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Expression of Epstein–Barr virus nuclear antigen-1 induces B cell neoplasia in transgenic mice

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The Epstein–Barr virus (EBV) nuclear antigen-1 (EBNA-1) is a pleiotropic protein which has been characterized extensively both biochemically and functionally. It is the only one of the identified latent protein-encoding genes to be consistently expressed in viral-associated endemic Burkitt's lymphoma cells. As such, it is the only candidate viral protein to possibly perform a maintenance function in the tumour pathology. Despite this, no oncogenic activity has been attributed to the protein in tissue culture assays. The experiments described here were initiated to explore the activity of the protein in B cells *in vivo*. EBNA-1 transgenic mice were generated with transgene expression directed to the B cell compartment using the mouse Ig heavy chain intron enhancer. Transgene expression was demonstrated in the lymphoid tissues of mice of two independent lines. Transgenic positive mice of both lines succumb to B cell lymphoma. The B cell tumours are monoclonal, frequently of follicular centre cell origin and remarkably similar to those induced by transgenic c-*myc* expression. These results demonstrate that EBNA-1 is oncogenic *in vivo* and suggest that the gene product may play a direct role in the pathogenesis of Burkitt's lymphoma and possibly other EBV-associated malignancies.

Keywords: B cell/EBNA-1/Epstein–Barr virus/lymphoma/transgenic mice

Introduction

Epstein–Barr virus (EBV) is associated with several human malignancies, including nasopharyngeal carcinoma, endemic Burkitt's lymphoma (BL) and the lymphomas to which immunosuppressed individuals are prone. In support of the premise that the virus plays a role in the pathogenesis of at least the B cell malignancies, primary human B cells are efficiently immortalized upon EBV infection in culture. Lymphoblastoid cell lines (LCLs) derived following infection express eight characterized viral protein-encoding genes (the latent genes), six encoding EBV nuclear antigens [EBNA-1, -2, -3A, -3B, -3C and -LP (or with the alternative nomenclature EBNA-1, -2, -3, -4, -6 and -5 respectively)] and two encoding latent membrane proteins [LMP-1 and LMP-2 (alternative nomenclature LMP and TP respectively)], as well as two small untranslated RNAs (EBER1 and EBER2). Most of the studies aimed at ascertaining the role of the viral proteins in cell transformation have been conducted using the accessible system of cultured B cells, either tumour derived or immortalized by EBV *in vitro*. As a consequence, certain latent genes (EBNA-2, EBNA-3A, EBNA-3C, EBNA-LP and LMP-1) have been apportioned an essential role in B cell transformation (Cohen *et al.*, 1989; Hammerschmidt and Sugden, 1989; Mannick *et al.*, 1991; Kaye *et al.*, 1993; Tomkinson *et al.*, 1993). However, the patterns of viral latent gene expression in cell culture and *in vivo* are profoundly different (Qu and Rowe, 1992; Chen *et al.*, 1995). Moreover, viral expression in tumours differs from *in vitro*-infected B cells. EBV-positive BL biopsies and BL phenotype cell lines exemplify this, because in these cells, EBNA-1 is the only latent protein that is detected consistently (Rowe *et al.*, 1987). EBNA-1 may therefore perform a critical function in the onset, progression or maintenance of this tumour.

EBNA-1 is expressed in all cells carrying the virus. It is a functionally pleiotropic protein, as well as a DNA binding protein for which a consensus binding site has been identified (Rawlins *et al.*, 1985; Reisman and Sugden, 1986, Ambinder *et al.*, 1990). The protein binds DNA as a homodimer, and both DNA binding and dimerization domains map to the C-terminus of the protein (Ambinder *et al.*, 1991; Chen *et al.*, 1994). EBNA-1 has an established role in the replication and maintenance of the episomal form of the viral genome, mediated by protein binding sites at the latent origin of replication (oriP, in the *Bam*HI C region of the genome; Yates *et al.*, 1984, 1985; Lupton and Levine, 1985; Chittenden *et al.*, 1989). OriP is composed of two essential elements, a site of dyad symmetry, containing four EBNA-1 binding sites at which latent replication is initiated, downstream of a repeated unit containing a further 20 copies of the binding site (Reisman *et al.*, 1985; Gahn and Schildkraut, 1989). The 20 repeat unit of OriP also functions as an enhancer in the presence of EBNA-1, with a proposed role in transactivation of the proximal latent promoters (Reisman and Sugden, 1986; Sugden and Warren, 1989; Wysokenski and Yates, 1989; Gahn and Sugden, 1995). Further, weaker EBNA-1 binding sites exist (in the *Bam*HI Q region of the genome) downstream of an alternate promoter for the exclusive expression of EBNA-1 in BL and nasopharyngeal carcinoma cells (Jones *et al.*, 1989; Ambinder *et al.*, 1990; Sample *et al.*, 1991; Schaefer *et al.*, 1991; Smith and Griffin, 1992; Nonkwelo *et al.*, 1995). This latter cluster of EBNA-1 binding sites can act as a negative regulatory element for expression in the presence of EBNA-1 (Sample *et al.*, 1992). A further activity of EBNA-1 is that it can bind to RNA, at least *in vitro*, and might therefore be capable of controlling expression at the post-transcriptional level (Snudden *et al.*, 1994). This

Table I. Summary of EμEBNA-1 transgenic lines

Line	Copy No.	Phenotype[a]	RNA[b]		Protein							Genotype[c]
			a	b	Spleen	Lymph node	Thymus	Liver	Brain	Heart	Kidney	
26	2	+++	+++	+++	+	+	–	–	–	–	–	+
59	1–2	++	–	++	+++	+++	+	–	+	–	–	++
60	1[r]	–	–	±	–	ND	–	–	–	–	–	++
61	10	–	–	–	–	ND	ND	ND	ND	ND	ND	+(Y)

(–) No expression detected; (+, ++, +++) relative levels of expression; ND = not determined; r, rearranged.
[a]The degree or absence of a phenotype is represented by + or – signs respectively.
[b]a, by Northern blot analyses of splenic and lymph node tissues; b, by RNase protection analyses of splenic and lymph node tissues.
[c](+) Hemizygous; (++) homozygous; (Y) Y chromosome insert.

combination of characteristics would certainly qualify the protein as a potential oncogene.

Despite the wealth of information concerning the activity of the protein, direct involvement in the oncogenic process has not been demonstrated previously. The requirement for the protein in the viral life cycle in latent infection has precluded the use of many of the functional assays for transformation in culture. Any method to inhibit the expression of EBNA-1 in EBV-positive cells should also inhibit viral presence, and would therefore measure entire viral activity instead of EBNA-1-specific activity. The expression of EBNA-1 in rodent fibroblasts or primary B cells in culture has not been demonstrated to induce a transformed phenotype; nor has it been shown to cooperate with other oncogenes to transform cells in culture.

Nevertheless, the compelling indications that EBNA-1 may perform an oncogenic function, at least in B cells, which is not accessible in culture, prompted this investigation into the action of the protein *in vivo* in transgenic mice. Transgenic mice were generated harbouring a transgene designed to direct EBNA-1 expression to the B cell compartment. The tissue-specific expression of EBNA-1 in mice of these lines induced a predisposition to B cell lymphoma, thereby demonstrating that EBNA-1 is indeed a viral oncogene. It is thus likely that the protein plays a significant role in the pathogenesis of B cell neoplasia, in accordance with the exclusive expression of this viral protein in endemic BL. The absence of detectable changes in cell growth following transfection of the gene in culture may reflect the lack of a suitable assay system and raises the possibility that the protein acts through a unique route which has been inaccessible to conventional analysis in culture, as discussed later.

