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Epstein–Barr virus nuclear antigen 2 is a transcriptional suppressor of the immunoglobulin μ gene: implications for the expression of the translocated c-*myc* gene in Burkitt's lymphoma cells

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A conditional mutant of Epstein–Barr virus nuclear antigen 2 (EBNA2) regulated by estrogen was employed to study the effect of EBNA2 on the cellular phenotype. Activation of EBNA2 in lymphoblastoid cell lines (LCLs) and in B cell lymphoma lines resulted in down-regulation of cell surface IgM and Ig-μ steady-state RNA expression. In LCLs, activation of EBNA2 is required for maintaining proliferation, whereas in Burkitt's lymphoma (BL) cell lines with t(8;14) translocations, activation of EBNA2 induces growth arrest. In these cells, Northern and nuclear run-on analyses revealed rapid simultaneous repression of Ig-μ and c-*myc* transcription as early as 30 min after activation of EBNA2. Since c-*myc* expression is under the control of the Ig heavy chain locus in BL cell lines with a t(8;14) translocation, we propose that Ig-μ and c-*myc* are down-regulated by EBNA2 through a common mechanism.

Keywords: Burkitt's lymphoma/c-*myc*/EBNA2/Epstein–Barr virus/immunoglobulin μ

Introduction

Epstein–Barr virus (EBV) is a lymphotropic herpes virus causing infectious mononucleosis, a self-limiting lymphoproliferative disease. EBV infects primary resting B cells, giving rise to lymphoblastoid cell lines (LCLs) proliferating unlimitedly *in vitro*, a process synonymously called immortalization or transformation. EBV is also associated with several human malignancies, including Burkitt's lymphoma (BL), nasopharyngeal carcinoma, lymphomas in immunocompromised individuals and Hodgkin's disease (for reviews, see Miller, 1990; Klein, 1994; Masucci and Ernberg, 1994).

In LCLs, only a subset of viral genes is expressed, coding for six nuclear proteins, three membrane proteins and two small non-polyadenylated nuclear RNAs. The minimal set of genes required for transformation is still unknown. The EBV nuclear antigen 2 (EBNA2) gene is deleted in the transformation-defective virus produced by the BL line P3HR1. It appears in two allelic forms (EBNA2A and EBNA2B) in natural isolates which share ~50% homology (Zimber *et al.*, 1986). EBNA2, together with EBNA-LP, is the first viral gene expressed after infection of primary B cells, and is a transcriptional activator of latent viral as well as cellular genes (CD21, CD23 and c-*fgr*) (Calender *et al.*, 1987; Wang *et al.*, 1987; Abbot *et al.*, 1990; Cordier *et al.*, 1990; Fahraeus *et al.*, 1990; Ghosh and Kieff, 1990; Knutson, 1990; Sung *et al.*, 1991; Woisetschlaeger *et al.*, 1991; Zimber-Strobl *et al.*, 1991, 1993; Jin and Speck, 1992; Ling *et al.*, 1993; Laux *et al.*, 1994a; Meitinger *et al.*, 1994). EBNA2 has been shown to exert its transactivating function, at least in part, by binding to a ubiquitously expressed cellular protein, RBP-Jκ, which binds to DNA in a sequence-specific manner (Grossmann *et al.*, 1994; Henkel *et al.*, 1994; Waltzer *et al.*, 1994; Zimber-Strobl *et al.*, 1994), and through interaction with transcription factors of the *ets* gene family (Spi-1, PU-1) (Laux *et al.*, 1994b; Johannsen *et al.*, 1995). Even though the transformation of primary B cells *in vitro* is strictly dependent on EBNA2 (Cohen *et al.*, 1989; Hammerschmidt and Sugden, 1989; Kempkes *et al.*, 1995), EBNA2 appears not to be expressed in BL tumors (Rowe *et al.*, 1986), Hodgkin's disease (Kanavaros *et al.*, 1993) and nasopharyngeal carcinoma (Fahraeus *et al.*, 1988; Young *et al.*, 1988), thus questioning its role in the development of these malignancies *in vivo*.

A consistent feature of BL cells is the transcriptional activation of the proto-oncogene c-*myc* by chromosomal translocation (Bornkamm *et al.*, 1988; Spencer and Groudine, 1991). The most frequent t(8;14) translocation fuses the c-*myc* gene locus on chromosome 8 to the constant region of the Ig heavy chain gene locus on chromosome 14. Since BL cells are assumed to proliferate through activation of the c-*myc* gene, the growth-promoting function of EBNA2 may not be required in the setting of BL.

