  NA</td>
</tr>
<tr>
<td>13</td>
<td>HK</td>
<td>III</td>
<td>$T_1N_0M_0$</td>
<td>Latent, $E^+, B^+$</td>
<td>1:1280 1:5120</td>
</tr>
<tr>
<td>14</td>
<td>HK</td>
<td>III</td>
<td>$T_1N_0M_0$</td>
<td>Latent, $E^+, B^+$</td>
<td>1:640 1:5120</td>
</tr>
<tr>
<td>15</td>
<td>HK</td>
<td>II</td>
<td>$T_2N_0M_0$</td>
<td>Latent, $E^+, B^+$</td>
<td>1:20  &gt;1:640</td>
</tr>
<tr>
<td>16</td>
<td>HK</td>
<td>III</td>
<td>$T_3N_0M_0$</td>
<td>Latent, $E^+, B^+$</td>
<td>&gt;1:320 &gt;1:640</td>
</tr>
<tr>
<td>17</td>
<td>HK</td>
<td>III</td>
<td>$T_1N_0M_0$</td>
<td>Latent, ($J^-$)</td>
<td>1:160  NA</td>
</tr>
<tr>
<td>18</td>
<td>HK</td>
<td>III</td>
<td>$T_1N_0M_0$</td>
<td>Latent, $E^+, B^+$</td>
<td>1:320 1:5120</td>
</tr>
<tr>
<td>19</td>
<td>HK</td>
<td>III</td>
<td>$T_1N_0M_0$</td>
<td>Latent, $E^+, B^+$</td>
<td>NA   NA</td>
</tr>
<tr>
<td>20</td>
<td>UK</td>
<td>NA</td>
<td>NA</td>
<td>Latent, $E^+, I^+$</td>
<td>1:5120 1:20480</td>
</tr>
<tr>
<td>21</td>
<td>HK</td>
<td>III</td>
<td>$T_2N_0M_0$</td>
<td>Latent, ($J^-$), $E^+, B^+$</td>
<td>1:40  NA</td>
</tr>
<tr>
<td>22</td>
<td>HK</td>
<td>III</td>
<td>$T_3N_0M_0$</td>
<td>Latent, $E^+$</td>
<td>1:80  NA</td>
</tr>
</tbody>
</table>