Results

Production of EμEBNA-1 transgenic mice and the phenotype of lymphoma

We generated 10 lines of transgenic mice with the transgene linking the EBNA-1 coding region of the B95-8 strain of EBV to the polyomavirus (Py) early promoter and mouse Ig heavy chain (IgH) intronic enhancer (Eμ; Wilson and Levine, 1992). Four of these lines are the focus of this study, denoted 26, 59, 60 and 61. Lines 26 and 59 harbour one or two copies of the transgene (with at least one intact) at an autosomal site, line 61 harbours multiple copies of the intact transgene (~10) with an inheritance pattern indicative of integration in the Y chromosome, while line 60 harbours a single rearranged copy with the EBNA-1 coding region interrupted, at an autosomal site (Table I). The zygotes used for microinjection were F_2 for strains C57Bl/6J and DBA/2J. Mice of these lines have been backcrossed to strain C57Bl/6 (up to the eighth backcross) without any change in phenotype. Mice of lines 59 and 60 were also successfully bred to homozygosity for the transgene. Transgene homozygous mice of line 26 showed poor viability and did not breed and the line is therefore maintained in the hemizygous state. Mice of line 26 have also been crossed into the strain Balb/cAnN without impact on the lymphoma phenotype described below.

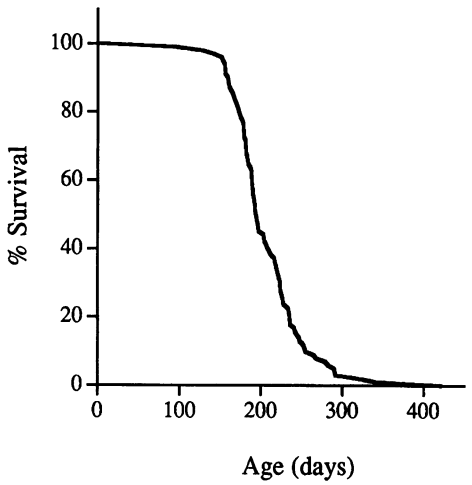

Fig. 1. Longevity of line 26 mice succumbing to lymphoma. The sample size is 61, representing five generations of mice from the initial strain mix B6D2 F_2 backcrossed into the strain C57Bl/6J. The mean is 200 days and median 235 days. No significant differences were apparent between the generations.

Mice of lines 26 and 59 succumb to B cell lymphoma, while mice of lines 60 and 61 display no pathological phenotype. The hallmark of the disease of transgene-positive mice of lines 26 and 59 is a massive enlargement of the spleen accompanied by massive enlargement of the liver and/or lymph nodes. The enlarged organs are replete with the neoplastic cells, and the blood contains large numbers of similar cells. Other tissues are often invaded. The typical pathological diagnosis is therefore neoplastic lymphoma with multi-organ involvement and associated leukaemia. The disease is 100% penetrant in transgene-positive mice of line 26, the mice succumbing within the age range of 4–12 months (Figures 1 and 2). Transgene-positive mice of line 59 demonstrate a dramatically longer latency to the age of onset of the disease (Table II). The

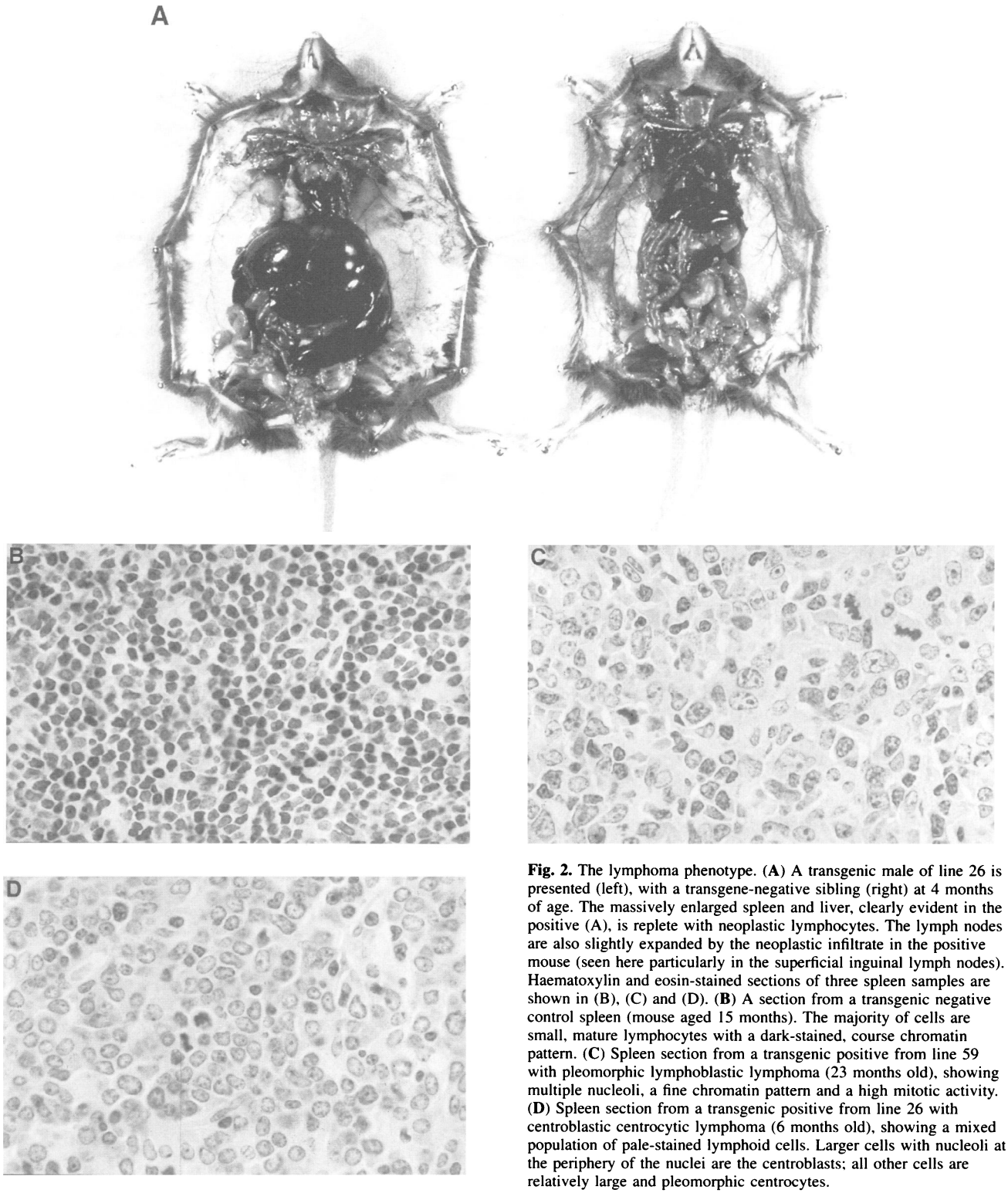

Fig. 2. The lymphoma phenotype. (**A**) A transgenic male of line 26 is presented (left), with a transgene-negative sibling (right) at 4 months of age. The massively enlarged spleen and liver, clearly evident in the positive (A), is replete with neoplastic lymphocytes. The lymph nodes are also slightly expanded by the neoplastic infiltrate in the positive mouse (seen here particularly in the superficial inguinal lymph nodes). Haematoxylin and eosin-stained sections of three spleen samples are shown in (B), (C) and (D). (**B**) A section from a transgenic negative control spleen (mouse aged 15 months). The majority of cells are small, mature lymphocytes with a dark-stained, course chromatin pattern. (**C**) Spleen section from a transgenic positive from line 59 with pleomorphic lymphoblastic lymphoma (23 months old), showing multiple nucleoli, a fine chromatin pattern and a high mitotic activity. (**D**) Spleen section from a transgenic positive from line 26 with centroblastic centrocytic lymphoma (6 months old), showing a mixed population of pale-stained lymphoid cells. Larger cells with nucleoli at the periphery of the nuclei are the centroblasts; all other cells are relatively large and pleomorphic centrocytes.