To understand better the role of EBNA2 in the process of B cell transformation, we have developed a conditional system in which the function of the EBNA2 protein can be switched on and off reversibly. To this end, making use of the inducible system developed by Picard *et al.* (1988) and Eilers *et al.* (1989), we have fused the hormone binding domain of the estrogen receptor (ER) to the N- or C-terminus of EBNA2, thus rendering the function of the EBNA2 protein dependent on the presence of estrogen. As expected, the chimeric EBNA2 proteins exerted their transactivating function on viral and cellular genes only in the presence of estrogen (Kempkes *et al.*, 1996a). Importantly, the EBNA2 fusion proteins were able to substitute for wild-type EBNA2 and to complement the EBNA2 defect of P3HR1 virus in primary B cell transformation. By removal of estrogen, it was thus possible to demonstrate that EBNA2 is not only required for initiation but also for maintenance of B cell transformation (Kempkes *et al.*, 1995). To our surprise, the same EBNA2–ER fusion protein, which is required for proliferation of

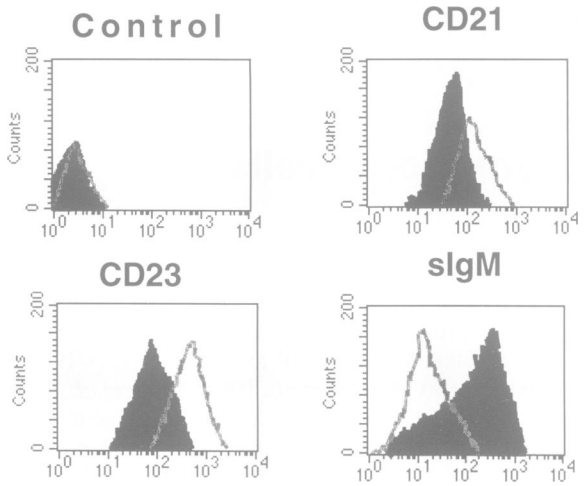

Fig. 1. Flow cytometric analysis of estrogen-treated (white histograms) and estrogen-deprived (black histograms) ER/EB2-5 cells for CD21, CD23 and surface IgM staining.

primary B cells, turned out to inhibit proliferation when stably expressed in established B cell lymphoma lines (Kempkes *et al.*, 1996b).

Here, we describe a novel function of EBNA2. It down-regulates surface IgM expression and transcription of the Ig-μ locus very efficiently. In BL cell lines with t(8; 14) translocations, down-regulation of Ig-μ expression is associated with concomitant transcriptional shut-off of the c-*myc* gene, reflecting the fact that c-*myc* is under the control of the Ig heavy chain locus in these cells. The function of EBNA2 as a negative regulator of Ig-μ transcription provides an explanation for the growth-inhibiting effect of EBNA2 in cells carrying a t(8;14) translocation.

Results

Surface IgM expression is up-regulated in conditionally transformed lymphoblastoid cells by removal of estrogen

By complementing the EBNA2 defect of the P3HR1 strain of EBV with EBNA2–ER fusions proteins, we have succeeded in transforming primary human B lymphocytes in a conditional fashion. In these cells, the function of EBNA2 can be regulated by addition or removal of estrogen: the cells grow in the presence of hormone and stop proliferation when estrogen is withdrawn (Kempkes *et al.*, 1995). To identify cellular targets which are regulated by EBNA2, we have analyzed the expression of cell surface markers in these cells before and after removal of estrogen. As shown in Figure 1, CD23 and, to a lesser extent, CD21 expression was down-regulated by switching off the function of EBNA2 by removal of estrogen. At the same time, however, IgM expression strongly increased. Up-regulation of IgM expression and down-regulation of CD21 and CD23 expression were reversible, since CD21 and CD23 expression increased and IgM expression decreased concomitantly when estrogen was re-added (data not shown).