(b) Normal nasopharyngeal biopsies

<table>
<thead>
<tr>
<th>Sample ref. no.</th>
<th>Origin*</th>
<th>Level of expression§</th>
<th>Patient diagnosis</th>
<th>Anti-VCA titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>001</td>
<td>HK</td>
<td>Latent, ($J^-$), $E^+$</td>
<td>Carcinoma of oesophagus</td>
<td>IgA  IgG</td>
</tr>
<tr>
<td>002</td>
<td>HK</td>
<td>Latent, ($J^-$), $E^+$</td>
<td>Carcinoma of tongue</td>
<td>&lt;1:5 1:1280</td>
</tr>
<tr>
<td>003</td>
<td>HK</td>
<td>Latent, ($K^-, J^-$), $E^+$</td>
<td>Carcinoma of oesophagus</td>
<td>&lt;1:5 1:640</td>
</tr>
<tr>
<td>005</td>
<td>HK</td>
<td>Latent, ($K^-, J^-$), $E^+$</td>
<td>Normal</td>
<td>&lt;1:5 1:320</td>
</tr>
<tr>
<td>006</td>
<td>HK</td>
<td>Latent, ($K^-, J^-$), $E^+$</td>
<td>Multi-metastatic melanoma</td>
<td>NA   NA</td>
</tr>
<tr>
<td>007</td>
<td>HK</td>
<td>Latent, ($K^-, J^-$), $E^+$</td>
<td>Carcinoma of oesophagus</td>
<td>NA   NA</td>
</tr>
<tr>
<td>009</td>
<td>HK</td>
<td>Latent, ($K^-, J^-$), $E^+$</td>
<td>Normal</td>
<td>&lt;1:5 1:1280</td>
</tr>
<tr>
<td>012</td>
<td>HK</td>
<td>Latent, ($K^-$), $E^+, B^+$</td>
<td>Carcinoma of breast</td>
<td>&lt;1:5 1:1280</td>
</tr>
<tr>
<td>016</td>
<td>HK</td>
<td>Latent, $E^+, B^+$</td>
<td>Carcinoma of colon</td>
<td>&lt;1:20 1:640</td>
</tr>
<tr>
<td>017</td>
<td>HK</td>
<td>Latent, $E^+$</td>
<td>Carcinoma of oesophagus</td>
<td>NA   NA</td>
</tr>
<tr>
<td>018</td>
<td>HK</td>
<td>Latent, ($J^-$), $E^+, I^+$</td>
<td>Normal</td>
<td>&lt;1:5 1:320</td>
</tr>
<tr>
<td>019</td>
<td>HK</td>
<td>Latent, ($J^-$), $E^+, I^+$</td>
<td>Normal</td>
<td>&lt;1:5 &gt;1:640</td>
</tr>
<tr>
<td>E 001</td>
<td>UK</td>
<td>Latent, ($J^-$), $E^+$</td>
<td>Normal</td>
<td>&lt;1:8 1:32</td>
</tr>
<tr>
<td>E 002</td>
<td>UK</td>
<td>Latent, ($J^-$), $E^+$</td>
<td>Normal</td>
<td>1:8 1:256</td>
</tr>
<tr>
<td>E 003</td>
<td>UK</td>
<td>Latent, ($J^-$), $E^+$</td>
<td>Normal</td>
<td>1:8 1:256</td>
</tr>
<tr>
<td>E 005</td>
<td>UK</td>
<td>Latent, ($J^-$), $E^+$</td>
<td>Normal</td>
<td>&lt;1:8 1:64</td>
</tr>
<tr>
<td>E 006</td>
<td>UK</td>
<td>Latent, ($J^-$), $E^+$</td>
<td>Normal</td>
<td>NA   NA</td>
</tr>
</tbody>
</table>

* Tumour origin: HK, Hong Kong; T, Tunisia; UK, England.
† WHO classification is as in Shanmugaratnam & Sabin (1978).
‡ Tumour staging. Primary tumour (T): $T_1$, tumour confined to one site of nasopharynx; $T_2$, tumour involving two sites (both posterio-superior and lateral walls) and $T_3$, extension of tumour into nasal cavity or oropharynx. Nodal involvement (N): (N.B. cervical nodes) $N_0$, no clinically positive node; $N_1$, single clinically positive homolateral node 3 cm or less in diameter; $N_2$, single clinically positive homolateral node more than 3 cm but not more than 6 cm in diameter, or multiple clinically positive homolateral nodes, none more than 6 cm in diameter; $N_3$, massive homolateral node(s), bilateral nodes or contralateral nodes. Metastases (M): $M_0$, no evidence of metastases.
§ Level of expression. 'Latent' refers to the level of expression involving four regions of the EBV genome (see Discussion). Where the expression differs from latent, the regions not expressed ($BamHI-K$ or $EcoRI-J$) are indicated in parentheses. Additional transcripts (from $EcoRI-E$, $BamHI-I$ or $EcoRI-H$) were also observed as noted.
∥ NA, Data not available.
LMP coding region was hybridized to RNA dot blots. All samples that were EBV-positive on the standard Southern blot expressed LMP region RNA whereas none of the EBV-negative samples expressed this region (data not shown).

**Lymphocytes from infectious mononucleosis patients**

Patients were defined clinically as having an EBV-associated mononucleosis by the presence of circulating antibodies to the Paul-Bunnel antigen and also to EBV early antigen. Four patients were studied and 'latent-type' EBV-specific expression was observed in all samples, although hybridization to EcoRI-J was observed in only two patients (Fig. 2, panel 3).

