Journal of General Virology (2001), **82**, 3051–3055. Printed in Great Britain SHORT COMMUNICATION

Elevated expression of c-*myc* in lymphoblastoid cells does not support an Epstein–Barr virus latency III-to-I switch

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Epstein–Barr virus (EBV) transforms primary B cells *in vitro*. Established cell lines adopt a lymphoblastoid phenotype (LCL). In contrast, EBV-positive Burkitt's lymphoma (BL) cells, in which the proto-oncogene c-*myc* is constitutively activated, do not express a lymphoblastoid phenotype *in vivo*. The two different phenotypes are paralleled by two distinct programmes of EBV latent gene expression termed latency type I in BL cells and type III in LCL. Human B cell lines were established from a conditional LCL (EREB2-5) by overexpression of *c-myc* and inactivation of EBV nuclear protein 2 (EBNA2). These cells (A1 and P493-6) adopted a BL phenotype in the absence of EBNA2. However, the EBV latency I promoter Qp was not activated. Instead, the latency III promoter Cp remained active. These data suggest that the induction of a BL phenotype by overexpression of c-*myc* in an LCL is not necessarily paralleled by an EBV latency III-to-I switch.

Burkitt's lymphoma (BL) is a human B-cell neoplasia that occurs classically as a childhood malignancy with high incidence in areas with holoendemic malarial infection (Nesbit *et al.*, 1999). BL cells are characterized by reciprocal chromosomal translocations involving the gene loci of the proto-oncogene c-*myc* (*myc*) and of immunoglobulin genes. The juxtaposition of immunoglobulin gene enhancers results in the oncogenic activation of *myc* (Henriksson & Lüscher, 1996).

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BL is frequently associated with Epstein–Barr virus (EBV), a lymphotropic γ-herpesvirus. Infection of primary B cells *in vitro* with EBV results in cell transformation and the establishment of lymphoblastoid cell lines (LCL) that express six viral latent nuclear antigens (EBNAs) and three latent membrane proteins (LMPs). In BL cells *in vivo*, the expression of EBV latent proteins is restricted to EBNA1 (Rickinson & Kieff, 1996). The two different programmes of EBV latent gene expression are termed EBV latency type I in BL and type III in LCL (Rowe *et al.*, 1987). Different promoters regulate EBNA1 expression in latency I and III. EBNA1 is expressed from the Qp promoter (Schaefer *et al.*, 1995) in BL whereas, in LCL, all EBNA genes are expressed from the Cp promoter. The individual mRNAs are generated by alternative splicing of long primary transcripts (Kieff, 1996).

Immortalization of B cells by EBV is assumed to be an important step during the pathogenesis of BL. Therefore, an intriguing question is which factors implement the restricted EBV gene expression programme found in BL; i.e. which factors induce the switch from EBV latency type III to type I. Several regulatory factors of the EBNA1 promoter Qp, active in BL cells, have been identified so far, among them viral proteins (EBNA1), immune response-dependent factors (IRFs, STATs) and cell-cycle regulating proteins (E2F, Rb) (Chen *et al.*, 1999; Davenport & Pagano, 1999; Nonkwelo *et al.*, 1997; Ruf & Sample, 1999; Schaefer *et al.*, 1997*b*; Zhang & Pagano, 1997, 1999). Methylation of the EBV genome has been demonstrated to be a prerequisite for the maintenance of latency type I by inhibiting the activation of the Cp promoter, which is active in LCL (Robertson, 2000). Until now, a latency III-to-I switch in B cells has not been observed *in vitro*.