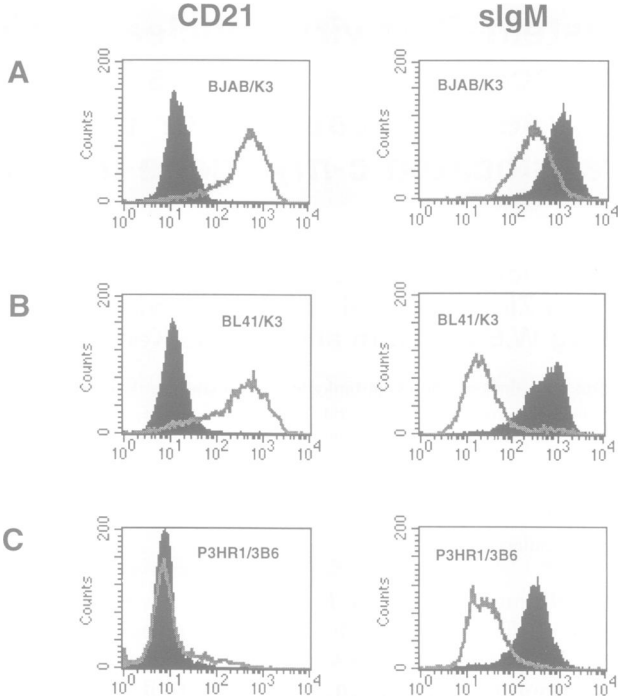

Fig. 2. Suppression of cell surface IgM by EBNA2 in B lymphoma cell lines. ER/EBNA2 transfectants (BJAB/K3, BL41/K3 and P3HR1/3B6) were kept for 2 days in the presence (white histograms) or absence (black histograms) of 1 μM β-estradiol and were then analyzed for cell surface CD21 and surface IgM expression by FACS analysis. 1×10^6 cells were incubated with excess unlabeled mouse mAbs recognizing human CD21 (BL13) or IgM (AF6). FITC-conjugated goat anti-mouse $F(ab)_2$ fragments were used as secondary antibody for staining of positive cells. Control cells were labeled with secondary antibodies only, but did not show any changes in response to estrogen (data not shown). Dead cells were identified and excluded from the analysis after propidium iodide staining (0.1 μg/ml).

EBNA2 down-regulates IgM and up-regulates CD21 and CD23 on the surface of stably transfected B cell lymphoma lines

Down-regulation of surface IgM may be mediated either by EBNA2 itself or by any of the viral downstream targets (e.g. the viral latent membrane proteins) which are induced by EBNA2. To discriminate between these possibilities, the gene encoding the ER–EBNA2 fusion protein was stably introduced into EBV-negative B cell lymphoma lines BL41 and BJAB, and, for comparison, into P3HR1 cells. In the stably transfected cell lines, activation of the TP1 or LMP1 promoter was strictly dependent on the presence of estrogen, indicating that the ER–EBNA2 protein is indeed functional in these cells (Kempkes *et al.*, 1996a). FACS analysis before and after addition of estrogen revealed a strong decrease of surface IgM expression upon the addition of estrogen (Figure 2). CD23 (not shown) and CD21 expression strongly increased as expected (except for CD21 in P3HR1 cells), confirming the notion that CD21 induction by EBNA2 is dependent additionally on the cellular background (Cordier and Bussat, 1993).

BJAB/K3 and BL41/K3 cells were analyzed further for a possible modulation of cell surface IgD, IgG, IgE or IgA after estrogen addition, but none of these molecules was found to be expressed before or after EBNA2 activation (data not shown). We conclude that down-regulation

