Epstein–Barr virus nuclear antigen 2 (EBNA2)–oestrogen receptor fusion proteins complement the EBNA2-deficient Epstein–Barr virus strain P3HR1 in transformation of primary B cells but suppress growth of human B cell lymphoma lines

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To develop a transformation system with a conditional Epstein–Barr virus nuclear antigen 2 (EBNA2) gene, we fused the hormone binding domain of the oestrogen receptor to the N or C terminus of EBNA2. In promoter transactivation as well as primary B cell transformation assays these chimeric EBNA2 proteins are able to substitute for wild-type EBNA2 in the presence of oestrogen. Here we provide evidence that this transformation is the result of double infection of a cell with two virions, the P3HR1 virus genome and a mini-EBV plasmid carrying the chimeric EBNA2 gene. Unexpectedly, expression of the same EBNA2–oestrogen receptor fusion protein in established human B cell lymphoma lines resulted in growth retardation or growth arrest upon the addition of oestrogen. By titrating the oestrogen concentration in these stably transfected cells, the growth retarding and the transactivating function of the chimeric proteins could not be dissociated. We propose that growth inhibition of established B cell lymphoma lines is a novel function of EBNA2 which has not been detected in the absence of an inducible system. It remains open whether the growth retarding property of the EBNA2–oestrogen receptor fusion protein in B cell lymphoma lines is due to unphysiologically high expression of the chimeric protein or to interference with a cellular programme driving proliferation in these cell lines.

Introduction

Epstein–Barr virus (EBV)-infected primary human B cells proliferate indefinitely in vitro, a phenomenon called immortalization or transformation. This process involves the complex interplay of a number of viral and cellular genes. EBNA2 is known to play a particularly important role in this process and is the first gene expressed in virus-infected primary B cells together with another viral gene product (EBNA-LP) (Allday et al., 1989; Rooney et al., 1989). EBNA2 serves as a master switch in that viral and cellular genes involved in transformation by EBV are turned on by EBNA2 (Abbot et al., 1990; Calender et al., 1987; Cordier et al., 1990; Fahraeus et al., 1990; Knutson, 1990; Sung et al., 1991; Wang et al., 1987; Woisetschlaeger et al., 1991; Zimber-Strobl et al., 1991). The mechanism of action of EBNA2 has just started to be elucidated. Transactivation of the TP1- (LMP2A-) and the BamHI-C promoters by EBNA2 is mediated by an EBNA2 responsive element (EBNA2RE) in the upstream region of these promoters. EBNA2 does not bind to DNA directly (Zimber-Strobl et al., 1993; Ling et al., 1993) but interacts with a cellular protein, RBP-Jκ (Matsumani et al., 1989) which binds to the conserved core sequence (CGTGGGAA) within the EBNA2REs in a sequence specific manner (Henkel et al., 1994; Grossman et al., 1994; Zimber-Strobl et al., 1994; Waltzer et al., 1994). Additional cellular proteins are likely to participate in the transactivation by EBNA2, since the EBNA2REs do not consist only of binding sites for RBP-Jκ. EBNA2 is absolutely required for initiation of transformation (Cohen et al., 1989; Hammerschmidt & Sugden, 1989). The transforming capacity is lost in
virus strains in which the EBNA2 gene is deleted, but can be restored if the EBNA2 open reading frame is restored.

Fusion of a given protein with the hormone binding domain of a steroid receptor can render the function of the protein dependent on the presence of hormone (Picard et al., 1988; Eilers et al., 1989). We have exploited this approach to develop a conditional system in which the function of the EBNA2 protein can be switched on and off. Fusion of EBNA2 with the hormone binding domain of the oestrogen receptor rendered the in which the function of the EBNA2 protein can be (Picard et al., 1988). The substrate for in vitro mutagenesis was a HindIII–SacI fragment with the EBV strain B95-8 sequence coordinates 49468–48849 (Baer et al., 1984) cloned in a Bluescript vector. A BamHI/EcoRI fragment from plasmid HE14 encompassing amino acids 282–595 of the human oestrogen receptor (Kumar et al., 1986) was inserted between these newly generated BgII and EcoRI sites.

