In vivo transcription of the Epstein–Barr virus (EBV) BamHI-A region without associated in vivo BARF0 protein expression in multiple EBV-associated disorders

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The in vivo expression of the Epstein–Barr virus (EBV) BamHI-A rightward transcripts (BARTs) as well as the putative BART-encoded BARF0 and RK-BARF0 proteins in various EBV-associated malignancies was investigated. RT-PCRs specific for the different splice variants of the BARTs and both a nucleic acid sequence-based amplification assay and an RT-PCR specific for the BARF0 ORF were used. Abundant transcription of BARTs was found in EBV-associated Hodgkin's lymphomas, Burkitt's lymphomas (BL), T-cell non-Hodgkin's lymphomas, post-transplant lymphoproliferative disorders, AIDS-related lymphomas and gastric carcinomas. Using RNA in situ hybridization (RISH), BARTs were detected within the neoplastic cells of these malignancies. BARTs encoding RK-BARF0 were not detected. The BARTs detected were shown possibly to encode the RPMS1 and BARF0 proteins, based on their splicing. However, BARTs actually harbouring the BARF0 ORF were detected only in specimens containing a relatively large number of EBV-positive cells. New monoclonal antibodies against the BARF0 protein were generated that efficiently recognized prokaryotic and eukaryotic recombinant BARF0. However, the BARF0 protein was not detected in clinical samples, nor in EBV-positive cell lines, even though these were positive for BARTs by RISH and/or BARF0 RNA in vitro analysis. Using immunoblot analysis, no antibodies against baculovirus-expressed BARF0 protein were detected in the sera of nasopharyngeal carcinoma patients, BL patients and Hodgkin's disease patients, patients with chronic EBV infection, infectious mononucleosis patients or EBV-positive healthy donors. Thus, BARTs containing the BARF0 ORF are expressed in vivo but the BARF0 protein cannot be detected and may be expressed only marginally. It is concluded that the BARF0 protein is unlikely to play a role in vivo in EBV-positive malignancies.

INTRODUCTION

Epstein–Barr virus (EBV) is a ubiquitous gammaherpes-virus capable of transforming B-lymphocytes in vitro. It is associated with several benign disorders, such as infectious mononucleosis (IM) and oral hairy leukoplakia (OHL), and also with certain malignant disorders (reviewed by Middeldorp et al., 2003), including B-cell lymphomas of the immunocompromised, endemic Burkitt's lymphoma (BL), nasopharyngeal carcinoma (NPC), 20–80 % of the cases of Hodgkin’s disease (HD) depending on histological subtype, peripheral T-cell non-Hodgkin’s lymphomas (PTCLs), especially those of the aero-digestive tract (De Bruin et al., 1994), and approximately 10 % of gastric carcinomas (GCs) (Takada, 2000).

EBV is transcriptionally active, though mostly present in a latent form in these malignancies. To date, at least three main types of EBV latent gene expression have been described (Middeldorp et al., 2003). Strikingly, only two types of transcripts are abundantly present in cell lines representing different latency types and in all EBV-associated diseases. These are the short EBV-encoded RNAs (EBERs) (Minarovits et al., 1992) and the rightward transcripts derived from the BamHI-A region of the EBV genome (BARTs) (Brooks et al., 1993).

Previous studies have demonstrated that transcription of the BamHI-A region is very complex. In C15 tumours, a
NPC that has been propagated in nude mice, many different transcripts have been detected. Interestingly, these transcripts are coterminal and harbour the BARF0 ORF at their 3' ends (Chen et al., 1992; Gilligan et al., 1991; Hitt et al., 1989; Karran et al., 1992; Smith et al., 1993). However, heterogeneous 3' processing of this region has also been described and most cDNA cloned transcripts appeared to be incomplete small products (Sadler & Raab-Traub, 1995; Smith, 2001). It was demonstrated that BARF0 is not essential for EBV-mediated transformation (Robertson et al., 1994) but it has been hypothesized that BARTs can act as antisense regulators for BamHI-A leftward transcripts, which are mostly associated with productive EBV infection (Baer et al., 1984). Thus, BARTs would play a role in the maintenance of latency.

The BARF0 ORF encodes a putative protein of 174 amino acids (Fries et al., 1997). By alternative splicing, the BARF0 ORF may be extended by 105 additional amino acids. The alternative ORF thus generated is called RK-BARF0 (Sadler & Raab-Traub, 1995). Recent in vitro transfection studies have indicated that the RK-BARF0-encoded protein might modulate Notch signalling. It was hypothesized further that the RK-BARF0 protein was involved in the activation of latent membrane protein 1 (LMP1) expression in EBV infections in the absence of EBV nuclear antigen 2 (EBNA2) through this Notch modulating effect (Kusano & Raab-Traub, 2001). To date, the contribution of the BARF0 and RK-BARF0 proteins to EBV oncogenic transformation and malignancy in vivo remains unclear.