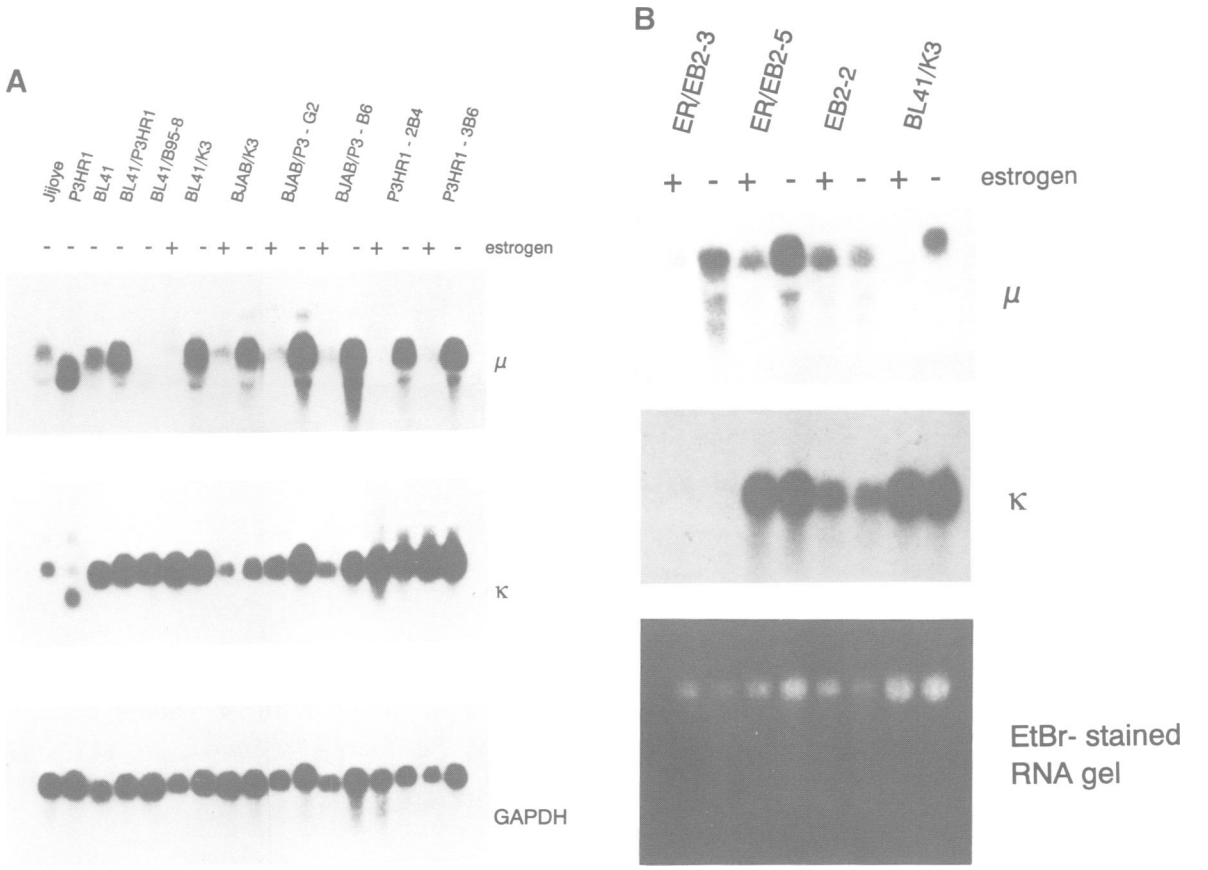

Fig. 3. Suppression of Ig-μ heavy chain expression by EBNA2 in B lymphoma cells and LCLs. Total cellular RNA was isolated from Jijoye, P3HR1, BL41, BL41/P3HR1, BL41/B95-8 and ER/EBNA2-transfected cell lines which had been kept for 2 days in the presence or absence of estrogen (**A**), or from LCL cell lines expressing the ER–EBNA2 protein (ER/EB2-3 and ER/EB2-5), or wild-type EBNA2 protein (EB2-2) treated with or deprived of estrogen for 4 days (**B**) and was analyzed for Ig-μ heavy chain, Ig-κ light chain and GAPDH RNA by Northern blot analysis.

of surface IgM expression is a function of EBNA2 and not of any of the viral target genes.

EBNA2 down-regulates Ig-μ RNA abundance

Northern blot analyses were performed to find out at which level IgM expression is down-regulated by EBNA2. By including RNA of paired cell lines differing in the presence and absence of the wild-type EBNA2 gene, Northern blot analysis also provided an answer to the question of whether IgM down-regulation is brought about not only by the ER–EBNA2 fusion protein but also by wild-type EBNA2. The paired cell lines included P3HR1 producing an EBNA2-defective virus and its parental cell Jijoye carrying a functional EBNA2 gene (EBNA2B allele) and BL41 cells stably infected/converted with P3HR1 virus (BL41/P3HR1) or the transformation-competent, EBNA2 wild-type positive B95-8 virus carrying the EBNA2A allele. Apparently, surface IgM expression was down-regulated by EBNA2 by decreasing the Ig-μ RNA abundance (Figure 3).

With the exception of BJAB/K3 cells, expression of Ig-κ light chain RNA was not affected by EBNA2 activation. Changes observed for Ig-κ RNA levels in P3HR1 virus-converted BJAB clones G2 and B6 stably transfected with ER/EBNA2 were accompanied by a decrease in glyceraldehyde phosphate dehydrogenase (GAPDH) RNA, and can, therefore, not be interpreted conclusively (Figure 3A and B).

Co-regulation of Ig-μ and c-myc expression in BL cell lines carrying the t(8;14) translocation

Switching on the function of EBNA2 induces proliferation of ER/EB 2-5 cells, but inhibits proliferation of stably transfected B cell lymphoma lines (Kempkes *et al.*, 1996b). We were therefore interested in whether c-*myc* expression is affected by the addition of estrogen in these cells.

Kinetic studies revealed that Ig-μ RNA levels in P3HR1/3B6 (Figure 4A) and BL41/K3 cells (not shown) started to decrease 6 h after addition of estrogen and then declined to constantly low levels after 2 days. Analysis of the same RNA samples also revealed an almost complete disappearance of c-*myc* RNA after EBNA2 activation. Suppression of c-*myc* RNA could be observed as early as 30 min after estrogen addition in P3HR1/3B6 cells (Figure 4B). Since Ig-μ and c-*myc* RNAs have different half-lives (Dani *et al.*, 1984; Jäck and Wabl, 1988), the delayed decrease of Ig-μ RNA can be attributed to its higher stability. Down-regulation of Ig-μ and c-*myc* RNA suggested that EBNA2 is targeting a common regulatory element which is driving Ig-μ as well as c-*myc* expression in cells carrying the t(8;14) chromosomal translocation. This is supported by the fact that, in BJAB/K3 cells lacking a t(8;14) translocation, the decrease of Ig-μ RNA after activation of EBNA2 followed similar kinetics to those observed for P3HR1/3B6 and BL41/K3 cells, whereas c-*myc* expression was not affected by the addition of estrogen in BJAB/K3 cells during the time course of the experiment (48 h) (Figure 4C).