The N-terminal fusion constructs (plasmids 294-6/N and p554-4 encoding ER/EBNA2) were generated by inserting an EcoRI site in front of the open reading frame of EBNA2 by in vitro mutagenesis of a HindIII–BamHI fragment (EBV strain B95-8 sequence coordinates 49468–48849) subcloned in a Bluescript vector using the oligonucleotide 5′ AACGCAAGAIAAGATGTGAGCAGGGAAACCCTCT 3′. The hormone binding domain of the oestrogen receptor from the plasmid HE14 was inserted into this EcoRI site as a PCR fragment. The sequence of the 5′ primer was 5′ GTAGTTGAAT 3′ and the sequence of the 3′ primer was 5′ GATGATGAATTCGACTGTGGCAGGGAAACCCTCT 3′. This PCR fragment included Kozak’s sequence and a start codon provided by HE14 and excluded the stop codon of HE14. The subsequent cloning steps of the p554 derivatives (Hammerschmidt & Sugden, 1989) regenerated EBV strain B95-8 sequence coordinates 13944–55775 with the following modifications being introduced: the NolI repeats were deleted and only two BamHI-W repeats were restored using p554, which carries a neomycin resistance gene and the terminal repeats and generated p554-3 and p554-4. For construction of 294-6/C and 294-6/N plasmids (Polack et al., 1984) was used as a vector regenerating EBV coordinates 39018–54364. The LMP1 and TP1 and TP2 promoter luciferase indicator plasmids have been described before (Laux et al., 1994; Zimmer-Strobl et al., 1993).

Methods

Cell lines and culture conditions. B lymphoma cell lines BL41 (Lenoir et al., 1985), BJAB (Klein et al., 1974), BJAB/P3HR1, BL41/P3HR1 (Calender et al., 1987), HH514 (Rabson et al., 1982), MCB, MCN (Cordier et al., 1990) and the M-ABA cell line (Crawford et al., 1979) were routinely kept in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 μg/ml penicillin, 100 μg/ml streptomycin and 1 mM-pyruvate. BPL2 is a lymphoblastoid cell line generated by infection of CD19-enriched human B cells with EBV strain B95-8. MCB and MCN are BL41/P3HR1 cells stably transfected with wild-type EBNA2 or vector control, respectively. HH514 is a subclone of B95-8 sequence coordinates 13944–55775 with deletions of the open reading frame of EBNA2. HH514 cells (1 × 10^5) were transfected with 10 μg pCMV BZLF1 (p509) and 10 μg EBNA2 (p554), ER/EBNA2 (p554-4) or EBNA2/ER (p554-3) expression plasmids by electroporation using a Bio-Rad gene pulser at 960 μlF and 250V in a total volume of 250 μl RPMI 1640 cell culture medium containing 10% fetal calf serum. After electroporation cells were kept on ice for 10 min and then resuspended in 10 ml RPMI 1640 cell culture medium with supplements. After 4 days the supernatant of the transfected cell was harvested and filtered through a 0.45 μm sterile filter. Ficoll-purified primary cord blood lymphocytes were infected with the filtered supernatants by overnight incubation. Infected cells, seeded in 96-well flat bottom plates (50000 cells per well) and cultivated on irradiated MRC5 fibroblast feeder layers (5000 rad) in the presence of oestrogen (1 μM-β-oestradiol) gave rise to cell lines after 4–6 weeks.

Southern blots. Cellular DNA was prepared and Southern blot hybridization carried out as described by Hammerschmidt & Sugden (1989).

DNA transfection and luciferase assays. ER/EBNA2 expression plasmid p554-4 was transfected in B lymphoma cell lines by electroporation as described before (Zimmer-Strobl et al., 1993). Two days after transfection G418 (BRL) was added to a final concentration of 1250 μg/ml (HH514 cells, BL41/P3HR1 and BJAB/P3HR1) or 800 μg/ml (BL41 and BJAB) to the culture medium. Growing cells were routinely kept in selection medium.