frequency of occurrence of the phenotype in line 59 has been studied by cross-sectional sampling of mice of different age and conducting a histopathological analysis, predominantly using splenic tissue (but also tissue from lymph nodes and the liver in many instances). The incidence of neoplastic, pre-neoplastic and hyperplastic pathology recorded for line 59 positive mice was significantly higher when compared with strain- and age-matched transgenic negative controls. In all, 43% (6/14) of line 59 mice sampled at 2 years of age had neoplastic lesions in the spleen, while 6% (1/17) of age-matched controls, housed under the same conditions, being given the same diet, and studied under the same sampling regime and in the same genetic backgrounds, had neoplastic lesions (Table II). Moreover, the single incidence of lymphoid neoplasia scored in the controls presented as a focal

Table II. The phenotype of line 59

Phenotype	Line 59				Controls			
	3–12	13–18	19+	24	3–12	13–18	19+	24
Neoplastic lymphoma	0	0	13	4	0	0	1	1
Early neoplasia	1	1	4	2	0	0	1	0
Splenic hyperplasia	3	5	12	2	1	0	2	2
Total number of mice	17	29	80	14	18	18	26	17
% Lymphoma[a]	6	3.5	21	43	0	0	7.7	6

Controls include mice from lines 60 and 61 and stock C57Bl/6. 19+, all mice >19 months old (including those at 24 months in the last column).
[a]The percentage of mice with lymphoma is derived from mice in the two phenotypic categories: neoplastic lymphoma and early neoplastic lesions.

mesenteric lymph node tumour, unlike the appearance of the tumours observed in lines 26 and 59 (Figure 2). Splenic hyperplasia has been detected in non-transgenic controls, but at a reduced frequency compared with line 59 mice. The mouse colony is housed under conventional non-barrier conditions and, as such, the cause of splenic hyperplasia is difficult to designate as either very early changes towards neoplasia or simply reactive to infection. Nevertheless, expansion of the splenic white pulp, with an associated increase in immature cell types, has been a consistent observation in spleen samples taken from line 26 mice prior to the development of neoplasia. Thus, in lines 26 and 59 this may represent an EBNA-1-induced pre-neoplastic stage.

The transplantation of splenocytes from the enlarged spleens of line 26 mice into syngeneic hosts established that these were frank, malignant tumours rather than massive proliferations of non-neoplastic cells. Rapidly growing tumours arose in the B6D2 F_1 hybrid recipients, being palpable 4–10 weeks after injection (either intravenous or intraperitoneal).

The morphology of the tumour cells from lines 26 and 59 varied from sample to sample and appeared to represent B cells at different stages of development. However, the tumours were always diffuse and not follicular in nature, and the lymphoma cell morphology was predominantly lymphoblastic or centrocytic/centroblastic (Figure 2). Interestingly, BL has been described as a proliferation of centroblasts (Gregory *et al.*, 1987).

A Southern analysis of genomic DNAs from lymphoma-invaded tissue from mice of both lines has demonstrated that all of the tumours analysed were monoclonal and had undergone rearrangements of the IgH locus (Wilson and Levine, 1992). Tumour samples from line 26 mice were analysed further to ascertain the genomic configuration of the Ig light chain loci and T-cell receptor loci. The κ light chain locus was monoclonally rearranged in 50% of samples from different mice, while no λ light chain rearrangements were detected (Wilson and Levine, 1992) and T-cell receptor β and γ chain rearrangements were never seen (data not shown). These data confirm the histopathological diagnosis of B cell tumours with variation in the stage of differentiation, although all have undergone IgH rearrangements.

Transcription of the EBNA-1 transgene

From RNase protection assays (Figure 3), it was found that the transgene is transcribed in line 26, at a lower level in line 59 and at a very low level in line 60. No transgene-specific transcripts have been detected in line

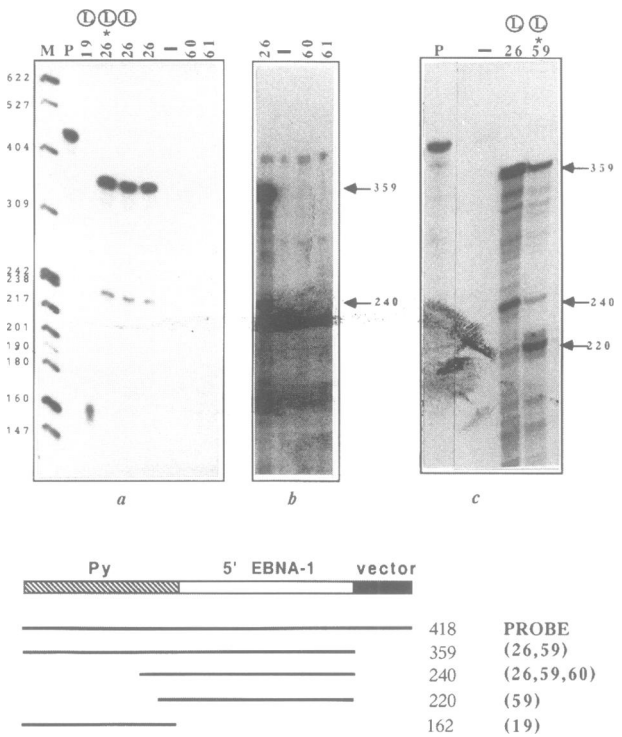

Fig. 3. Transgene-specific transcription. RNase protection assays using 5′ transgene sequences as a probe. The intact probe (track P, 418 nucleotides) and sizes of the major protected fragments are indicated and depicted below. Total RNA samples derived from spleens were used from mice of the lines 26, 59, 60 and 61 as well as transgene-negative controls (–). Also as a control, a sample derived from an unrelated transgenic line has been included (line 19), which harbours a Py promoter linked gene (without EBNA-1), thereby protecting the 162 nucleotide Py-only fragment. An asterisk (*) above the tracks indicates that the sample was derived from an individual homozygous for the transgene. L indicates that the sample was derived from a mouse with lymphoma. (**a**) and (**b**) Overnight and 1 week exposures (respectively), comparing levels of expression between lines 26 and 60. (**c**) Comparison between lines 26 and 59. Samples were normalized for loading using a GAPDH probe in conjunction with the transgene probe, as described in Materials and methods.

61 samples. The steady state transgene message levels are such that only transcripts in line 26 samples are detectable by Northern blotting using total RNA from spleen or lymph node tissues, with expression in line 59 and line 60 samples being below the resolution of this assay (as are the EBNA-1 transcripts in the EBV-positive human B cell line controls; Figure 4). There is some variability in the levels of detectable transgene-specific transcripts in the tumour-bearing tissue samples from line 26 and line 59 mice, presumably reflecting the extent of tumour cell

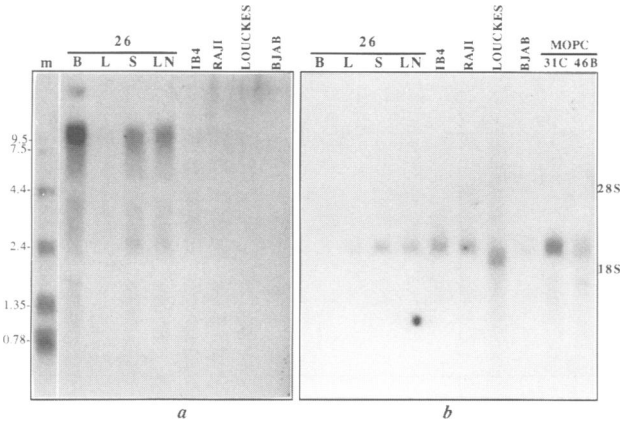

Fig. 4. Expression in line 26. Northern blots of 20 μg total RNA samples isolated from a tumour-bearing line 26 mouse from lymphoma-positive tissues: liver (L), spleen (S) and lymph node (LN), and lymphoma-free brain (B). RNA samples from cell lines are indicated: IB4 (B95-8 strain EBV-positive human B cell line), Raji (EBV-positive BL cell line), Louckes (EBV-negative BL cell line), Bjab (EBV-negative variant BL cell line) and MOPC (two mouse plasmacytoma cell lines; 31C and 46B). (**a**) Transgene-specific transcripts were detected using a 3′ EBNA-1 fragment of the transgene plasmid pEμEBNA-1 as a probe. (**b**) c-*myc* expression was detected following striping of the blot in (a) and probing with an equal concentration and c.p.m. mix of the radiolabelled mouse and human 420 bp *Pst*I fragments of the c-*myc* gene exon II. The marker track is a 20 μg sample of transgene negative RNA, including a radiolabelled RNA ladder (BRL system).