In order to investigate the genetic events that contribute to the pathogenesis of BL, we overexpressed the proto-oncogene *myc* in the conditional LCL EREB2-5 (Pajic *et al.*, 2000; Polack *et al.*, 1996). This cell line carries the EBNA2 deletion mutant P3HR-1 and an expression plasmid for an EBNA2–oestrogen receptor fusion protein, which renders EBNA2 function and EBV immortalization dependent on the presence of oestrogen (Kempkes *et al.*, 1995). Ectopic overexpression of *myc* in

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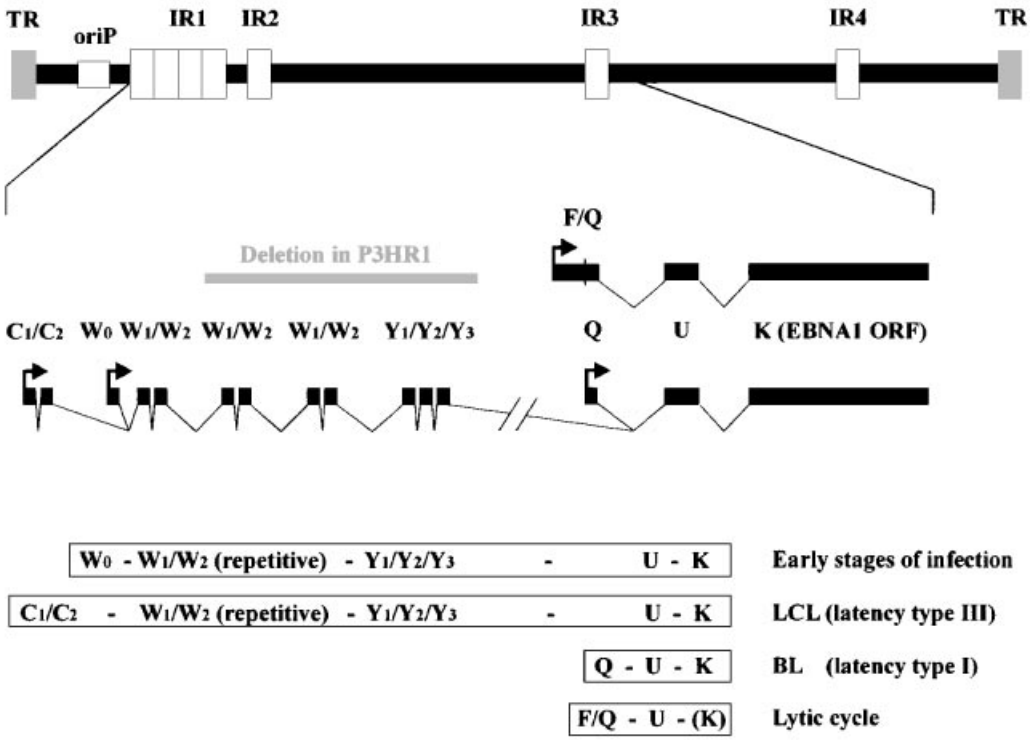

Fig. 1. Schematic illustration of typical EBNA1 transcripts generated during the various stages of EBV infection in B cells. The viral genome is shown in linear form at the top. The positions of major elements like the origin of replication (oriP) and the terminal (TR) and internal (IR) repeats are indicated. Exons are depicted as black bars. The shaded bar indicates the deletion in the genome of the EBV mutant virus P3HR-1 that was used as the helper virus in the establishment of the cell lines EREB2-5, A1 and P493-6. At the bottom, the exon composition of the various EBNA1 transcripts is summarized.

EREB2-5 cells allows the establishment of cell lines that proliferate independently of EBNA2 and express features typical of BL cells (*in vivo* phenotype) (Polack *et al.*, 1996). Here, we asked whether the observed switch from an LCL phenotype to a BL phenotype was accompanied by an EBV latency III-to-I switch. The activities of the EBNA promoters Qp and Cp were analysed in the cell lines A1, in which *myc* is overexpressed under the control of Igκ enhancer elements (Polack *et al.*, 1996), and P493-6, in which *myc* expression can be controlled by a tetracycline-responsive promoter (Pajic *et al.*, 2000).