The literature is inconclusive about the expression of BARF0 and RK-BARF0 at the protein level and only indirect evidence is available: the presence of the polyadenylation signal 5’ to the stop codon renders actual translation unlikely (Karran et al., 1992; Sadler & Raab-Traub, 1995; Smith et al., 2000) but occasional antibody (Gilligan et al., 1991) and CTL (Kienzle et al., 1998) responses to recombinant BARF0 and RK-BARF0 protein have been described in a limited number of individuals. These data suggested that synthesis of these proteins takes place in vivo. However, the only antibody reagent directly detecting the BARF0 protein described thus far was shown recently to cross-react with cellular proteins (Kienzle et al., 1999b; Schroder et al., 2002). In addition, a recent in vitro study (Kienzle et al., 1999a) showed the existence of splice variants, which would give rise to at least four RK-BARF0 isoforms that would abolish the BARF0 start codon. Therefore, further evidence is required to confirm actual BARF0 or RK-BARF0 protein expression in vivo.

At least two other putative ORFs have been demonstrated in BARTs and these have been designated RPMS1 and A73 (Karran et al., 1992; Smith et al., 2000). RPMS1 encodes a putative nuclear protein (Chen et al., 1999) that is partially homologous to EBNA2 (Smith et al., 1993) and it is tempting to speculate that RPMS1 acts as a substitute for EBNA2, for example, in latency types I and II. Although transcription of RPMS1 in latently infected B-cells from healthy donors was found recently (Chen et al., 1999), neither transcription of RPMS1 in patient samples nor expression of RPMS1 in tumour biopsies at the protein level have been reported (Zhang et al., 2001).

In this study, we have aimed to unravel the in vivo transcription of the putative BARF0, RK-BARF0 and RPMS1 ORFs encoded in BARTs and to demonstrate expression of BARF0 and RK-BARF0 at the protein level in biopsy material from different EBV-associated disorders. For this purpose, we have used RT-PCR analysis specific for BARTs in general, as well as for RPMS1 and the different splice variants of RK-BARF0. Moreover, transcription of the ORF encoding BARF0 was studied by nucleic acid sequence-based amplification (NASBA) and RT-PCR (see Fig. 1). To determine whether BART transcription actually takes place in the neoplastic cells of the different malignancies, we used RNA in situ hybridization (RISH). To study BARF0 and RK-BARF0 expression at the protein level, new monoclonal antibodies (mAbs) were generated and used in immunohistochemistry and immunostaining of Western blots. Moreover, the presence of serum antibodies directed against the recombinant BARF0 protein was determined in patients with EBV-associated disease and healthy donors using immunoblot analysis.

**METHODS**

**Cell lines.** The EBV-positive Bl. cell lines Raji and HH514, the lymphoblastoid cell lines JY and B95-8 and the EBV-negative Bl. cell line Ramos were cultured in RPMI medium containing 10% FCS at 37 °C and 5% CO2 in a humidified atmosphere.

**Clinical material.** All clinical material in this study was selected on the basis of an EBV-positive status of the tumour cells as determined by EBER1/2 RISH (see below) unless specified otherwise. Formalin-fixed, paraffin-embedded material of two nasal and one gastrointestinal PTCLs, two NPCs, eight GCS, 26 HDs, one IM case, one OHL, four post-transplant EBV-associated lymphoproliferative disorders (PTLDs) (including snap-frozen material of the same tumours), one AIDS-related lymphoma (ARL) and seven Bl. samples, as well as additional snap-frozen material of two nasal and two nodal PTCLs, four GCS and four HD samples, were obtained from the tissue archives of the Department of Pathology, Vrije Universiteit Medical Center, Amsterdam, and have been described previously (Brink et al., 1997a, b; Hayes et al., 1999). Material from two Bls and a C15 tumour was provided by B. Griffin (London, UK) and P. Busson (Villejuif, France), respectively.

Well-characterized sera from NPC (n=8), Bl (n=8) and IM (n=13) patients, from patients with a chronic EBV infection (n=3) or EBV-positive HD (n=7), and sera of EBV seropositive (n=2) and seronegative (n=2) healthy donors were obtained from the archives of the Department of Pathology, Vrije Universiteit Medical Center, Amsterdam. All sera were stored at −20 °C and have been analysed previously by standard serological techniques (Meij et al., 1999; Middeldorp & Herbrink, 1988; van Grunsven et al., 1993).