contribution to the sample. Nevertheless, a transgene-specific transcript(s) of >9.5 kb is detectable in spleen samples of all line 26 mice tested by Northern analysis and not in any line 59 sample (Figure 4). Surprisingly, quantification of the overall level of 5′-protected transcript fragments assayed by RNase protection [using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal sample control] revealed only a 2-fold higher level in a line 26 tumour-bearing spleen sample compared with a homozygous line 59 tumour-bearing spleen sample (Figure 3C). Detection of the 5′-protected transcript in a line 60 spleen sample was ~100-fold lower than in line 26 (Figure 3B). The higher level of transgene-specific transcripts in splenic samples from line 26 compared with line 59 is supported further using the method of RT-PCR, in which line 59 RNA samples consistently require more rounds of amplification than line 26 RNA samples to detect the products (data not shown).

The >9.5 kb transcript detected in line 26 samples by Northern analyses is larger than the entire tandem transgene insert in this line, which is contained within an 8.5 kb *Bcl*I genomic fragment. Thus, this message must be a hybrid with cellular sequences. Transcription is detectable specifically in spleen and lymph node samples, as well as in the brain (Figure 4). However, no expression at the protein level is detected in the brain (Figure 5). Transcription in the brain without protein expression is not an unusual observation in transgenic studies.

Transgene transcription initiation sites have been mapped by RNase protection using a Py-5′-EBNA-1 fragment as a probe. The transcript initiation sites are the same for line 26 and line 59 samples, but they are differentially utilized between the lines (Figure 3). Three major protected fragments are detected. A 359 nucleotide fragment results from protection of the entire transgene sequences of the 5′ probe and is indicative of upstream initiation at an unknown site(s). Two further fragments of ~240 nucleotides and a doublet at 220 nucleotides represent initiation within the Py early promoter ~10 and 30 bp 3′ to the TATA box (respectively). In line 26 samples, the upstream start site (359 fragment) is the principal start site, with the 240 nucleotide Py start site used to a lesser degree and the 220 nucleotide fragment seen as a minor component. In contrast, in line 59 samples, the 220 nucleotide fragment(s) represents the primary start site, with upstream-initiated transcripts (359 fragment) detected at 2-fold lower levels and the 240 nucleotide fragment seen as the minor component. The relative start site usage is consistent between samples from different mice of the two lines, irrespective of age or degree of tumour development. This is exemplified by the three line 26 spleen samples shown in Figure 3a, which were derived from a transgene homozygous, tumour-bearing mouse aged 6.5 months [26*(L)], a hemizygous, tumour-bearing mouse aged 7 months [26(L)] and a hemizygous mouse prior to tumour development aged 3 months (26). All demonstrate the same pattern of expression, with the sample from the homozygous mouse predictably showing 2-fold higher levels of expression. The only protected fragment observed in the line 60 sample is the 240 nucleotide fragment (Figure 3b). The differential usage of transcription initiation sites is likely to be influenced by both the configuration of the integrated transgenes and the cellular sequences at the sites of insertion.

The observed levels of steady state RNA correlate with the degree of the neoplastic phenotype. Transcription is highest in line 26 samples, the line with the most penetrant phenotype. Transcription is lower in samples from line 59 with a phenotype of longer latency. Transcription from a coding region-rearranged transgene is barely detectable in samples from line 60 without pathological phenotype, and no transcription is detectable in samples from line 61 with normal phenotype.

EBNA-1 expression

Expression of the protein product has been examined using anti-EBNA-1 antisera. Tissue extracts were immunoprecipitated with either a monoclonal antiserum (Aza2E8; Hearing *et al.*, 1985) or a polyclonal human serum (WS). Immunoprecipitated products were visualized by Western blotting using the polyclonal serum WS (Figure 5). Protein extracts were tested from spleen, lymph nodes, thymus, brain, liver, heart and kidney. No EBNA-1 expression was observed in any tissue of the phenotypically negative lines 60 and 61, in agreement with the transcriptional studies. Expression of EBNA-1 of the correct size for the B95-8 strain (88 kDa) was detected in tissues of lines 26 and 59 (Figure 5). Results from a preliminary single-strand conformation polymorphism analysis of the integrated transgenes revealed no differences in the coding regions (data not shown). They support the idea that the protein expressed in both lines is wild type for the EBV strain B95-8. EBNA-1 expression in line 26 mice is restricted to spleen and lymph nodes and is expressed in mice of all ages analysed (from the earliest time point studied at 1 month old, prior to tumour development). Expression could only be detected in other tissues following tumour