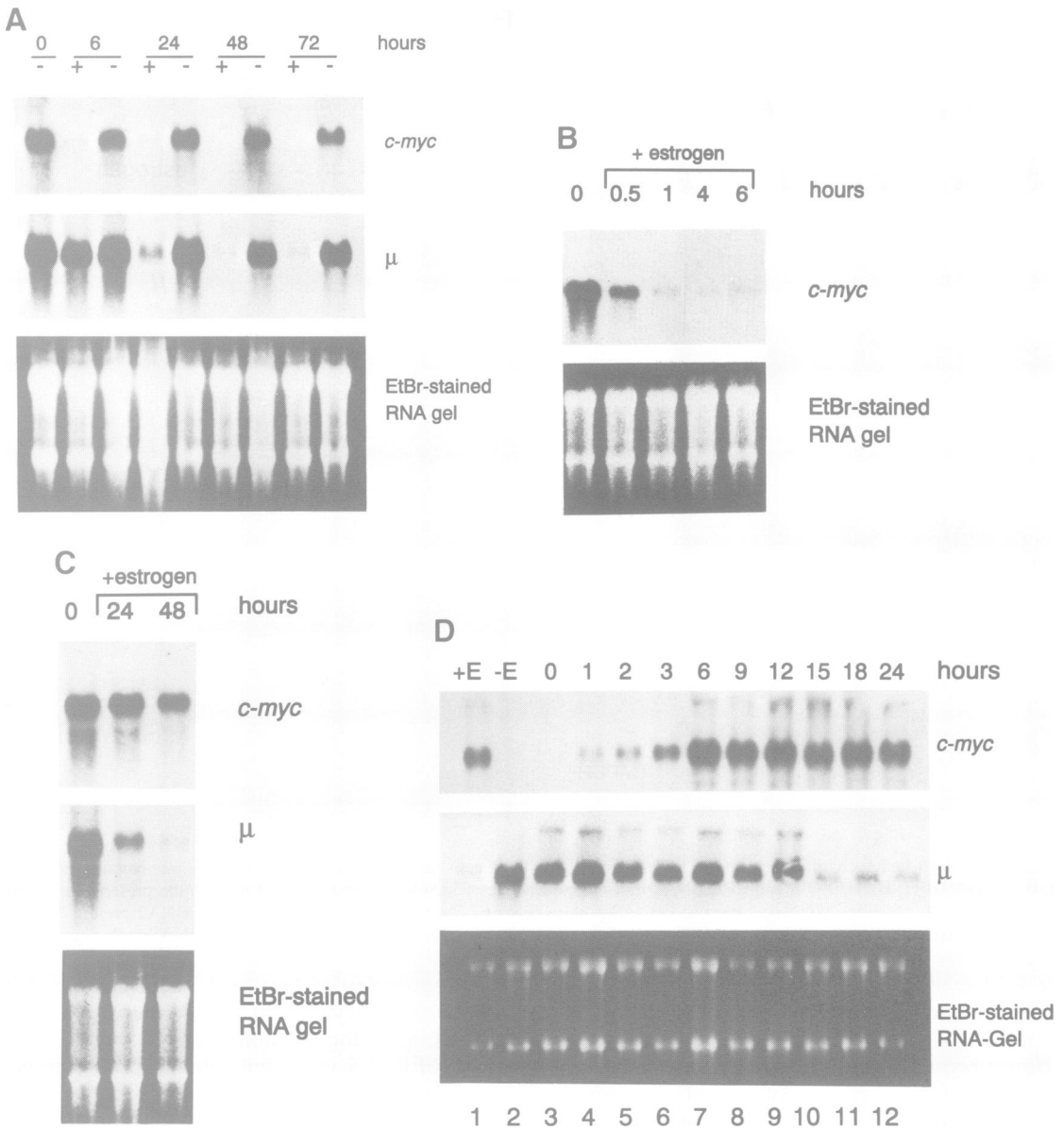

Fig. 4. Co-regulation of Ig-μ and c-*myc* gene expression in ER/EBNA2-transfected BL cells. P3HR1/3B6 (**A** and **B**) and BJAB/K3 (**C**) cells were treated with estrogen (+) for the indicated time periods and studied for expression of Ig-μ and c-*myc* RNA by Northern analysis. The ethidium bromide-stained gels are shown at the bottom of each panel. In estrogen-dependent LCLs, Ig-μ and c-*myc* expression are regulated in an opposite manner by estrogen (**D**).

In conditionally transformed lymphoblastoid cells, c-*myc* expression was regulated by estrogen in the opposite way to that in ER/EBNA2-transfected BL cells. c-*myc* was expressed in these cells in the presence of estrogen and was switched off if hormone was withdrawn (Figure 4D). Re-addition of hormone led to a rapid reappearance of c-*myc* RNA, which was detectable as early as after 1 h, and reached its full level of expression after 6 h. Ig-μ RNA decreased gradually over the time course of the experiment (Figure 4D).