Qp- and Cp-initiated EBNA1 transcripts can be detected and distinguished by RT–PCR (Schaefer *et al.*, 1996; Schlager *et al.*, 1996). Reverse transcription was performed with 5·0 µg total RNA, 0·5 µg oligo(dT) primer, 0·5 mM dNTPs, 200 U reverse transcriptase (Superscript II, Gibco BRL), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 50 mM Tris–HCl (pH 8·3) and 20 U RNase inhibitor (RNasin) in 20 µl at 42 °C for 50 min. The reaction was stopped by heating at 70 °C for 15 min.

A 1/50 aliquot of the RT reaction was subjected to 25 cycles of PCR. For amplification of Qp-initiated transcripts, PCR was performed with 5 pmol primer, 0·1 mM dNTPs, 2·5 U *Taq* DNA polymerase, 1 mM $MgCl_2$, 0·1% Triton X-100 and 10 mM Tris–HCl (pH 9·0) in 50 µl. Each thermocycle was composed of 1 min denaturation at 94 °C, 30 s primer hybridization at 58 °C and 1 min synthesis at 72 °C. For amplification of Cp-initiated transcripts, PCR was performed with 10 pmol primer, 0·2 mM dNTPs and 5 U *Taq* DNA polymerase (Promega) in 1·5 mM $MgCl_2$, 0·1% Triton X-100 and 10 mM Tris–HCl (pH 9·0). The PCR programme was as above except that the primer hybridization and DNA synthesis phases were extended to 1 and 3 min, respectively.

PCR products were resolved by agarose gel electrophoresis and subjected to Southern blotting according to standard protocols (Sambrook *et al.*, 1989). Blots were hybridized with a ^{32}P-labelled U-exon probe. Radiolabelling of DNA was performed by random-primed labelling according to the protocols of the manufacturer.

A Q–K primer pair was applied for the detection of Qp activity in A1 and P493-6 cells (Schaefer *et al.*, 1995). During the lytic cycle, the Fp promoter generates transcripts that overlap Qp-initiated transcripts and may mimic Qp activity in RT–PCR analysis (Fig. 1). Therefore, we controlled for the presence of lytic, Fp-initiated EBNA1 transcripts by a separate PCR with an F–K primer pair. Neither Qp- nor Fp-initiated EBNA1 transcripts were present in A1 and P493-6 cells. In control cells, these transcripts could be detected easily (Fig. 2). In order to exclude the possibility that the negative results in our cellular system were due to point mutations in the primer-binding sites, we sequenced the respective regions in A1 cells.