Phosphatase treatment and immunostaining of proteins. Nuclear protein extracts were prepared as described (Zimmer-Strobl et al., 1993). For Western blot analysis 5 μg of the nuclear extracts treated for 2 h at 37 °C with calf intestine phosphatase (11 units; Sigma), white
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Results

EBNA2–oestrogen receptor fusion protein complementing the EBNA2 defect of P3HR1 virus in primary B cell transformation is provided in trans

We have shown recently that EBNA2–oestrogen receptor fusion proteins are able to substitute for wild-type EBNA2 in primary B cell transformation by EBV in the presence of oestrogen (Kempkes et al., 1995b). Withdrawal of hormone from conditionally transformed lymphoblastoid cells induced growth arrest indicating that EBNA2 is required not only for initiation but also for maintenance of B cell transformation by EBV. For these experiments, transforming virus preparations had been generated by transfecting into P3HR1 cells the chimeric EBNA2 gene cloned onto a mini-EBV vector carrying a viral episomal and lytic origin of replication (Hammerschmidt & Sugden, 1988; Yates et al., 1984). The defective phenotype resulting from the deletion in the P3HR1 virus strain can be cured either by complementation or recombination (Hammerschmidt & Sugden, 1989; Kempkes et al., 1995a). In the former case, double infection with a P3HR1 virion and a packaged concatameric derivative of the mini-EBV plasmid gives rise to immortalized B cell clones. Complementation has been predominantly observed with p554 mini-EBV derivatives expressing a functional EBNA2 allele (Hammerschmidt & Sugden, 1989). Alternatively, the deletion in P3HR1 is healed as the result of a double recombinational event between the P3HR1 virus genome and mini-EBV plasmid DNA giving rise to a single species of viral DNA in the immortalized cell clone. Recombinations presumably arise during concomitant lytic replication of both DNAs in the HH514 packaging cell line.

To analyse the status of viral DNAs in the B cell clones which arose from infections with virus stocks containing P3HR1 and p554 derived mini-EBVs, Southern blot hybridization was carried out with cellular DNA from individual cell clones. All eight clones were found to harbour P3HR1 helper virus DNA together with mini-EBV DNA (Fig. 1). This result is supported by four specific fragments which were visualized in the autoradiogram. The 4-1 kbp BamHI fragment is indicative of the EBNA2 deletion fragment of P3HR1 virus and is present in all clones including the packaging cell line HH514. The fragments of 14-8, 4-6 and 1-5 kbp are characteristic of the mini-EBV plasmid p554-4, indicating that not only the chimeric EBNA2 gene (4-6 and 1-5 kbp fragments) but also the plasmid backbone of this plasmid (14-8 kbp fragment) is present in all ER/EB2 clones. Similarly, the corresponding fragments of p554 (14-8, 6-0 and 1-8 kbp) are all present in the control cell line EB2-2 (Fig. 1 a). The size of the 14-8 kbp BamHI fragment which is characteristic of the plasmid backbone of p554 and its derivatives shows some variability, as can be seen in the cell clone ER/EB2-2. These size variations are due to different copy numbers of the terminal repeats which are located in this fragment (Hammerschmidt & Sugden, 1989). The remaining signals of 9-2 and 9-6 kbp are indicative of the BamHI-C fragment and the fragment carrying the second lytic origin of replication in the P3HR1 virus genome, respectively, which is also detected by this probe (Bornkamm et al., 1982). Additional off-size bands seen in clones ER/EB2-1, -5 and -7 might indicate rearrangements of the viral genomes which may occur during lytic replication in the helper cell line. The results clearly indicate that all cell lines carry two different species of virus DNA. No clone could be found which arose from a single infection with a P3HR1 virus with its deletion healed by recombination as has been observed previously (Hammerschmidt & Sugden, 1989). We conclude that transformation of primary B cells with P3HR1 virus and the chimeric EBNA2–oestrogen receptor gene is, as a rule, the result of a double infection of a cell with two virions giving rise to complementation in trans.