Suppression of Ig-μ and c-myc transcription by EBNA2 in BL cells

To examine whether repression of the Ig-μ and c-*myc* genes by EBNA2 occurs at the transcriptional or post-transcriptional level, nuclear run-on experiments were performed with P3HR1/3B6 cells. As early as 30 min after addition of estrogen, the transcription signals for a c-*myc* cDNA probe and two probes of the Ig-μ constant locus decreased 4- to 5-fold, and then remained low (Figure 5). This time course identified Ig-μ as an immediate response gene of EBNA2. In addition, the simultanuous rapid repression of c-*myc* transcription in P3HR1/3B6 cells is in line with the possibility that Ig-μ and c-*myc* repression by EBNA2 act through a common target in the IgH locus.

Suppression of c-myc transcription in BL cells is followed by down-regulation of DNA synthesis

The c-*myc* oncogene has been shown to be involved in induction of DNA synthesis and cell growth (Marcu *et al.*, 1992). When P3HR1/3B6 and BL41/K3 cells were treated

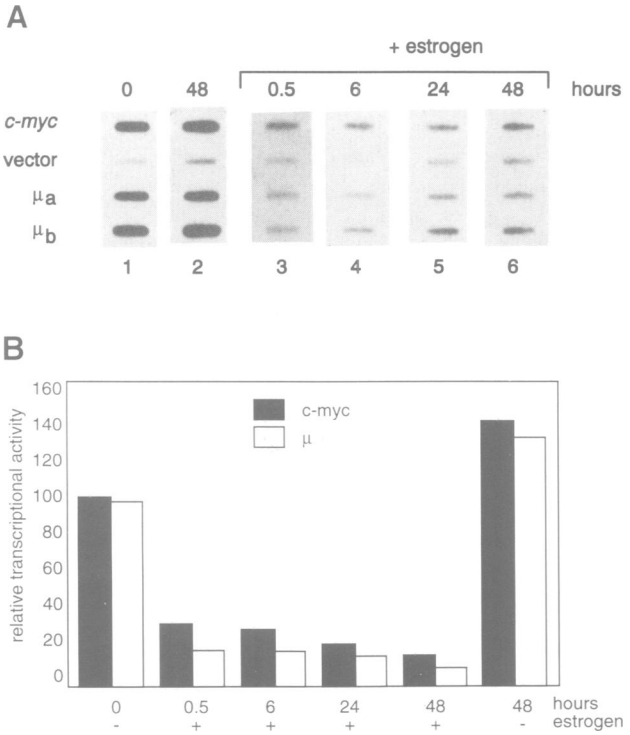

Fig. 5. Suppression of Ig-μ and c-*myc* RNA occurs at the transcriptional level. Nuclei were prepared from cells cultured in the presence (+) or absence (–) of estrogen for the indicated times. (**A**) Nuclear run-on RNAs were hybridized to c-*myc* and μ gene probes or vector DNA. (**B**) Quantitative evaluation of hybridization signals.

with estrogen for 48 h, a decline in DNA synthesis was observed, as measured by the reduction in [^{3}H]thymidine incorporation (Figure 6). At the same time, [^{3}H]thymidine incorporation had remained unchanged in BJAB/K3 cells, suggesting that down-regulation of c-*myc* expression is indeed responsible for the decrease in DNA synthesis 48 h after addition of estrogen.

A growth-retarding effect has, however, also been observed for BJAB/K3 cells in which c-*myc* is not switched off immediately after turning on EBNA2 function. Kinetic analysis revealed that growth retardation occurred in BJAB/K3 cells with considerable delay, as compared with P3RH1/3B6 and BL41/K3 cells. Moreover, BJAB/K3 cells were found to undergo DNA fragmentation between days 2 and 4 (data not shown), indicative of programed cell death (apoptosis). The loss in viability of BJAB/K3 cells may thus explain the failure to proliferate continuously after activation of EBNA2 (Kempkes *et al.*, 1996b).

Discussion

Studying the phenotype of lymphoblastoid cells conditionally transformed with the aid of a hormone-regulatable EBNA2–ER fusion protein, we have shown that EBNA2 is regulating IgM, CD21 and CD23 expression in a reciprocal fashion. IgM is down-regulated by EBNA2 whereas CD21 and CD23 expression is induced. Down-regulation of IgM by EBNA2 takes place at the level of transcription regulation. It is not an artifactual property of the fusion protein since triplets of EBV-negative BL cells converted by either the EBNA2-defective P3HR1

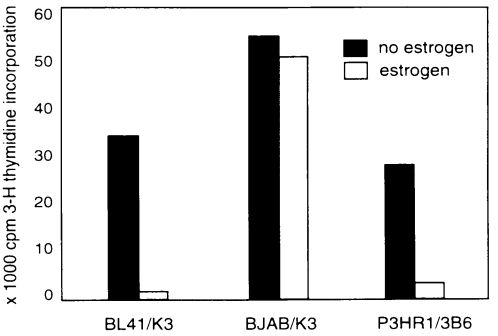

Fig. 6. Decline of DNA synthesis after suppression of c-*myc* expression in BL cell lines. BL41/K3, BJAB/K3 and P3HR1-3B6 (25 000 cells/well) were cultivated in the presence of estrogen for 2 days, pulsed for 4 h with [^{3}H]thymidine (0.5 μCi/well) harvested and analyzed for [^{3}H]thymidine incorporation (Kempkes *et al.*, 1995). The results are given in c.p.m.