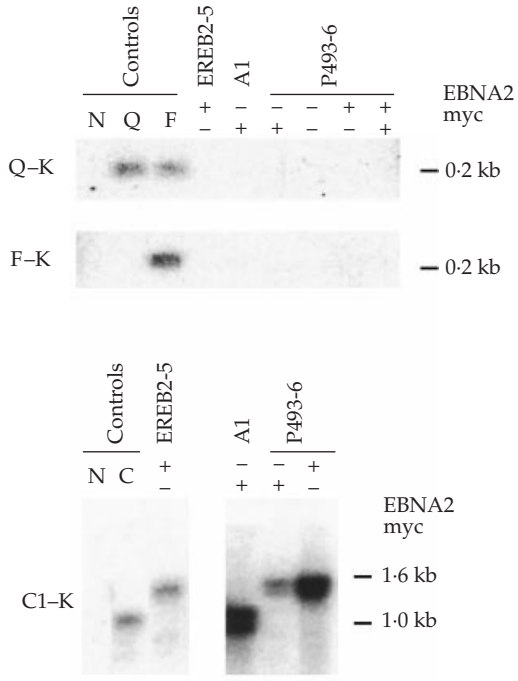

Fig. 2. EBNA1 is expressed from Cp, and Qp-initiated EBNA1 transcripts are absent in EREB2-5, A1 and P493-6 cells. EREB2-5 cells were cultured in the presence of EBNA2 function. A1 and P493-6 cells were grown in the absence of EBNA2 function and the presence of elevated *myc* expression. In addition, EBNA2 activity and *myc* expression were altered in P493-6 cells for 4 days as indicated. RT–PCR was performed with a 5′ primer in exon Q (Q primer: 5′ AAGGCGCGGGATAGCGT 3′), F (F primer: 5′ ATATGAGCTCGGTGAGGCCACGCTT 3′) or C1 (C1 primer: 5′ CACTACAAGACCTACGCC 3′) and a 3′ primer in the K exon (K primer: 5′ CCCCTCGTCAGACATGAT 3′). The results were analysed on a Southern blot, which was hybridized with a ^{32}P-labelled U-exon probe derived from an EBV cosmid by restriction with *Hin*cII (bases 66722–68619 of the B95-8 viral genome). N, Negative control (DG75; EBV-negative BL line); Q, positive control for Qp-initiated EBNA1 transcription (BL29; EBV-positive BL line, latency I); F, positive control for Fp-initiated EBNA1 transcription [HH514; EBV-positive BL line P3HR-1, in which the lytic cycle was induced by treatment with sodium butyrate and 12-*O*-tetradecanoylphorbol 13-acetate (TPA)]; C, positive control for Cp-initiated EBNA1 transcription (Jijoye; EBV-positive cell line, latency III). The positions of size markers are shown.

No mutations were found in the sequences covering the F, Q and K primer-binding sites (data not shown).

As Qp was not responsible for EBNA1 transcription in A1 and P493-6 cells, we asked whether EBNA1 transcription was driven by Cp (Fig. 1). A C1–K primer pair was applied for the detection of Cp-initiated EBNA1 transcripts. In A1 and P493-6 cells, two transcripts could be detected that differed in size by about 200 bp (Fig. 2). Additionally, the transcripts in A1 cells were about 400 bp smaller than those in P493-6 cells. As the size of a W-exon unit is 198 bp, these differences could reflect different contents of W-exon units. Activation of EBNA2 resulted in increased expression of EBNA1 (Fig. 2 and data not shown). This observation was in agreement with previous reports that EBNA2 enhances Cp activity (Evans *et al.*, 1996). The LCL EREB2-5, the parental line of A1 and P493-6, expressed Cp-initiated EBNA1 transcripts of uniform size, whereas the EBV-positive BL line BL29 (latency I) did not show any Cp activity (data not shown).

In LCL, Cp-initiated transcripts are spliced to generate the mRNA of all EBNA genes (Rickinson & Kieff, 1996). We asked whether the observed Cp activity in A1 and P493-6 cells would also result in the expression of other EBNAs and analysed the expression of EBNA3 transcripts (EBNA3A, B and C). PCR was performed with a 5′ primer in the W2 exon that is common for all EBNA3 transcripts and 3′ primers in the unique EBNA3A, B or C coding regions. In EREB2-5, A1 and P493-6 cells, transcripts of EBNA3A, B and C were detected. The results of the EBNA3C analysis are shown in Fig. 3 as a representative of all EBNA3 genes.

Our observation that *myc* induces the expression of a surface antigen pattern characteristic of BL cells and downregulates surface molecules expressed on EBV-immortalized LCL contradicts the results of previous studies (Cutrona *et al.*, 1995; Hotchin *et al.*, 1990; Lombardi *et al.*, 1987). However, the activities of the EBV promoters Cp and Qp were not tested in those studies. We now show that the EBV latency I promoter Qp is not active in A1 or P493-6 cells or in the parental LCL EREB2-5. Instead, the EBV latency III promoter Cp drives transcription of EBNA1 and EBNA3 genes in these cells. This transcription is independent of EBNA2. The data presented suggest that the elevated expression of *myc* in LCL is not sufficient to promote an EBV latency III-to-I switch.