**Plasmid clones and production of riboprobes.** A BamHI-A transcript-specific clone was generated by cloning the A3/A4 RT-PCR product (Fig. 1) from Raji mRNA into pBluescript using RT-PCR primers carrying recognition sites for EcoRI and BamHI, respectively. A BARF0 ORF-specific clone was generated by direct
cloning of the PCR product of the B95-8 BARF0 ORF including the first start codon and the stop codon (EBV nt 160459–160994; Table 1) into pCRscriptII (Stratagene). DNA sequence analysis of the clone excluded mutations incorporated by PCR. EBER1/2 clones have been described previously (Herbst et al., 1992). For the generation of digoxigenin-labelled riboprobes, in vitro transcription was performed using the RiboProbe system (Promega). After precipitation, probes were dissolved in 50 mM RNase-free water and 200 mM formamide containing 50 μg yeast tRNA ml⁻¹ (final concentration of probe RNA: 10 ng ml⁻¹). The integrity of the probes was determined by agarose gel electrophoresis. Probes were transferred to nylon membranes via capillary blotting in 10⁻⁶ SSC and labelling efficiencies were determined by detection with alkaline phosphatase (AP)-conjugated anti-digoxigenin antibodies (Roche) and NBT/BCIP (Sigma), according to the manufacturers’ protocols.

RNA isolation and RT-PCR. RNA was isolated from 10 cryosections of 5 μm thickness using 1 ml RNAzol (Biotecx Laboratories). The purity and concentration of the RNA were determined spectrophotometrically and the integrity of the RNA was determined by agarose gel electrophoresis, the presence of 18S/28S rRNA bands being an index for high-quality RNA. DNase treatment of the samples was performed according to the manufacturer’s protocol (Promega). Reverse transcription and subsequent PCR were performed as described previously (Brink et al., 1997a). PCR products were analysed on 1.5% agarose/Tris/borate/EDTA gels, transferred to nylon filters by alkaline Southern blotting and hybridized to specific [³²P]ATP-labelled oligonucleotide probes to determine their specificity. RT-PCR primers and probes are listed in Table 1. As a positive and RNA quality control, RT-PCR for snRNP U1A, a low-abundance RNA transcript, was performed on all samples as described previously (Bijl et al., 1995). All samples were analysed by multi-primed RT-PCR for mRNA encoding EBNA1 (driven by the Cp, Wp or Qp promoters), LMP1, LMP2A, LMP2B and the BARTs (A3/A4), according to Brink et al. (1997a). To analyse the alternative splices as described by Kienzle et al. (1999a), primers were designed to cross the various putative spliced regions (acceptors at nt 160728, 160689 and 160679, respectively).

NASBA. NASBA is an isothermal amplification technique that allows direct ORF-specific mRNA amplification in the presence of the coding genomic DNA. As a consequence, for the analysis of non-spliced transcripts, DNase pretreatment is no longer required. The sensitivity of NASBA and RT-PCR assays are comparable (Brink et al., 1998). BARF0 ORF-specific NASBA was optimized and performed as described previously for other EBV messenger targets (Hayes et al., 1999). Using this assay, between 10 and 100 in vitro-generated copies of BARF0 cRNA could be detected or one JY copy. NASBA products were analysed by agarose gel electrophoresis, transferred to nylon membranes via capillary blotting in 10× SSC and hybridized to a specific ³²P-labelled oligonucleotide probe. Primers and probes are listed in Table 1. NASBA specific for transcripts encoding the snRNP U1A protein was performed as a control on all samples.

RISH. RISH was performed on paraffin sections as described previously (Jiwa et al., 1993). Briefly, hybridization mixtures consisted of 50% formamide (Merck), 10% dextran sulphate (Eppendorf 5 Prime), 0-2× SSC (0-5× SSC for EBER RISH) and probe diluted 1:50 (1:100 for EBER RISH). Slides were hybridized overnight at 55°C. To remove excess probe, slides were rinsed three times for 15 min each in 0-05× SSC (0-1× SSC for EBER) at 55°C. Bound probe was detected using mouse anti-digoxigenin antibody followed by sheep anti-mouse antibody and alkaline phosphatase-conjugated secondary antibody. Bound tertiary antibody was detected using NBT/BCIP.
Table 1. Sequences of oligonucleotide primers and probes