virus or the EBNA2-competent B95-8 virus show the corresponding pattern of Ig RNA and surface IgM expression, with IgM being high in EBV-negative and P3HR1-converted BL cells and low in B95-8 convertants. Furthermore, Jijoye cells harboring an EBNA2-expressing virus express low amounts of Ig RNA whereas its clonal derivative, the P3HR1 cell line which is characterized by a deletion of the EBNA2 reading frame in the EBV genome, expresses high levels. The finding that EBNA2 is down-regulating IgM expression provides an explanation for the long standing observation that EBV-negative BL cells in culture tend to have higher levels of Ig expression than their EBV-positive counterparts (Magrath *et al.*, 1980; Benjamin *et al.*, 1982; Cohen *et al.*, 1987).

Since EBNA2 is a key regulator of the other viral nuclear as well as membrane antigens in EBV-transformed cells, the effect on IgM expression might be mediated either by EBNA2 or any of the viral downstream targets of EBNA2. To discriminate between these possibilites, the gene encoding the conditional EBNA2 fusion protein was stably introduced into various EBV-negative and P3HR1 virus-converted B cell lymphoma lines. Switching on EBNA2 function in these cells by addition of estrogen led to a decrease in surface IgM and μ RNA expression similar to that which is observed in estrogen-deprived ER-EB2-5 cells upon re-addition of estrogen. Ig-κ expression appeared not to be affected by EBNA2, except in BJAB cells.

The following points led us consider the possibility that EBNA2 is not only down-regulating IgM but also c-*myc* expression in the stably transfected BL41, BL41/P3HR1 and P3HR1 cells: (i) the target of EBNA2 for down-regulation of IgM is presumably located in the Ig heavy chain locus; (ii) EBNA2 induces growth arrest when its function is switched on in stably transfected lymphoma lines and (iii) due to the chromosomal t(8;14) translocation, the Ig heavy chain locus is governing c-*myc* expression in BL41, BL41/P3HR1 and P3HR1 cells.

In fact, we could show that switching on EBNA2 efficiently down-regulates c-*myc* expression within ~1 h. The different time course of c-*myc* and Ig-μ RNA decrease observed after addition of estrogen merely reflects the different stabilities of the RNA, with a half-life of ~10–15 min for c-*myc* RNA and ~3 h for Ig-μ RNA (Dani *et al.*, 1984; Jäck and Wabl, 1988). Nuclear run-on analysis

provided evidence for the fact (i) that Ig-μ and c-*myc* are indeed down-regulated by EBNA2 at a transcriptional level and (ii) that Ig-μ and c-*myc* transcription are affected by EBNA2 simultaneously. This strongly suggests that EBNA2 is mediating its effect on Ig-μ and c-*myc* expression through a common target, presumably a transcription factor or a transcription factor complex which is involved in Ig heavy chain but not light chain gene expression. The suggestion that EBNA2 is affecting Ig-μ and c-*myc* expression through a common target is strengthened by the fact that, in BJAB cells lacking a t(8;14) translocation, Ig-μ and c-*myc* expression are not affected simultaneously by EBNA2. The comparison of the ER–EBNA2-expressing clones BL41-K3 and P3HR1-3B6 with the corresponding BJAB-K3 transfectant revealed, furthermore, that down-regulation of c-*myc* is presumably responsible for the immediate arrest of [^{3}H]thymidine incorporation. BJAB-K3 cells, which do not show the immediate down-regulation of c-*myc* expression upon addition of estrogen, continue to incorporate [^{3}H]thymidine over the time course of the experiment (48 h). It should be noted, however, that also in BJAB-K3 cells in which c-*myc* expression is not affected directly by EBNA2, the cell number does not increase significantly when the function of EBNA2 is switched on. The number of replicating cells may be equal to the number of dying cells under these conditions. This effect of the ER–EBNA2 fusion protein on BJAB cells may be explained by the assumption that EBNA2 is inhibiting proliferation when expressed at an unselectedly high level (transfected cells express a non-functional fusion protein in the absence of estrogen), or that the EBNA2 function has to be balanced by other viral gene products such as, for example, EBNA3C (Le Roux *et al.*, 1994; Marshall and Sample, 1995; Robertson *et al.*, 1995) or LMP1 in order to promote proliferation.