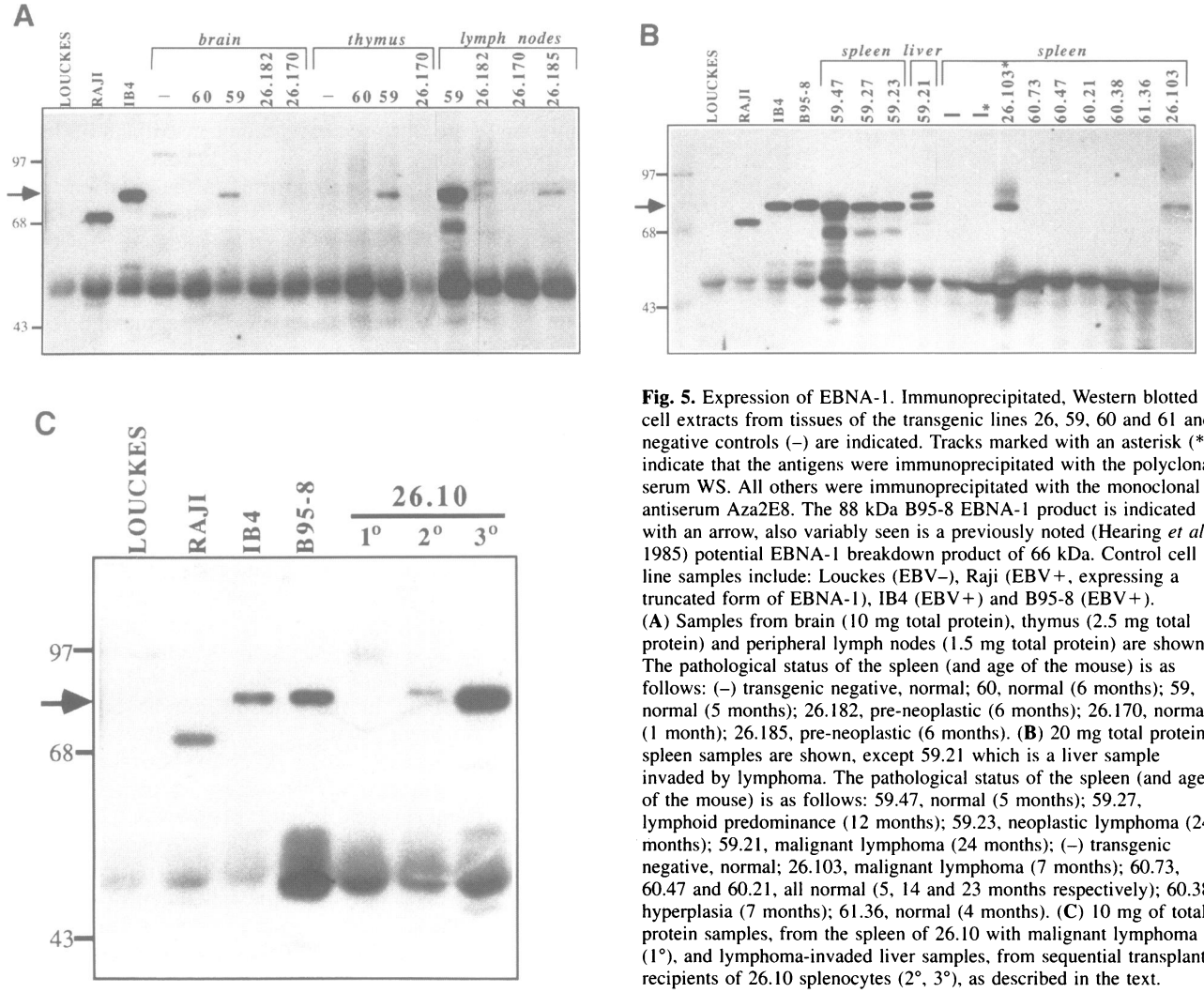

Fig. 5. Expression of EBNA-1. Immunoprecipitated, Western blotted cell extracts from tissues of the transgenic lines 26, 59, 60 and 61 and negative controls (–) are indicated. Tracks marked with an asterisk (*) indicate that the antigens were immunoprecipitated with the polyclonal serum WS. All others were immunoprecipitated with the monoclonal antiserum Aza2E8. The 88 kDa B95-8 EBNA-1 product is indicated with an arrow, also variably seen is a previously noted (Hearing *et al.*, 1985) potential EBNA-1 breakdown product of 66 kDa. Control cell line samples include: Loucke (EBV–), Raji (EBV+, expressing a truncated form of EBNA-1), IB4 (EBV+) and B95-8 (EBV+). (**A**) Samples from brain (10 mg total protein), thymus (2.5 mg total protein) and peripheral lymph nodes (1.5 mg total protein) are shown. The pathological status of the spleen (and age of the mouse) is as follows: (–) transgenic negative, normal; 60, normal (6 months); 59, normal (5 months); 26.182, pre-neoplastic (6 months); 26.170, normal (1 month); 26.185, pre-neoplastic (6 months). (**B**) 20 mg total protein spleen samples are shown, except 59.21 which is a liver sample invaded by lymphoma. The pathological status of the spleen (and age of the mouse) is as follows: 59.47, normal (5 months); 59.27, lymphoid predominance (12 months); 59.23, neoplastic lymphoma (24 months); 59.21, malignant lymphoma (24 months); (–) transgenic negative, normal; 26.103, malignant lymphoma (7 months); 60.73, 60.47 and 60.21, all normal (5, 14 and 23 months respectively); 60.38, hyperplasia (7 months); 61.36, normal (4 months). (**C**) 10 mg of total protein samples, from the spleen of 26.10 with malignant lymphoma (1°), and lymphoma-invaded liver samples, from sequential transplant recipients of 26.10 splenocytes (2°, 3°), as described in the text.

development if they showed significant tumour invasion (data not shown). The absence of detectable EBNA-1 in the thymus indicates that expression is confined to the B cell compartment in this line. Relatively high levels of EBNA-1 were also detected in spleen and lymph node samples (pre- and post-tumour development) of line 59 mice, with a low level of expression in brain and thymus samples. Liver, heart and kidney samples were negative and only showed expression if they were significantly invaded with tumour cells, post tumour development (e.g. Figure 5B, track 59.21). A more slowly migrating EBNA-1 serum-reactive product was also variably detected in samples from lines 26 and 59 (26.182, 26.10.1°, 26.103*, 59.21). This species may be a modified form of EBNA-1, such as the phosphorylated product. Interestingly, although it is not detected consistently, so far it has only been observed in tumour-bearing samples, which is perhaps a reflection of the proportion of cells actively cycling. The conformation-sensitive Aza2E8 reacts less well with the slower migrating EBNA-1 species (compare 26.103* with 26.103).

Using equivalent protein concentrations (within tissue types), steady state levels of protein precipitated with the monoclonal antiserum Aza2E8 (visualized with WS by blotting) were higher in line 59 than in line 26 samples. However, the monoclonal antiserum Aza2E8 does not recognize all of the EBNA-1 in a given sample, as demonstrated by the increase in precipitated product using the polyclonal serum WS from a split sample (compare 26.103 with 26.103*). Moreover, the monoclonal antiserum Aza2E8 is conformation sensitive and does not recognize denatured EBNA-1 on a Western blot (Hearing *et al.*, 1985). Nevertheless, the apparent higher abundance of EBNA-1 protein in line 59 samples compared with line 26 samples is consistently observed using several alternative polyclonal and monoclonal antisera (data not shown). The analysis has been conducted at the level of whole tissues and, as such, it remains to be determined if the higher detectable protein levels in line 59 samples reflect higher levels per cell or expression in a wider range of cell types and stages than line 26.

There is an absolute correlation between the presentation of lymphoma phenotype and the expression of EBNA-1, in that the phenotypically negative lines (60 and 61) do not express the transgene protein product while the lymphoma-predisposed lines (26 and 59) show lymphoid expression of EBNA-1. However, unlike the levels of steady state transcripts, there is an inverse relationship between the levels of detectable EBNA-1 and the penetrance of disease between the two lines 26 and 59.

Splenocytes from line 26 tumour-bearing mice have been transplanted into syngeneic hosts by intravenous or

intraperitoneal injection. Transplantation resulted in the fully malignant spread of the tumour in the recipient. Splenocytes from mouse 26.10 were transplanted into a syngeneic host, and either neoplastic lymphoma nodules from the liver of this host were analysed for EBNA-1 expression (Figure 5C, 2°) or the cells were again passaged *in vivo* and the subsequent lymphoma nodules from the liver analysed for expression (Figure 5C, 3°). These results demonstrate that tumours sequentially passaged *in vivo* maintain, and possibly show an increased, EBNA-1 expression.

The c-myc locus

The neoplastic cells of BL and mouse plasmacytoma bear characteristic translocations between the c-*myc* locus and an Ig locus (most commonly the IgH locus), which are believed to result in the deregulation of c-*myc* expression. While these translocations are usually detectable at the level of a Southern analysis of the c-*myc* locus in sporadic BL and mouse plasmacytoma, they are not detectable in EBV-associated endemic BL. This is because the rearrangements in sporadic BL and mouse plasmacytoma commonly involve the 5′ sequences of the c-*myc* gene (promoter, first exon or first intronic regions), while in endemic BL the translocation is frequently at a distance of many kb from the c-*myc* gene (reviewed in Rabbitts and Rabbitts, 1989).

The lymphoma phenotype described here for both EBNA-1-expressing lines 26 and 59 bears a striking resemblance to that described for Eμmyc transgenic mice (Adams *et al.*, 1985) and is unlike other tumour phenotypes which arise in transgenic mice harbouring different oncogenes linked to the IgH enhancer, including Bcl-2, v-*able*, Pim-1, SV40 LT, *ras*, Py-TAgs and EBV LMP-1 (Suda *et al.*, 1987; McDonnell *at al.*, 1989; van Lohuizen *et al.*, 1989; Rosenbaum *et al.*, 1990; our unpublished observations). Taken together with the involvement of the c-*myc* locus in BL, a preliminary investigation of the status of c-*myc* was conducted using line 26 tumour samples.

In all EBNA-1 transgenic tumour samples tested from line 26 mice, the 20 kb *Eco*RI genomic fragment spanning the murine c-*myc* locus was intact, with no 5′ gene rearrangements detected (data not shown). The potential presence of distant translocations involving the c-*myc* gene in these transgenic tumour samples awaits to be investigated by a karyotypic analysis.