**NPC biopsy material**

Twenty NPC biopsies were analysed; 16 were from Hong Kong, three from Tunisia and one from England and three different patterns of complexity of EBV-specific expression were found. Firstly, in two of the biopsies (not shown) the pattern was like that seen in BL biopsies, EBV-positive lymphoblastoid cell lines or normal lymphocytes from seropositive individuals and IM patient lymphocytes. In this 'latent type' expression, transcription is seen from the BamHI-W, -Y, -H, and -K fragments and from EcoRI-J. In the second pattern, seen in most (15) NPC biopsies (Fig. 2, panels 6 and 8), we consistently observed additional hybridization to the EcoRI-E fragment and usually a weak signal from BamHI-B. This is not detected in latent infection of lymphoid cells (Fig. 2, panel 3; Van Santen et al., 1981; Arrand & Rymo, 1982). The third, most complex pattern showed, in addition to the 'latent-type' transcripts, strong hybridization to EcoRI-E and additional hybridization to BamHI-I and -B and sometimes to EcoRI-H (Fig. 2, panel 7). It is known that EBV-specific nuclear RNA in virus-infected cells is of greater complexity than cytoplasmic RNA (see King et al., 1980). It is therefore conceivable that the non-latent transcripts observed in some NPCs are due to nuclear contamination of the cytoplasmic preparation. However, our analysis of nuclear RNAs in the tightly latent Putko and Namalwa cell lines (data not shown) failed to show hybridization to regions that specified these non-latent transcripts, indicating that these RNAs are unlikely to be nuclear precursors. The extent of transcription from the EcoRI-J fragment varied considerably between different biopsies. As an extreme example, NPC samples 7 and 11 (Fig. 2, panels 6 and 8) showed similar levels of hybridization to BamHI-W but no EcoRI-J-specific transcription was detectable in no. 11, whereas no. 7 showed a significant level of J-RNA. Hybridization of NPC biopsy RNA to the individually cloned J-RNA genes (Jat & Arrand, 1982) showed that in all J-RNA-positive samples the abundance of J-RNA 1 was significantly greater than that of J-RNA 2 (Fig. 3).

The hybridization results for individual biopsies are summarized in Table 1.

**Serological and histological data from NPC patients and non-diseased individuals**

Anti-VCA IgA titres were obtained for the majority of NPC patients, and for most of the control individuals from whom nasopharyngeal biopsies were taken (Table 1). All 20 NPC patients were VCA-IgA-positive, whereas only two of 18 normal individuals showed a positive response, and at a very low titre. All the normals for whom data were available showed positive VCA-IgG titres. There was no obvious correlation between serum antibody titres and complexity of viral RNA expression in both NPC and normal patients (see Table 1a and b).

For some of the biopsies data were available concerning the state of differentiation of the tumour, using a WHO classification (Shanmugaratnam & Sabin, 1978) and clinical staging (Table 1a). All the tumours for which data were available were of the poorly differentiated type (WHO class II or III), which is known to be associated with EBV. The data on tumour staging indicate the degree of tumour invasiveness and number of metastases (Table 1). This does not correlate with the extent of EBV transcription in the tumour or anti-VCA titres.

**Discussion**

In this study, EBV transcription in NPC biopsy specimens and in the lymphocytes of IM patients is examined and compared with previous analyses of BL biopsies and EBV-positive lymphoblastoid cell lines. These earlier studies have consistently revealed only limited viral gene
**EBV-specific RNA expression**

Fig. 3. Comparison of the relative amounts of J-RNA I and J-RNA II in NPC biopsy tissue. Fragments of DNA containing the J-RNA genes were excised by cleaving plasmid pJJ1 (contains J-RNA gene 1) with EcoRI plus HindIII (lane 1), plasmid pJJ2 (contains J-RNA gene 2) with EcoRI plus BamHI (lane 2) and plasmid pJF (contains J-RNA genes 1 and 2) with EcoRI plus SacI (lane 3). The fragments were separated by electrophoresis on a 0.8% agarose gel. (a) The pattern of fragments after ethidium bromide staining and visualization under u.v. light. DNA was transferred to a nitrocellulose membrane and hybridized with cytoplasmic RNA labelled *in vitro* from NPC biopsy no. 9 (b). Seven NPC biopsies were examined in this way and in all cases the level of J-RNA I greatly exceeded that of J-RNA II.