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<th>Oligonucleotide</th>
<th>Use</th>
<th>Sequence (5'→3')</th>
<th>Position*</th>
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<td>BamHI-A transcripts A3/A4 splice†</td>
<td>A3</td>
<td>Sense: agagaccagggcttgtaaaca</td>
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<td></td>
<td>A4</td>
<td>Antisense: aaccagccctcttccgag</td>
<td>159194–159175</td>
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<td></td>
<td>PA</td>
<td>Probe: aagacgttgaggccagcttg</td>
<td>157359–157378</td>
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<td>Transcripts encoding all isoforms of RK-BARF0 but not BARF0</td>
<td>RKBF0-s</td>
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<td>RKBF0-p</td>
<td>Probe: cgctgaggactgcaaactcc</td>
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<td>160688–160679/160376–160367</td>
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<td>RPMS1-p1n</td>
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<td>RPMS1-p2n</td>
<td>Antisense: accaaccagggctacgctg</td>
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<td>RPMS1-p</td>
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<td>BF0NAS-PL</td>
<td>Probe: gagaacgcgtgatgctgctcgcctgc</td>
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<td>Primers for cloning the BARF0 ORF</td>
<td>BARF0ORF1</td>
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<td>BARF0ORF2</td>
<td>Antisense: ttaatggaagagaatcaatagcttg</td>
<td>160994–160970</td>
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</table>

*As in the EMBL EBV sequence (B95-8), except for RPMS1-p1n, where the coordinates of EMBL HEHS4RAJ (Raji insertion) are given.
†A3/A4 oligonucleotides have been described previously by Brooks et al. (1993).
‡In combination with the RKBF0-fl antisense oligonucleotide, RKBF0-p and RKBF0-as served as sense primer and probe, respectively.
§The RKBF0-acc antisense oligonucleotides were used in combination with the RKBF0-s sense oligonucleotide and the RKBF0-p probe.
||The RPMS1 oligonucleotides have been described previously by Chen et al. (1999).
by biotinylated rabbit anti-mouse antibody (Dako) and streptavidin–biotin complex (SABC) (Dako) and visualized using DAB/nickel [for EBER, catalysed reporter deposition (CARD) (Kerstens et al., 1995) signal enhancement and aminoethylcarbazol visualization were used].

**Generation of recombinant BARF0 proteins and anti-BARF0 antibodies.** Prokaryotic recombinant BARF0 protein was generated by culturing *Escherichia coli* transformed with the BARF0-pCRscriptII plasmid described above until late exponential phase, followed by induction using 10 mM IPTG for 4 h. Eukaryotic recombinant BARF0 protein was generated by cloning the BARF0 ORF from the BARF0-pCRscriptII into the baculovirus genome using the FastBac system (Gibco-BRL). *Spodoptera frugiperda* 9(S9) cells were cultured with the recombinant virus for 48 h using an m.o.i. of 0.5. In addition, Flag-epitope-tagged RK-BARF0 and control vector lysate from H1299 epithelial cells was kindly provided by N. Raab-Traub, Chapel Hill, NC, USA (Kusano & Raab-Traub, 2001). mAbs were generated by standard polyclonal glycol fusion techniques using spleen cells from BALB/c mice immunized with synthetic peptides derived from either the BARF0 N-terminal region (BARF0-N, 5′ATATPLPCTDSMAALRPYELRLKHyG) or the C-terminal region (BARF0-C, 15′DGTYAPKAQAQIQGQFLQPMGVRHApH)5. Specific antibody activities were checked by determination of reactivity with the respective peptides and with the recombinant *E. coli* BARF0 in ELISA tests (Meij et al., 1999). Polyclonal antibodies to N- and C-terminal regions were raised in New Zealand White rabbits by immunization with Freund’s adjuvant and the BARF0-N and BARF0-C peptides coupled to keyhole limpet haemocyanin.

**Immunoblotting.** Bacterial pellets (from 1 ml culture after IPTG induction) or cell pellets (~1.5 × 10⁶ cultured cells) were suspended in sample buffer (0.2 M Tris/HCl, 4 % SDS, 18 % glycerol, 2 % β-mercaptoethanol and 0.004 % bromophenol blue) and lysed by sonication and heating at 100 °C for 5 min. Samples were subjected to electrophoresis on SDS-PAGE gels and equal protein loading was confirmed by Coomassie brilliant blue staining of the gels. Proteins were transferred to nitrocellulose filters (Schleicher & Schuell) by electroblotting for 1 h in 192 mM glycine, 2.5 mM Tris/HCl and 20 % methanol.

Filters were blocked with PBS containing 5 % non-fat dried milk, 5 % FCS and 0.05 % Tween-20 and incubated with primary antibodies against BARF0, EBNAl (OT1X) (Chen et al., 1993) or LMP1 (S12) (Jiwa et al., 1993) followed by horseradish peroxidase-conjugated rabbit anti-mouse antibody (Dako) or AP-conjugated anti-mouse antibody (Dako). For the detection of antibodies in human sera, these filters were incubated at 1:10 dilution in PBS containing 5 % non-fat dried milk, 5 % FCS and 0.05 % Tween-20 and bound antibodies were detected using horseradish peroxidase-conjugated rabbit anti-human IgG antibodies (Dako) as described by Meij et al. (1999). Antibody complexes were visualized using 0.05 % 4-chloro-1-naphthol, 0.01 % H₂O₂ or DAB/Cobalt. AP was visualized using NBT/BCIP (Kirkegaard & Perry Laboratories).