Kerr *et al.* (1992) studied somatic cell hybrids and observed a downregulation of Wp/Cp and an upregulation of Qp in hybrid cell lines that had lost the LCL phenotype and acquired the phenotype of the non-B cell fusion partners. However, all cell hybrids that retained the LCL-like phenotype also kept the EBV latency III programme. This was always the case when a BL cell line was fused with an LCL, showing that the LCL phenotype was dominant over the BL phenotype (Wolf *et al.*, 1993).

In contrast, our system allows the switching from an LCL to a BL phenotype in the same cells. Similar phenotypic changes were observed after the transition of Jijoye BL cells to the P3HR-1 daughter cell line. Jijoye cells show an LCL phenotype, including the EBV latency III transcription programme, whereas P3HR-1 cells have acquired an EBNA2 deletion and have lost the expression of typical LCL surface antigens. EBNA1 is expressed exclusively from Wp in P3HR-1 cells. This is in contrast to the cell system used here. However, the factors that determine the use of Wp rather than Cp are unknown in P3HR-1 cells. It is also unclear whether there is a difference in virus promoter regulation if EBNA2 is expressed from an episome *in trans*, as in our cellular system (Kempkes *et al.*, 1996), or from the same genome *in cis*. This can be overcome by generating recombinant EBV with a conditional EBNA2 based on the EBV B95-8 strain (Delecluse *et al.*, 1998, 1999).

In another analysis, cells of an EBV-negative BL line were infected with the P3HR-1 virus (Schlager *et al.*, 1996). The