 expressing four sectors of the EBV genome (Rymo, 1979; Dambaugh *et al.*, 1979; Van Santen *et al.*, 1981, 1983; Arrand & Rymo, 1982; Arrand *et al.*, 1983; Weigel & Miller, 1983). The BamHI-K fragment specifies the nuclear antigen EBNA 1 (Summers *et al.*, 1982), the BamHI-W,Y,H region encodes another nuclear antigen EBNA 2 (Dambaugh *et al.*, 1984; Dillner *et al.*, 1985a, b; Mueller-Lantzsch *et al.*, 1985; Rymo *et al.*, 1985) and an mRNA for a membrane protein is transcribed from the EcoRI-D fragment (Fennewald *et al.*, 1984; Hennessy *et al.*, 1984; Hudson *et al.*, 1985; Mann *et al.*, 1985). The fourth region is within the last 1 kb of the EcoRI-J fragment and specifies two small, non-polyadenylated, RNA polymerase III transcripts referred to as J-RNAs or EBERs, which are not translated and whose function is unclear (Arrand & Rymo, 1982; Jat & Arrand, 1982; Rosa *et al.*, 1981). The results of the present study show that no difference is apparent between viral gene expression in BL cells, the lymphocytes of IM patients and those NPC biopsies which show the least complex pattern of EBV-specific RNA. This suggests that no disease-specific EBV expression occurs and that the apparently virus-associated transformation of epithelial cells involves the same viral genes that appear to be associated with lymphocyte immortalization. We are confident that the
transcription analysis is of sufficient sensitivity to detect low abundancy EBV-specific transcripts. This is supported by the observation of virus-specific RNA in lymphocytes from normal seropositive donors, although the anti-VCA titres of those donors tested (1:256) implies that they may have above average numbers of EBV-positive circulating B lymphocytes.

Two variations in the extent of EBV-specific RNA expression were observed in the NPC biopsies. Firstly the relative level of expression of the J-RNAs differed between individual samples (Fig. 2, panels 6 and 8). A similar phenomenon has been observed previously in BL biopsies (Rymo, 1979). The two small RNAs are differentially expressed in latently infected lymphoid cells where J-RNA 1 expression far exceeds that of J-RNA 2 (Jat & Arrand, 1982). The same is true in NPC biopsies (Fig. 3). The transcriptional control mechanisms responsible for these differences in levels of expression are not clear.

The second variation was in the overall complexity of EBV-specific expression in the different samples. As noted above, some tumours showed a latent pattern of RNA expression, others expressed additional RNA sequences homologous to the EcoRI-E and BamHI-B fragments (Fig. 2, panels 6 and 8), and the rest exhibited a still higher complexity of virus-specific gene expression (Fig. 2, panel 7). This variation in extent of transcription is possibly due to differences in the intrinsic level of virus production in the nasopharyngeal epithelium (see below) and/or differences in the proportion of tumour cells in the biopsies. Unfortunately the samples were too small both to extract RNA and to perform microscopical analysis. However, microscopy of sections of additional NPC biopsies revealed quite extensive variation in the proportion of tumour tissue in the samples.

Raab-Traub et al. (1983) also observed both latent and 'activated' transcription in NPC biopsies, although the 'activated' transcription, in nude mice-passaged tumours, was more analogous to 'abortive' rather than 'productive' EBV infection. This may seem surprising, since mature virus may be detected in NPC tissue grown in nude mice (Glaser et al., 1976; Trumpet et al., 1976).