**Immunohistochemistry.** Snap-frozen sections of 5 μm thickness were mounted on SuperFrost Plus slides, air-dried, fixed in 4 % paraformaldehyde in PBS for 5 min and rinsed three times in PBS for 5 min. Endogenous peroxidase activity was quenched by incubation in 0.3 % H₂O₂ in PBS followed by three 5 min rinses in PBS. Slides were preincubated with normal rabbit serum prior to incubation with the specific mAb. Bound antibody was detected using a biotinylated rabbit anti-mouse antibody (Dako) and SABC, and visualized using DAB (for the cytospins of S9 cells, we used AP-conjugated rabbit anti-mouse and new fuchsin staining). LMP1 immunohistochemistry was performed using the S12 mAb and the same three-step detection (Jiwa et al., 1995); EBNAl immunohistochemistry was performed using the rat 2B4 mAb (Grässer et al., 1994) diluted 1:100, biotinylated goat anti-rat antibody and SABC with CARD enhancement (Oudejans et al., 1996).

**RESULTS**

All tumour samples included in this study were analysed for EBER1/2 RISH with a positive result. The presence of coding transcripts (EBNA1, LMP1, LMP2A and LMP2B) in RNA extracts from frozen materials of these tumours was analysed by multiprimed RT-PCR. All samples were positive for low-abundance snRNP U1A mRNA and revealed the expected pattern of EBV transcription indicating high-quality RNA (Brink et al., 1997a, b; Hayes et al., 1999).

**Detection of BARTs in situ**

Using RISH on archival paraffin-embedded material of various EBV-associated malignancies, we found positive results for A3/A4 spliced BARTs in neoplastic cells in 10 of 26 HDs, 1 of 1 ARL, 3 of 3 extranodal PTCLs, 3 of 7 BLs, 2 of 2 NPCs and 3 of 8 GCs. The BART riboprobe showed cytoplasmic staining (Fig. 2). Hybridization of consecutive slides showed signals in the same cells that showed nuclear staining with the EBER1/2 riboprobe. For both BARTs and EBER1/2, positive staining was found occasionally in small lymphoid cells. Hybridization with the sense riboprobe was always negative in these samples. Hybridization with an antisense probe specific for the housekeeping mRNA β-actin (van den Brule et al., 1991) showed positive staining of >90 % of cells in these samples, in both neoplastic and non-neoplastic cells. In samples that did not show any A3/A4 BART signal, the β-actin RISH was also negative when tested, indicating that RNA degradation was the most likely cause of the negative results. The reduced sensitivity of A3/A4 BART and β-actin RISH compared with EBER1/2 RISH may be explained by different levels of transcription for the respective targets.

**Analysis of BamHI-A transcripts in EBV-associated disorders by RT-PCR**

The results are summarized in Table 2. The sensitivities of the different RT-PCRs were equal to those described previously for other targets, allowing the detection of ~10–100 RNA copies of in vitro-transcribed RNA or one lymphoblastoid cell line equivalent (Brink et al., 1997a). All samples tested showed good RNA quality, as determined by the visibility of 18S/28S rRNA bands on the gel and the detection using RT-PCR of RNA from the housekeeping gene U1A, which is expressed at a low level.

We detected A3/A4 spliced BamHI-A transcripts by RT-PCR in all samples tested (Fig. 3A). Moreover, transcripts possibly encoding the RPMS1 ORF were also detected in all samples (Fig. 3B). These results are in agreement with data published previously. Although the results of RT-PCR cannot be interpreted quantitatively, it was interesting to...
note that the signal intensities of the A3/A4 spliced transcripts in the different samples strongly resembled those of RPMS1-encoding transcripts indicating equal transcription levels.

Using RT-PCR assays specific for full-length RK-BARF0 transcripts and transcripts spliced in the manner described by Kienzle et al. (1999a), we could not detect these transcripts in any of the samples tested, except in one HD sample that showed clear transcription of RK-BARF0 with the 160376–160689 (acceptor 2) splice (Table 2), whereas EBNA1 (Qp promoter driven), LMP1 and LMP2A mRNA were readily detectable in all HD samples (Brink et al., 1997a, b; Hayes et al., 1999).