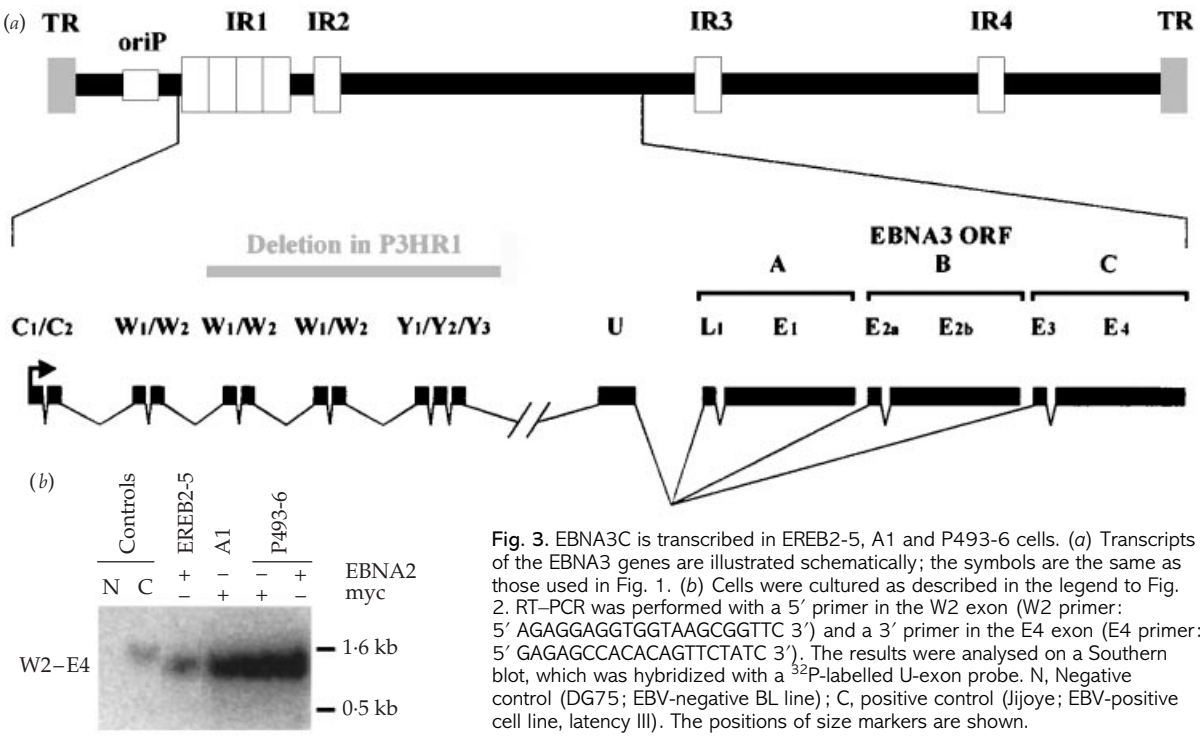

Fig. 3. EBNA3C is transcribed in EREB2-5, A1 and P493-6 cells. (*a*) Transcripts of the EBNA3 genes are illustrated schematically; the symbols are the same as those used in Fig. 1. (*b*) Cells were cultured as described in the legend to Fig. 2. RT–PCR was performed with a 5′ primer in the W2 exon (W2 primer: 5′ AGAGGAGGTGGTAAGCGGTTC 3′) and a 3′ primer in the E4 exon (E4 primer: 5′ GAGAGCCACACAGTTCTATC 3′). The results were analysed on a Southern blot, which was hybridized with a ^{32}P-labelled U-exon probe. N, Negative control (DG75; EBV-negative BL line); C, positive control (Jijoye; EBV-positive cell line, latency III). The positions of size markers are shown.

activities of the EBNA promoters were monitored during a time-course of several hours after infection. It was observed that both Qp and Cp were activated. These data suggested that the environment of a BL cell might support the activity of both EBV latency I (Qp) and III (Cp) promoters and that the P3HR-1 EBV strain does not contain defective Qp or Cp promoters.

We intended to construct an EBV latency III-to-I switch *in vitro* by overexpressing *myc* and inactivating EBNA2 in parallel. It is striking that the EBNA transcription programme characteristic of LCL remained active despite the enormous phenotypic changes. Repression of Wp and Cp by methylation has been demonstrated to be a prerequisite for Qp activation in B cells (Schaefer *et al.*, 1997*a*). It is likely that the EBV genome is not methylated in the same way in A1 and P493-6 cells as in BL cells. At present it is not clear which factors trigger methylation of EBV promoters in B cells. Our data imply that the overexpression of *myc* and inactivation of EBNA2 are not sufficient for Wp/Cp methylation.

It has been reported that elevated levels of EBNA1 can inhibit Qp activity (Sample *et al.*, 1992). A1 and P493-6 cells expressed higher levels of EBNA1 protein than did EREB2-5 cells. However, the levels of EBNA1 protein in A1 and P493-6 cells were comparable with levels found in BL phenotype I cells Rael and MutuI. Additionally, MutuIII cells, representing Mutu cells in EBV latency III, expressed levels of EBNA1 protein comparable to those of EREB2-5 cells (data not shown). These data suggest that the higher EBNA1 protein levels in A1 and P493-6 cells may not be inhibitory for Qp activity.

EBNA3A, -B and -C transcripts were also detected in A1 and P493-6 cells. However, the presence of EBNA3 proteins could not be verified, due to the lack of antibodies that recognize EBNA3 proteins of the EBV type 2 P3HR-1 strain specifically. Members of the EBNA3 protein family have been demonstrated to modulate EBNA2-dependent transcription and are also likely to contribute to transcriptional regulation in the absence of EBNA2 (Cludts & Farrell, 1998; Marshall & Sample, 1995). It remains unclear whether EBNA3 proteins could contribute to the activation of Cp in A1 and P493-6 cells. The answer to these questions could be provided by establishing a similar cell system based on a B95-8 recombinant EBV, for which the appropriate reagents are available.

This work was supported by the Deutsche Forschungsgemeinschaft (La948/2-2 and SFB455) and the Fonds der Chemischen Industrie.

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Received 26 March 2001; Accepted 9 August 2001