The c-*myc* gene is expressed in line 26 tumour samples to a degree comparable with EBV-positive human B cell lines (including BL derived) and mouse plasmacytoma cell lines, as determined by a Northern analysis (Figure 4B). Similar levels of c-*myc* transcripts were detectable in the clonal cell samples of Raji and IB4, as in line 26 lymphoma-positive splenic tissue, using the 420 bp *Pst*I (exon II) homologous fragment of both the human and murine c-*myc* genes as probes together. Whether the expression of c-*myc* is deregulated in specific tumours or whether EBNA-1 exerts an effect on the expression of c-*myc* independent of tumour development is the subject of further investigation.

Discussion

Results have been presented from four independent mouse lines transgenic for EμEBNA-1. Expression is detected predominantly in the B cell compartment in two of these lines (26 and 59). Mice of both of these lines succumb to the phenotype of monoclonal B cell lymphoma. Mice of the other two lines (60 and 61) do not express the protein and have no pathological phenotype. The correlation of transgene expression and lymphoma phenotype is absolute, revealing that the expression of the viral gene EBNA-1 can predispose B cells to lymphoma. As such, EBNA-1 may be regarded as a viral oncogene. Therefore its consistent expression in EBV-associated malignancies raises the possibility that the protein plays a significant role in the onset, progression and/or maintenance of these tumours. Previous studies have indicated that the viral DNA replication activity of the protein is species specific (Yates *et al.*, 1985), being restricted to human and primate cells. Fortunately, the oncogenic activity can be manifested in the mouse and as such this model provides a valuable inroad to dissection of the mechanism of action of the protein in this role. The notable lack of growth-promoting properties attributed to EBNA-1 in tissue culture assays may be indicative of an unusual mode of action in disease progression.

Lymphomas develop in the two transgenic mouse lines presented here with different latency periods. This difference is reflected in the levels of steady state transgene-specific RNA detected in splenic tissues. Mice of line 26 have a higher tumour incidence at an earlier age of onset and express higher levels of transgene RNA than mice of line 59. Differences in the expression levels of transgenes between lines is a common finding and is usually attributable to the influence of adjacent cellular sequences at the site of insertion as well as integrated transgene configuration. The observation of a cellular/transgene hybrid message in line 26 samples is a further suggestion of cellular sequence involvement in the transgene transcription patterns of this line, although no potential hybrid protein products were detected. Further subtle differences in transcript start site usage were found between line 26 and line 59 samples, which could influence message translation efficiency. The primary transcript initiation site(s) in line 26 samples was found to be upstream of the probe used and could incorporate ATGs upstream of the EBNA-1 start codon. The primary transcript initiation site observed in line 59 samples maps to the major RNA start site of Py early viral transcripts within the Py promoter (30 bp 3′ to the TATA box) and does not incorporate any ATGs upstream of the EBNA-1 start codon.

The apparent levels of steady state protein in tissues from mice of the two lines are inversely related to the disease penetrance, suggesting that the difference in disease penetrance between the two lines may not be explained simply by differing expression levels. However, unlike RNA protocols, in which probes can hybridize to all transcripts present in a sample, protein extraction and epitope recognition by antisera are highly dependent on the state of the protein. It is feasible that the protocols and antisera used are biased towards the detection of forms of EBNA-1 which may not be complexed to DNA and/or proteins. It is therefore not clear if the protein levels detected represent the absolute levels or, more importantly, the levels of active EBNA-1. Discerning the activity of EBNA-1 in tissues from the two lines (such

as the transcriptional transactivation capability) will be important in investigating the mechanism of oncogenicity and is currently the subject of further investigation.

At this stage, it cannot be ruled out that the cellular sequences at the site of transgene insertion in either of the two lines exert more effect on the phenotype than just influencing the expression patterns of the transgene. It is feasible that the insertional mutation of a cellular locus contributes to the difference in disease latency. However, while the latency differs between the two lines, the lymphoma pathology is the same and must therefore be attributable to the transgene EBNA-1.

The similarity between the EBNA-1-induced lymphomas and those arising in transgenic Eμmyc mice is suggestive of the possibility that the oncogenic action of EBNA-1 is mediated through c-*myc*. However, if this is at least in part true, it is unlikely to be purely a result of direct transactivation of the c-*myc* gene, because (i) no consensus EBNA-1 binding sites are present within the human or murine c-*myc* promoters and (ii) EBNA-1 has not been shown to replace c-*myc* in its function in tissue culture assays. Therefore, the similarity between EμEBNA-1 and Eμmyc transgenic phenotypes may instead reflect a cooperative mechanism in oncogenesis between EBNA-1 and c-*myc*.

One possible way in which EBNA-1 could exert an oncogenic effect, which would be entirely consistent with the lack of growth-promoting activity in cultured cells, is through interfering with the progress of B cell differentiation. If B cell maturation was blocked or delayed at the stage of Ig gene rearrangement, in a state permissive for continued cell cycling, it is easy to envisage how this state could predispose to lymphomagenesis. Some credence can be lent to such an idea by the recent observation that in EBV-positive BL cell lines, latent EBV expression (in the absence of LMP-1 expression) correlates with increased transcription of the recombination-activating genes 1 and 2 (RAG1 and RAG2; Kuhn-Hallek *et al.*, 1995). RAGs are normally expressed during a narrow window of lymphocyte development, and it is intriguing to correlate this observation with the previous findings concerning the c-*myc*/Ig translocation breakpoints in BL. The predominant site for breakage at the IgH locus in EBV-associated endemic BL is within the joining (J) region, presumably having taken place during V–D–J joining. In EBV-negative sporadic BL, breakpoints are frequently at the switch regions, suggesting that they occur in more mature cells undergoing the Ig class switch (reviewed in Rabbitts and Rabbitts, 1989). The induction of RAG expression by EBV, either directly or as a consequence of delayed cell maturity, could result in genomic instability. Interestingly, all of the EμEBNA-1 lymphomas have undergone IgH rearrangements, as detected using a J-region probe.

The transgenic mice developed here will allow an analysis of the EBNA-1-induced cell phenotype prior to the onset of malignant disease as the predisposing event. Furthermore, the secondary event(s) cooperating with EBNA-1 action, which results in monoclonal malignancies in these mice, can be discerned and its relevance to EBV-associated disease explored.

Materials and methods

Construction of plasmids and transgenic mice

EBNA-1-encoding sequences of the B95-8 strain were isolated from plasmid p429(K^+) (Lupton and Levine, 1985) from the *Bam*HI to *Pvu*II (the latter converted to *Eco*RI) sites (EBV nucleotides 107 565–110 176). They were then ligated through the *Bam*HI site to a converted *Bgl*II site 5′ of the Py promoter and IgH enhancer, as described for pEμLMP (Wilson *et al.*, 1990). To remove ATGs within the EBV sequences upstream of the EBNA-1 start codon, the *Bgl*II–*Nco*I fragment of this plasmid (EBV nucleotides 107 565–108 064) was replaced with the *Sau*3A–*Nco*I fragment of the same plasmid (EBV nucleotides 107 930–108 064) to generate the plasmid pEμEBNA-1. Thus, pEμEBNA-1 incorporates the murine IgH intronic enhancer, the Py origin of replication and the early promoter (TATA and cap site, as described; Wilson *et al.*, 1990), and EBV sequences from 20 nucleotides upstream of the EBNA-1 start codon to 234 nucleotides 3′ of the EBNA-1 poly (A) addition signal. An EμEBNA-1 3.2 kb *Xba*I–*Aat*II linear fragment was used for microinjection of the zygote pronuclei (Wilson and Levine, 1992). The 359 bp fragment from *Apa*I within the Py promoter (Py nucleotide 3) to *Sma*I within the 5′ EBNA-1 coding region (EBV nucleotide 108119) was subcloned into pBluescript (Stratagene) at the *Eco*RV site for RNase protection analyses; it was denoted pPy5′EB-1.