In contrast to the earlier study (Raab-Traub et al., 1983) we have examined normal nasopharyngeal biopsies as control tissue samples. Analysis of EBV-specific transcription in these controls shows RNA which corresponds to that from those regions of the genome which are expressed during latent infection of lymphocytes. In addition, as with the NPC biopsies, samples of normal nasopharyngeal tissue frequently contain RNA which is homologous to the EcoRI-E fragment of EBV (Fig. 2, panel 5). This fragment of the genome specifies a highly abundant mRNA which encodes a protein that is recognized by anti-VCA serum (Hummel & Kieff, 1982a, b; Seibl & Wolf, 1985) and is probably the 150K major capsid protein of the virion (Dolyniuk et al., 1976). The EcoRI-E fragment is part of a 13-5 kb region of the EBV genome that transforms monkey epithelial cells in vitro (Griffin & Karran, 1984). Also, we observe transcription from the BamHI-B fragment in 15 of 20 NPC biopsies, but in only three of 18 normal biopsies (see Table 1 a and b). Sequence comparisons with herpes simplex virus indicate that the EBV-specific DNase maps to this fragment (E. Littler et al., unpublished observation) and an antibody response to this enzyme has been proposed as a diagnostic marker for NPC (Chen et al., 1985).

The BamHI-I region expressed in a proportion of NPC biopsies (e.g. Fig. 2, panel 7), is also heavily transcribed during productive infection (Hummel & Kieff, 1982b). One interpretation of our data would be consistent with a model in which the majority of EBV DNA-containing normal nasopharyngeal epithelial cells express only latent viral genes but relatively few cells support productive infection. The sensitivity of our experiments may be such that from this minor population of cells we would detect only the most abundant of the productive cycle RNA, such as that for the major capsid protein transcribed from EcoRI-E.

There is other evidence to support the existence of a productive virus infection in the nasopharynx. Lemon et al. (1977) identified epithelial cells containing EBV genomes in throat washings from IM patients, and Yao et al. (1985) demonstrated that healthy seropositive individuals shed mature EBV into the buccal fluid from a site in the nasopharynx. Although it is tempting to propose the occurrence of a productive virus infection in nasopharyngeal epithelial cells on the basis of the results presented here, further evidence is required. We are currently
attempts to confirm the hypothesis by using EBV-specific monoclonal antibodies on tumour tissue in situ.

The observation that the three EBV-associated diseases share at the minimum a 'latent-type' expression implies that this limited viral transcription is sufficient to induce cell immortalization (by analogy with viral expression in B-lymphocytes immortalized in vitro). It is very interesting, however, that certain transcripts are more readily detectable in NPC tissue than in normal nasopharyngeal tissue. This is unlikely to be due to less EBV RNA in the normal biopsies, since other EBV-specific transcripts are present at levels comparable to those in NPC tissues (e.g. BamHI-W and EcoRI-E transcripts; Fig. 2, panel 5). It is also significant that NPC patients have elevated IgA titres against VCA and early antigen (Henle & Henle, 1976; Zeng et al., 1982) whereas normal individuals do not. This indicates that there is some difference in the state of the EBV infection in NPC patients as compared with normal individuals. It is particularly intriguing to note that 17 of 20 NPC biopsies expressed the small J-RNAs whereas only three of 18 normal nasopharyngeal samples contained detectable amounts of these species (Table 1). Recently it has been shown that small RNAs appear to be essential for cell transformation by herpesvirus saimiri (Murthy et al., 1986). Alternatively, by analogy with the adenovirus virus-associated RNAs which stimulate virus-specific translation (Thimmappaya et al., 1982; Reichel et al., 1984; Siekierka et al., 1985; Bhat & Thimmappaya, 1985) the EBV J-RNAs may activate or enhance EBV-specific protein synthesis. The presence of EBV-specific transcripts in all of the normal nasopharyngeal biopsies examined in this study once again raises the question of whether EBV infection plays an active role in NPC or whether the viral genome is merely a passenger. The serological data and the association of certain EBV transcripts with NPC suggest that this is not the case. Indeed, it is possible that the BamHI-B, -I and EcoRI-J transcripts may play a role in the progression of EBV-containing nasopharyngeal epithelial cells from the normal to the hyperproliferative state of NPC.

We thank Dr S. Barton, Kenmore Medical Centre, Wilmslow, for blood samples from IM patients. This work was supported by the Cancer Research Campaign (U.K.).

REFERENCES


EBV-specific RNA expression


(Received 27 August 1986)