**Transcription of the BARF0 ORF in EBV-associated disorders by NASBA and RT-PCR**

All samples tested showed the presence of amplifiable RNA and the absence of inhibiting factors, as shown by U1A

**Fig. 2.** RISH demonstrating A3/A4 spliced BamHI-A transcripts in Reed–Sternberg cells of a EBV-positive HD (A), in neoplastic epithelium of a NPC (B) and in neoplastic cells of an ARL (C). Hybridizations with sense riboprobes (all DAB/nickel staining with nuclear fast red counterstaining) were negative. EBER RISH served as positive control (aminoethylcarbazol with haematoxylin counterstaining). EBER RISH gave clear nuclear staining, while A3/A4 RISH resulted in cytoplasmic staining. Sense controls were negative. In (A) and (C), dispersed tumour cells can be seen.
Table 2. Analysis of BamHI-A transcripts in EBV-associated disorders

W, Weak result; +, positive result; −, negative result; ND, not determined.

<table>
<thead>
<tr>
<th>Sample</th>
<th>No.</th>
<th>A3/A4</th>
<th>RPMS1</th>
<th>A3/A4</th>
<th>RPMS1</th>
<th>RT-PCR</th>
<th>RK-BARF0</th>
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<td>PTCL (extranodal)</td>
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<td>+</td>
<td>+</td>
<td>w</td>
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Fig. 3. RT-PCR analysis of A3/A4 spliced BamHI-A transcripts (A), RPMS1-encoding BamHI-A transcripts (B) and NASBA analysis of transcripts encoding the (RK-)BARF0 ORF (C) in diverse EBV-associated disorders (see Table 2). Water (W) was included as a negative control.
Expression of BARF0 and RK-BARF0 proteins in vivo

The newly generated 4H7 mAb was used to detect the BARF0 or RK-BARF0 proteins in snap-frozen material from EBV-positive disorders (one HD, one NPC, one BL, one nodal anaplastic large cell lymphoma and one nodal PTCL). Using the 4H7 mAb in immunohistochemistry, we found no signal in any of these samples, whereas EBNA1 immunohistochemistry using the 2B4 mAb (Oudejans et al., 1996) was positive in neoplastic cells of these samples (data not shown). In addition, the HD sample showed clear staining for LMP1 using the S12 mAb (Jiwa et al., 1995). Cytosplains of the EBV-positive cell lines Raji and JY were also negative for BARF0 and RK-BARF0 using either 4H7 or 6C1 mAbs but were positive for EBNA1 and LMP1. When Raji and JY cells and the C15 tumour were subjected to SDS-PAGE and immunoblot analysis using the 4H7 and 6C1 mAbs, no BARF0-related signal was found, although it was possible to detect EBNA1 and LMP1 protein using the OT1X and S12 mAbs (data not shown). Fig. 4 shows the expression of EBNA1 and LMP1 in the C15 tumour at the RNA and protein level. Strips were cut from a single blot ensuring that equal amounts of C15 protein were analysed. Similarly, a single RNA extract was analysed simultaneously for each target. The low copy number U1A RNA transcript was used as a cellular RNA control (Fig. 4B). Whereas BARF0 transcripts could be detected clearly by RT-PCR and NASBA analyses, immunoblotting using either monoclonal or polyclonal antibodies gave consistent negative results in C15 tumour cell extracts. Baculovirus-expressed BARF0 protein was detected readily by either 4H7 or 6C1 mAbs in parallel immunostaining experiments (Figs 4 and 5).

The reactivity of the 4H7 mAb with paraffin-embedded baculovirus-expressed BARF0 material allowed us to perform BARF0 immunohistochemistry on the paraffin-embedded HDs, NPCs and the ARL that had been found to be positive for BARTs in the RISH analysis (Fig. 2). Moreover, paraffin-embedded material was available for the BARF0 NASBA-positive PTLDs (Fig. 3C, samples 7–10). First, immunohistochemistry was performed with and without antigen retrieval (0·1 M citrate buffer, pH 6, in a pressure cooker for 10 min), using various dilutions of the primary antibody with and without CARD signal amplification (data not shown). In paraffin-embedded Sf9/BARF0 cells that were used as a positive control, clear staining was observed using undiluted 4H7 mAb without antigen retrieval and without CARD signal enhancement. Using antigen retrieval, a much stronger signal was obtained in these cells, even when the primary antibody was diluted up to 500 times. However, using this optimized procedure, even with antigen retrieval and using undiluted primary antibody and CARD signal enhancement, we were not able to detect BARF0 protein in the clinical samples tested (one nasal PTCL, two NPCs, three HDs, one ARL, four PTLDs, one IM and one OHL), nor in paraffin-embedded Raji or JY cells, despite detectable BARF0 RNA expression.
Detection of human antibody responses against recombinant BARF0