The observation that EBNA2 is down-regulating IgM expression as well as c-*myc* expression in cells with a t(8;14) translocation not only provides an explanation for the apparent inefficiency of using EBV-transformed cells for monoclonal antibody (mAb) production, it also has important implications for the role of EBV in the development of lymphomas in immunocompromised individuals as well as BL. First, a number of EBV-associated lymphomas in immunocompromised individuals are apparently associated with plasmocytoid differentiation. Our data confirm and extend the observation of Wendel-Hansen *et al.* (1987) indicating that Ig and EBNA2 expression in one cell are mutually exclusive. In EBV-induced lymphoproliferations in SCID mice, cells producing high levels of Ig have lost the capacity to proliferate. EBNA2-positive cells within the lymphoproliferation should therefore represent the stem cell compartment of proliferating cells from which a proportion of the cells is driven into differentiation. Importantly, cellular factors must exist which are able to override the proliferation program imposed by the virus. As also suggested from the SCID model, plasmacytoid differentiation may be linked closely to virus production. Continuous virus production and reinfection of new cells may increase the risk that a predisposed cell may become infected and may finally give rise to the development of a tumor.

The fact that EBNA2 is down-regulating not only IgM but also c-*myc* expression in cells carrying the chromosomal t(8;14) translocation is apparently also important for Burkitt's lymphomagenesis. Non-expression of viral antigens in BL cells *in vivo* (except EBNA1) has, up until now, been attributed solely to selection of cells which can escape the surveillance of cytotoxic T cells directed towards viral antigens. Our data indicate that this is not the only mechanism which is operating. EBNA2 also has a direct anti-proliferative effect, at least for cells with t(8;14) translocations. Whether this is also true for cells with t(2;8) and t(8;22) variant translocations has still to be seen. Switching on the viral genome by azacytidine (AzaC) treatment *in vivo*, as proposed by Robertson *et al.* (1994), should therefore not only induce a cytotoxic T cell response to BL cells, but should also have a direct anti-proliferative effect. AzaC treatment may therefore be an interesting alternative for treatment of cases in which conventional chemotherapeutic strategies have failed.

Materials and methods

Cell lines and culture conditions

The estrogen-dependent cell lines ER/EB2-3 and ER/EB2-5 are LCLs which have been established by co-infection of primary B cells by P3HR1 virus and a mini EBV plasmid expressing ER–EBNA2 which is complementing the EBNA2 defect of P3HR1 virus *in trans* (Kempkes *et al.*, 1995). The estrogen-independent EB2-2 cell line was generated in parallel using wild-type EBNA2 to complement the EBNA2 defect of the P3HR1 virus. BL41 is an EBV-negative BL cell line with a t(8;14) translocation (Lenoir *et al.*, 1985), BL41/P3HR1 and BL41/B95-8 are BL41 cells stably infected (converted) with P3HR1 and B95-8 virus, respectively (Calender *et al.*, 1987). BJAB is an EBV-negative B lymphoma cell line (Klein *et al.*, 1974). BJAB/P3 is a BJAB cell line stably infected with P3HR1 virus. P3HR1 is a single cell clone of the EBV-positive BL cell line Jijoye carrying a t(8;14) translocation (Hinuma *et al.*, 1967). Jijoye produces an EBNA2 competent-, and P3HR1 cells an EBNA2-defective virus (Bornkamm *et al.*, 1982). The ER–EBNA2-expressing lymphoma cell lines are described in detail elsewhere (Kempkes *et al.*, 1996b). All cell lines were cultivated in RPMI1640 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin and streptomycin and 1 mM pyruvate. β-Estradiol (Merck, Darmstadt) was added to the cell culture medium at a final concentration of 1 μM.

FACS analysis and [^{3}H]thymidine incorporation

Cells (1×10^6) were incubated with an excess of unlabeled mouse mAbs recognizing CD21 (BL13) and surface IgM (AF6) (Dianova, Hamburg). Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse $F(ab)_2$ fragments (DAKO, Hamburg) were used as secondary antibody for staining of positive cells. Dead cells were identified and excluded from the analysis after propidium iodide staining (0.1 μg/ml). Samples were analyzed using a FACSCAN (Becton and Dickinson). The [^{3}H]thymidine incorporation assay was carried out as described elsewhere (Kempkes *et al.*, 1995).

Northern blot and nuclear run-on analyses

Northern and nuclear run-on analyses were performed as described (Eick and Bornkamm, 1989). The c-*myc* cDNA probe has been described in Albert *et al.* (1994). The 1.2 kb *Eco*RI–*Eco*RI fragment and the 0.9 kb *Eco*RI–*Sac*I fragment of the constant region, described in Eick *et al.* (1985), were used as Ig-μ gene probes, and the 2.5 kb *Eco*RI–*Eco*RI fragment of the constant region as the Ig-κ gene probe (Hieter *et al.*, 1980). The GAPDH probe is an end-labeled oligonucleotide complementary to the last 50 nucleotides of the mRNA (Allen *et al.*, 1987).

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