Transgenic mice were generated using C57Bl/6×DBA/2 F_2 zygotes, as described previously (Wilson *et al.*, 1990). Of 58 mice born, 12 were positive for the EμEBNA-1 transgene, 10 of which were bred into lines in the strain C57Bl/6. Eight lines displayed autosomal inheritance of the transgene, including lines 26, 59 and 60, while line 61 showed Y-linked inheritance of the transgene; another line (64) demonstrated X-linked inheritance of the transgene. Four of the lines are presented here (26, 59, 60 and 61); the remaining six lines, without pathological phenotype and not expressing the transgene (Wilson and Levine, 1992), were not studied further.

Tail genomic DNA was prepared and tested by Southern blotting for founder mice and by slot blotting for subsequent generations, as described previously (Wilson *et al.*, 1990).

RNA analyses

Total RNA was isolated from snap-frozen tissues and cell lines by acid guanidinium thiocyanate–phenol–chloroform extraction, essentially as described by Chomczynski and Sacchi (1987). Northern blots were performed using 20 μg total denatured RNA per sample, which was electrophoresed in formaldehyde gels and electroblotted as described previously (Wilson *et al.*, 1990). A 1 kb *Bst*XI–*Eco*RI 3′ EBNA-1 transgene fragment was used as a probe (avoiding the cross-reactive EBNA-1 internal repeat), labelled by the random primer method and hybridized. Blots were washed at high stringency, as described previously (Wilson *et al.*, 1990). Exon II *Pst*I fragments (420 bp) of human and murine c-*myc* genes were used as probes for c-*myc* expression. The size marker track was a 20 μg sample of transgene-negative RNA, including a radiolabelled RNA ladder (BRL system). Samples were quantitatively normalized by film densitometric comparison, with the expression of the riboprotein gene RL32, as described previously (Wilson *et al.*, 1990).

For RNase protection, pPy5′EB-1 (described above) was digested with *Eco*RI (5′ to the insert) and treated with proteinase K. We used 2 μg to generate a radiolabelled T3-polymerized RNA probe employing [^{32}P]UTP. Probes were treated with DNase and purified by acid phenol extraction, chloroform extraction and two ethanol precipitations. 2×10^5 c.p.m. purified probe were solution hybridized to each 40 μg denatured RNA sample at 65°C (TM-12°C). Single-stranded RNA was digested with RNase A and RNase T1. Proteinase K-treated, phenol-extracted samples were ethanol precipitated, denatured in 98% formamide loading buffer at 95°C and separated on 5% denaturing acrylamide gels; 2×10^5 c.p.m. undigested probes were used as control samples. Samples were quantitatively normalized by including a radiolabelled GAPDH probe in the reaction (Ambion), with the *Dde*I-digested probe protecting a 149 nucleotide fragment readily separable from the EBNA-1-protected fragments. Protected fragments were quantified by phosphorimage analysis.

Protein analysis

Whole-cell extracts were prepared by the homogenization of snap-frozen tissues or cell pellets in non-ionic detergent lysis buffer [1% NP40, 100 mM Tris, pH 8.8, 100 mM NaCl, 2% protease inhibitor aprotinin (Sigma), 5 mM protease inhibitor phenylmethylsulfonyl fluoride (Sigma)] at 4°C. Samples were centrifuged at 10 000 *g* for 20 min at 5°C, and the supernatants were collected for immunoprecipitation. Protein concentrations were measured using a Bio-Rad assay, and aliquoted samples were made up to 1 ml in lysis buffer using 10–20 mg total protein from the spleen, 10 mg total protein from the brain, 2.5 mg total protein from the thymus, 1.5 mg total protein from the lymph node and

5×10^6 cells from the cell lines. In instances where insufficient protein was obtained from individual normal lymph node or thymus extracts, extracts were pooled from different sibling mice, maintaining transgenic status, age and pathological diagnosis for a given sample. Samples were precleared with protein-A–Sepharose (Sigma) bound to rabbit anti-mouse IgG (Capel). EBNA-1 antigens were immunoreacted from precleared samples with 200 µl monoclonal tissue culture supernatant (Aza2E8), followed by rabbit anti-mouse IgG (Capel); in samples (-*) and 26.103* EBNA-1 antigens were immunoreacted with 20 µl polyclonal human serum WS. Proteins were precipitated with protein-A–Sepharose. Precipitates were washed once with non-ionic detergent buffer and once with Tris-buffered saline. Immunoprecipitated proteins were separated by reducing SDS–PAGE (7.5%) and electroblotted as described previously (Wilson *et al.*, 1990). Transferred immobilized EBNA-1 antigens were visualized with WS serum, used at 1% total volume in PBS containing 5% non-fat milk, followed by ^{125}I–protein-A incubation also in PBS containing 5% non-fat milk. As such, the Igs used for immunoprecipitation were detected in all samples.

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References

Adams,J.M., Harris,A.W., Pinkert,C.A., Corcoran,L.M., Alexander,W.S., Cory,S., Palmiter,R.D. and Brinster,R.L. (1985) The c-*myc* oncogene driven by immunoglobulin enhancers induces lymphoid malignancy in transgenic mice. *Nature*, **318**, 533–538.

Ambinder,R.F., Shah,W.A., Rawlins,D.R., Hayward,G.S. and Hayward, S.D. (1990) Definition of the sequence requirements for binding of the EBNA-1 protein to its palindromic target sites in Epstein–Barr virus DNA. *J. Virol.*, **64**, 2369–2379.

Ambinder,R.F., Mullen,M., Chang,Y.N., Hayward,G.S. and Hayward, S.D. (1991) Functional domains of Epstein–Barr virus nuclear antigen EBNA-1. *J. Virol.*, **65**, 1466–1478.

Chen,F., Zou,J.-Z., Di Renzo,L., Winberg,G., Hu,L.-F., Klein,E. and Klein,G. (1995) A subpopulation of normal B cells latently infected with Epstein–Barr virus resembles Burkitt lymphoma cells in expressing EBNA-1 but not EBNA-2 or LMP1. *J. Virol.*, **69**, 3752–3758.

Chen,M.R., Zong,J. and Hayward,S.D. (1994) Delineation of a 16 amino acid sequence that forms a core DNA recognition motif in the Epstein–Barr virus EBNA-1 protein. *Virology*, **205**, 486–495.

Chittenden,T., Lupton,S. and Levine,A.J. (1989) Functional limits of *ori*P, the Epstein–Barr virus plasmid origin of replication. *J. Virol.*, **63**, 3016–3025.

Chomczynski,P. and Sacchi,N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal. Biochem.*, **162**, 156–159.

Cohen,J.I., Wang,F., Mannick,J. and Kieff,E. (1989) Epstein–Barr virus nuclear protein 2 is a key determinant of lymphocyte transformation. *Proc. Natl Acad. Sci. USA*, **86**, 9558–9562.

Gahn,T.A. and Schildkraut,C.L. (1989) The Epstein–Barr virus origin of plasmid replication, *ori*P, contains both the initiation and termination sites of DNA replication. *Cell*, **58**, 527–535.

Gahn,T.A. and Sugden,B. (1995) An EBNA-1-dependent enhancer acts from a distance of 10 kilobase pairs to increase expression of the Epstein–Barr virus LMP gene. *J. Virol.*, **69**, 2633–2636.

Gregory,C.D., Tursz,T., Edwards,C.F., Tetaud,C., Talbot,M., Caillou,B., Rickinson,A.B. and Lipinski,M. (1987) Identification of a subset of normal B cells with a Burkitt's lymphoma (BL)-like phenotype. *J. Immunol.*, **139**, 313–318.

Hammerschmidt,W. and Sugden,B. (1989) Genetic analysis of immortalizing functions of Epstein–Barr virus in human B lymphocytes. *Nature*, **340**, 393–397.