Immunoblot analysis was used to detect antibody responses against the recombinant baculovirus-expressed BARF0 in the sera of patients with different EBV-associated diseases and healthy donors (Fig. 6). As a positive control for the quality and reactivity of the sera, antibody responses against viral proteins expressed by the HH514 cell line were determined (Fig. 6A) (Meij et al., 1999; Middeldorp & Herbrink, 1988; van Grunsven et al., 1993). As a negative control, we used a blot prepared with extracts from Sf9 cells infected with wild-type baculovirus (Fig. 6C). Although antibody responses against known EBV proteins as produced by the HH514 cell line were detected clearly in the sera from various patients and EBV seropositive donors, we were not able to detect any anti-BARF0-specific reactivity in these sera (Fig. 6B). Parallel experiments using mouse or rabbit sera from animals immunized with the BARF0 protein or peptides clearly allowed the detection of BARF0-specific antibody staining (Fig. 6B, lane 1; see also Fig. 5). The same sera were also analysed by indirect immunofluorescence on cytopsins of Sf9 cells infected with BARF0 recombinant baculovirus and cultured for 0, 24 and 48 h (left to right). An increase in the proportion of infected cells and protein expression levels with time can be seen. Results were visualized with AP/new fuchsine staining, with haematoxylin counterstaining.
of (human) antibody binding was related to conformational epitopes.

**DISCUSSION**

In this study, we have analysed the expression of the EBV BamHI-A region in diverse EBV-associated disorders. Our results showed, as detected by A3/A4 RT-PCR and RISH, that this region is transcribed in all types of EBV-associated disorders. This transcription is regardless of cellular origin, as indicated by the finding that both lymphoid and epithelial disorders were positive. Moreover, the BamHI-A region was transcribed regardless of the expression of other EBV latent genes. For example, BamHI-A RNA expression was demonstrated in BLs, which usually display the restricted latency type I expression pattern, and in HD and T-cell non-Hodgkin’s lymphoma (T-NHL), which display the latency type II pattern, as well as in PTLDs, which usually show expression of all known EBV latent genes (latency type III). These findings are in line with previous studies in which BARTs were found in all latency types in vitro (Brooks et al., 1993) and in vivo (Oudejans et al., 1995). Furthermore, we have shown using RISH that BamHI-A transcription actually occurs within the (EBER1/2-positive) neoplastic cells of the EBV-associated disorders. This was demonstrated for NPCs in a previous study (Gilligan et al., 1991); our findings confirm and extend these observations. The fact that BARTs are present in the cytoplasm of neoplastic
cells (Fig. 2) indicates that these transcripts or the putative encoded proteins may play a role in maintenance and/or growth of these neoplastic cells. Interestingly, in contrast to the non-coding EBER1/2 RNAs, which showed a nuclear localization, the BARTs RISH revealed a cytoplasmic staining pattern, suggesting active transport of mature mRNAs with possible translation.

The BARTs putatively encode multiple protein species, such as RPMS1, A73, BARF0 and RK-BARF0. Given the putative homology between RPMS1 and EBNA2 (Smith et al., 1993) and the fact that RPMS1 transcripts have been found previously in resting B-cells (Chen et al., 1999), it is tempting to speculate that RPMS1 acts as a substitute for EBNA2 in other circumstances where EBNA2 is not expressed (for example, HDs and BLs). However, we have shown that transcripts encoding RPMS1 are detectable in various types of EBV-associated disorders, regardless of their origin or their latency type, thus including cases with detectable EBNA2 expression. Moreover, the signal intensities obtained with RPMS1 RT-PCR closely resembled those obtained with the A3/A4 RT-PCR. This suggests that transcripts detected with the A3/A4 RT-PCR, which have an exon V–VI–VII splice and thus possibly encode BARF0 but not RK-BARF0, also contain the RPMS1 ORF. This is in line with the previous finding that RK-BARF0-encoding transcripts never contain the RPMS1 ORF (Sadler & Raab-Traub, 1995). Interestingly, we were not able to detect any transcripts encoding either full-length RK-BARF0 or the recently reported (Kienzle et al., 1999a) splice variants of RK-BARF0 in our samples (with the exception of one HD sample). Moreover, this suggests that the signals obtained with the NASBA analysis are caused by the presence of V–VI–VII-spliced BARF0-encoding transcripts only and not by RK-BARF0-encoding transcripts. The fact that we find this signal only in a subset of clinical samples with relatively high numbers of EBV-positive cells may reflect the relatively low abundance of transcripts containing the entire BARF0 ORF and relatively high abundance of truncated transcripts, as reported previously (Sadler & Raab-Traub, 1995; Smith, 2001; Smith et al., 2000). Alternatively, it may reflect yet another splicing pattern.