Stable expression of ER/EBNA2 in B cell lymphoma lines

To study the molecular events following EBNA2 activation, the gene encoding the EBNA2–oestrogen receptor fusion protein was also stably introduced into established B cell lymphoma lines. Since the N-terminal and C-terminal fusion proteins were similarly active in transactivation and transformation (Kempkes et al., 1995b), the following experiments were carried out with one of the fusion proteins only (ER/EBNA2). BL41, BL41/P3HR1, BJAB, BJAB/P3HR1 and P3HR1 (subclone HH514) cells were transfected with the ER/EBNA2 expression plasmid p554-4 and selected by G418. Expression of the ER/EBNA2 fusion protein was studied by immunostaining of Western blots using the
Fig. 1. Southern blot analysis of lymphoblastoid cell lines transformed by P3HR1 virus complemented with ER/EBNA2 encoding constructs. (a) Primary lymphocytes were infected with the supernatant of p554/p509 or p554-4/p509 co-transfected HH514 cells (a subclone of the P3HR1 cell line) and permanently growing lymphoblastoid cell lines were isolated. The cell line EB2-2 was generated after infection with the supernatant of p554 transfected HH514 cells, whereas the cell lines ER/EB2-1 to ER/EB2-8 were generated after infection with p554-4 transfected HH514 supernatants. Total cellular DNA from these cell lines and from HH514 cells was isolated and digested with BamHI and analysed by the Southern blot technique. The plasmid pM780-28 was used as a probe. (b) Schematic presentation of the BamHI restriction pattern of the constructs p554 and p554-4 and the probe pM780-28 (EBV B95-8 strain coordinates 35448–54364). In the P3HR1 virus this probe detected the BamHI W repeats (3-1 kb), the fragment containing the right-hand duplicated segment of about 9.2 kb, and the BamHI-H fragment of about 4-1 kbp with the deletion of EBNA2 and parts of the EBNA-LP gene. Three additional fragments were detected in the case of the cell line EB2-2, which was generated by infection of primary B cells with supernatants containing P3HR1 virus and packaged p554 plasmid: (i) the 6-0 kb BamHI-H fragment spanning the EBNA2 wild-type gene, (ii) the 1-8 kb BamHI-Y fragment and (iii) the 14-8 kb plasmid backbone of p554. In the case of the cell lines ER/EB2-1 to ER/EB2-8, which had all been generated by infection of primary B cells with supernatants containing the p554-4 plasmid,
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EBNA2-R3 monoclonal antibody (Kremmer et al., 1995). As shown in Fig. 2(a) for the BL41/P3HR1-9A transfectant, the antibody detected a protein of apparent molecular mass 120 kDa as compared to the molecular mass of 86 kDa of wild-type EBNA2. Addition of oestrogen for 24 h resulted in a marked increase in abundance of the fusion protein which exceeded the amount of EBNA2 detected in the human lymphoblastoid cell line BPL2 and was similar to the amount of EBNA2 protein seen in the marmoset cell line M-ABA. The oestrogen-mediated increase in the amount of EBNA2 protein presumably reflects the fact that EBNA2 enhances its own transcription from the BamHI-C promoter.

The increase in the amount of the EBNA2 protein was accompanied by a shift in the electrophoretic mobility indicating post-translational modification. Treatment of BPL2 nuclear extracts with white potato phosphatase (WPP), but not with calf intestine phosphatase (CIP), resulted in increased electrophoretic mobility of wild-type EBNA2 protein (Fig. 2b). When nuclear extracts of oestrogen induced BL41/P3HR1-9A cells were treated with WPP, ER/EBNA2 migrated at the same position as the chimeric protein in the absence of oestrogen. No further change in the electrophoretic mobility of the ER/EBNA2 protein was seen after treatment of extracts of uninduced cells. We thus conclude that the ER/EBNA2 protein is phosphorylated after ligand binding and that this phosphorylation is sensitive to WPP treatment. Since both wild-type EBNA2 and the human oestrogen receptor are known to be phosphoproteins (Ali et al., 1994; Grässer et al., 1991), EBNA2 as well as the hormone binding domain could be the target for phosphorylation within the chimeric protein. The functional relevance of this phosphorylation is still unknown. All ER/EBNA2 transfectants, which expressed the ER/EBNA2 fusion protein, were functional since transactivation of the LMP1 and TP1 promoter luciferase reporter constructs transiently transfected into these cells was dependent on oestrogen (B. Kempkes, unpublished results).

**Activation of EBNA2 function in B cell lymphoma lines induces growth arrest**

Addition of oestrogen to cell lines stably transfected with EBNA2–oestrogen receptor fusion constructs had a dramatic effect on proliferation of the cells. Fig. 3(a) shows the result of an experiment in which cell proliferation was followed semiquantitatively in an MTT assay (Mosmann, 1983). The kinetics of proliferation was studied in more detail by counting trypan blue