Hearing,J.C., Lewis,A. and Levine,A.J. (1985) Structure of the Epstein–Barr virus nuclear antigen as probed with monoclonal antibodies. *Virology*, **142**, 215–220.

Jones,C.H., Hayward,S.D. and Rawlins,D.R. (1989) Interaction of the lymphocyte-derived Epstein–Barr virus nuclear antigen EBNA-1 with its DNA-binding sites. *J. Virol.*, **63**, 101–110.

Kaye,K.M., Izumi,K.M. and Kieff,E. (1993) Epstein–Barr virus latent membrane protein 1 is essential for B-lymphocyte growth transformation. *Proc. Natl Acad. Sci. USA*, **90**, 9150–9154.

Kuhn-Hallek,I., Sage,D.R., Stein,L., Groelle,H. and Fingeroth,J.D. (1995) Expression of recombination activating genes (RAG-1 and RAG-2) in Epstein–Barr virus-bearing B cells. *Blood*, **85**, 1289–1299.

Lupton,S. and Levine,A.J. (1985) Mapping genetic elements of Epstein–Barr virus that facilitate extrachromosomal persistence of Epstein–Barr virus-derived plasmids in human cells. *Mol. Cell. Biol.*, **5**, 2533–2542.

Mannick,J.B., Cohen,J.L., Birkenbach,M., Marchini,A. and Kieff,E. (1991) The Epstein–Barr virus nuclear protein encoded by the leader of the EBNA RNAs is important in B-lymphocyte transformation. *J. Virol.*, **65**, 6826–6837.

McDonnell,T.J., Deane,N., Platt,F.M., Nunez,G., Jaeger,U., McKearn,J.P. and Korsmeyer,S.J. (1989) *bcl*-2–immunoglobulin transgenic mice demonstrate extended B cell survival and follicular lymphoproliferation. *Cell*, **57**, 79–88.

Nonkwelo,C., Henson,E.B.D. and Sample,J. (1995) Characterization of the Epstein–Barr virus Fp promoter. *Virology*, **206**, 183–195.

Qu,L. and Rowe,D.T. (1992) Epstein–Barr virus latent gene expression in uncultured peripheral blood lymphocytes. *J. Virol.*, **66**, 3715–3724.

Rabbitts,T.H. and Rabbitts,P.H. (1989) Molecular pathology of chromosomal abnormalities and cancer genes in human tumours. In Glover,D.M. and Hanes,B.D. (eds), *Oncogenes*. IRL Press, Oxford, UK, pp. 67–111.

Rawlins,D.R., Milman,G., Hayward,S.D. and Hayward,G.S. (1985) Sequence-specific DNA binding of the Epstein–Barr virus nuclear antigen (EBNA-1) to clustered sites in the plasmid maintenance region. *Cell*, **42**, 859–868.

Reisman,D. and Sugden,B. (1986) Transactivation of an Epstein–Barr viral transcriptional enhancer by the Epstein–Barr viral nuclear antigen 1. *Mol. Cell. Biol.*, **6**, 3838–3846.

Reisman,D., Yates,J. and Sugden,B. (1985) A putative origin of replication of plasmids derived from Epstein–Barr virus is composed of two *cis*-acting components. *Mol. Cell. Biol.*, **5**, 1822–1832.

Rosenbaum,H., Harris,A.W., Bath,M.L., McNeal,J., Webb,E., Adams, J.M. and Cory,S. (1990) An Eµ–v-*abl* transgene elicits plasmacytomas in concert with an activated *myc* gene. *EMBO J.*, **9**, 897–905.

Rowe,M., Rowe,D.T., Gregory,C.D., Young,L.S., Farrell,P.J., Rupani,H. and Rickinson,A.B. (1987) Differences in B cell growth phenotype reflect novel patterns of Epstein–Barr virus latent gene expression in Burkitt's lymphoma cells. *EMBO J.*, **6**, 2743–2751.

Sample,J., Brooks,L., Sample,C., Young,L., Rowe,M., Gregory,C., Rickinson,A. and Kieff,E. (1991) Restricted Epstein–Barr virus protein expression in Burkitt lymphoma is due to a different Epstein–Barr nuclear antigen 1 transcriptional initiation site. *Proc. Natl Acad. Sci. USA*, **88**, 6343–6347.

Sample,J., Henson,E.B.D. and Sample,C. (1992) The Epstein–Barr virus nuclear protein 1 promoter active in type I latency is autoregulated. *J. Virol.*, **66**, 4654–4661.

Schaefer,B.C., Woisetschlaeger,M., Strominger,J.L. and Speck,S.H. (1991) Exclusive expression of Epstein–Barr virus nuclear antigen 1 in Burkitt lymphoma arises from a third promoter, distinct from the promoters used in latently infected lymphocytes. *Proc. Natl Acad. Sci. USA*, **88**, 6550–6561.

Smith,P.R. and Griffin,B.E. (1992) Transcription of the Epstein–Barr virus gene EBNA-1 from different promoters in nasopharyngeal carcinoma and B-lymphoblastoid cells. *J. Virol.*, **66**, 706–714.

Snudden,D.K., Hearing,J., Smith,P.R., Grässer,F.A. and Griffin,B.E. (1994) EBNA-1, the major nuclear antigen of Epstein–Barr virus, resembles 'RGG' RNA binding proteins. *EMBO J.*, **13**, 4840–4847.

Suda,Y., Aizawa,S., Hirai,S.-i., Inoue,T., Furuta,Y., Suzuki,M., Hirohashi,S. and Ikawa,Y. (1987) Driven by the same Ig enhancer and SV40 T promoter, *ras* induced lung adenomatous tumors, *myc* induced pre-B cell lymphomas and SV40 large T gene a variety of tumors in transgenic mice. *EMBO J.*, **6**, 4055–4065.

Sugden,B. and Warren,N. (1989) A promoter of Epstein–Barr virus that can function during latent infection can be transactivated by EBNA-1, a viral protein required for viral DNA replication during latent infection. *J. Virol.*, **63**, 2644–2649.

Tomkinson,B., Robertson,E. and Kieff,E. (1993) Epstein–Barr virus nuclear proteins EBNA-3A and EBNA-3C are essential for B-lymphocyte growth transformation. *J. Virol.*, **67**, 2014–2025.

van Lohuizen,M., Verbeek,S., Krimpenfort,P., Domen,J., Saris,C., Radaszkiewicz,T. and Berns,A. (1989) Predisposition to lymphomagenesis in *pim*-1 transgenic mice: cooperation with c-*myc* and N-*myc* in murine leukemia virus-induced tumors. *Cell*, **56**, 673–682.

Wilson,J.B. and Levine,A.J. (1992) The oncogenic potential of Epstein–Barr virus nuclear antigen 1 in transgenic mice. *Curr. Topics Microbiol. Immunol.*, **182**, 375–384.

Wilson,J.B., Weinberg,W., Johnson,R., Yuspa,S. and Levine,A.J. (1990) Expression of the BNLF-1 oncogene of Epstein–Barr virus in the skin of transgenic mice induces hyperplasia and aberrant expression of keratin 6. *Cell*, **61**, 1315–1327.

Wysokenski,D.A. and Yates,J.L. (1989) Multiple EBNA1-binding sites are required to form an EBNA1-dependent enhancer and to activate a minimal replicative origin within *ori*P of Epstein–Barr virus. *J. Virol.*, **63**, 2657–2666.

Yates,J.L., Warren,N., Reisman,D. and Sugden,B. (1984) A *cis*-acting element from the Epstein–Barr viral genome that permits stable replication of recombinant plasmids in latently infected cells. *Proc. Natl Acad. Sci. USA*, **81**, 3806–3810.

Yates,J.L., Warren,N. and Sugden,B. (1985) Stable replication of plasmids derived from Epstein–Barr virus in various mammalian cells. *Nature*, **313**, 812–815.

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