Using newly generated anti-BARF0 monoclonal and polyclonal antibodies with defined reactivity against native or denatured BARF0 protein, we were not able to detect the BARF0 protein in any of the EBV-positive cell lines and clinical samples tested, even when these had proved to be positive for the encoding transcripts in RISH, RT-PCR or NASBA analysis. mAbs were raised using a peptide that is localized upstream of the NASBA primer pair within the putative BARF0 ORF. Therefore, a positive NASBA result is likely to coincide with positive immunostaining, except when the protein is not translated at all or is processed with a high turnover in combination with a low expression level. The fact that the polyadenylation signal of all BamHI-A transcripts containing the BARF0 ORF precedes the stop codon has led previously to the hypothesis that BARF0 and RK-BARF0 are not translated (Karran et al., 1992; Sadler & Raab-Traub, 1995; Smith et al., 2000) and our results support this notion. Previously, BARF0 and RK-BARF0 protein expression was observed only in in vitro expression systems. However, for BARF0 and RK-BARF0 protein detection, thus far only anti-tag antibodies have been used. In initial experiments, polyclonal anti-BARF0 antibodies were used, which were shown later to cross-react with a human protein of the same size (Fries et al., 1997; Kienzle et al., 1999b; Schroder et al., 2002). The absence of BARF0 and RK-BARF0 protein in EBV-transformed cell lines in vitro and in the neoplastic cells of EBV-associated malignancies in vivo supports the finding that the protein is dispensable for EBV-mediated transformation (Robertson et al., 1994).

When mAbs are generated using synthetic peptides, there is a possibility that they do not recognize the full-length protein. However, this is unlikely for the newly generated anti-BARF0 mAbs in this study, since they do recognize the eukaryotic recombinant protein (in baculovirus-expressed BARF0) in both its linear and its native full-length conformation. In addition, these mAbs recognize the Flag-tagged RK-BARF0 protein expressed in the human carcinoma cell line H1299 (Kusano & Raab-Traub, 2001). Moreover, differences in the glycosylation pattern of the recombinant and the native protein can be ruled out because no glycosylation sites are present in the region where the 4H7 and rabbit antibodies bind. Furthermore, parallel staining for EBNA1 and LMP1 using mAbs yielded a positive result in most tissues and cell lines examined.

Previous studies in a limited number of individuals have shown antibody (Gilligan et al., 1991) and CTL responses (Kienzle et al., 1998) directed against in vitro-generated BART-encoded polypeptides and overexpressed BARF0, respectively, suggesting that BARF0 may be expressed in vivo. However, we were unable to detect any antibody responses against the baculovirus-expressed recombinant BARF0 protein using sera from patients with different EBV-associated syndromes. This discrepancy may reflect technical differences between the recognition of full-length BARF0 and in vitro-generated polypeptides as used by Gilligan et al. (1991). It cannot be excluded that BARF0 expression occurs during the primary or productive phase of infection, yielding BARF0-specific memory B- and T-cells. Our finding that an IM biopsy was also negative for BARF0 by immunohistochemistry suggests that, if BARF0 protein expression occurs during primary infection, it would be a very early feature or expression would occur, at best, at very limited levels. In addition, no BARF0 protein was detected by immunoblot analysis on an OHL biopsy or using HH514 cells chemically induced into early antigen or viral capsid antigen phase (Fig. 6A, lane 1) (Middeldorp & Herbrink, 1988; van Grunsven et al., 1993). In addition, the lack of detectable anti-BARF0 antibody responses in IM sera, NPC sera and even in sera from patients with chronic EBV infection that otherwise react with a wide spectrum of
EBV proteins, especially from early antigen phase, support our in situ data that BARF0-encoding transcripts do not lead to the production of protein in vivo.

The RPSM1 protein may still play an important role in EBV-positive malignancies. However, it is unlikely that RPSM1 acts as an exclusive substitute for EBNA2 because BARTs encoding RPSM1 were detected in all clinical samples tested, including PTLDs, which are known to express EBNA2. The expression of RPSM1 at the protein level remains to be investigated.

In conclusion, our data show that, in vivo, BARTs specifically encoding RK-BARF0 cannot be detected. However, BARTs do contain (part of) the BARF0 ORF, but BARF0 protein expression and antibody responses against BARF0 were not detected. This indicates that the putative BARF0 protein may not be expressed at all or may at best have a high turnover combined with a very low expression level. Therefore, in vivo, the BARF0 protein itself may not have a role in established EBV-positive malignancies. On the other hand, a role for the BARF0 RNA as an antisense regulator cannot be excluded.

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