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**Fig. 2.** Phosphorylation and increase of ER/EBNA2 abundance after ligand binding. Nuclear extracts (5 μg) of the LCL cell line BPL2, the marmoset cell line M-ABA and the ER/EBNA2 transfectant BL41/P3HR1-9A either untreated or after oestrogen induction were separated on 8% SDS-polyacrylamide gels run under reducing conditions and analysed for EBNA2 expression by immunostaining with the monoclonal antibody EBNA2-R3. (a) The transfectants expressed an EBNA2 fusion protein of about 120 kDa compared to the 86 kDa wild-type EBNA2 seen in BPL2 and M-ABA. Addition of oestrogen resulted in increase of EBNA2 abundance and alteration in the electrophoretic mobility of the construct indicating post-translational modification of the ER/EBNA2 protein. (b) Nuclear protein extracts (5 μg) from oestrogen induced or non-induced BL41/P3HR1-9A and BPL2 cells were treated with calf intestine phosphatase (CIP) or white potato phosphatase (WPP) and then analysed for EBNA2 expression as described in (a). Treatment of extracts of oestrogen induced BL41/P3HR1 cells with WPP resulted in increased electrophoretic mobility of the ER/EBNA2 molecule. The additional faster migrating proteins of about 80–90 kDa seen after treatment of cell extracts are proteolytic degradation products of the ER/EBNA2 chimeric proteins.

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The 1.8 kb and 6.0 kb fragments are replaced by 1.5 kb and 4.6 kb fragments. The fragment X which contains the ER domain is not detected by the probe. Additional off-size bands seen in the HH514 cellular DNA and in the clones ER/EB2-1, -5 and 7 might stem from recombination events. Variable faint bands of high molecular mass might possibly indicate cross-hybridization of the probe with other viral fragments.
positive and negative cells after seeding the cells for 1, 2, 3 and 4 days in the presence and absence of oestrogen. Fig. 3(b) shows one representative experiment out of a total of three. Addition of oestrogen dramatically suppressed proliferation in cells stably transfected with the ER/EBNA2 gene but not in control cells. The number of viable cells remained fairly constant throughout the course of the experiment (between 85 and 98%) for all cell lines except for BL41/K3 cells after treatment with oestrogen. Viability of BL41/K3 cells decreased to 60% at day 2 and 25% at day 4 after addition of oestrogen. Flow cytometric analysis for propidium iodide staining on the one hand and granularity on the other (Cotter et al., 1992; Eissner et al., 1995) revealed no signs of apoptotic cell death in oestrogen-treated BL41/K3 cells (data not shown).

Growth arrest or growth retardation was seen in all ER/EBNA2 expressing B cell lymphoma lines irrespective of the presence of the P3HR1 EBV genome. As a control, we tested BL41/P3HR1 cells transfected with wild-type EBNA2 (MCB) or a vector plasmid (MCN). Oestrogen had no effect on the growth kinetics of both of these cell lines (Fig. 3b).

Suppression of growth and transcriptional activation by ER/EBNA2 cannot be dissociated

ER/EBNA2 dramatically suppressed proliferation of B cell lymphoma lines in the presence of oestrogen. This finding was unexpected since EBNA2 is essential for primary B cell transformation. We next investigated whether the growth suppressing and the transactivating function of ER/EBNA2 can be dissociated by titrating the oestrogen concentration. Proliferation and transactivation of the TP1 promoter was quantified for two ER/EBNA2 transfected cell lines (BL41/K3 and BJAB/K3) at different concentrations of β-oestradiol. Fig. 4 shows one representative experiment out of a total of three. It was apparently impossible to dissociate both effects. Dose response curves measuring TP1 promoter
effects of conditional EBNA2

Fig. 3. Oestrogen induces growth arrest in ER/EBNA2 expressing B cell lymphoma lines as revealed by MTT assay (a) and measuring the proliferation kinetics (b). B cell lymphoma lines BL41, BJAB and HH514 and the corresponding ER/EBNA2 transfectants BL41/K3, BL41/P3HR1-5E, BJAB/K3, BJAB/P3HR1-B6 and HH514-3B6 were seeded at 0.25 x 10^6 cells/ml in the presence or absence of oestrogen ('E'). Parallel cultures were assayed for MTT conversion on day 0 immediately after seeding and on day 4 (a). To measure the proliferation kinetics, BL41, HH514, BL41/P3HR1 and the corresponding transfectants BL41/K3, HH514-3B6, BL41/P3HR1-5E, MCB (EBNA2) and MCN (vector control) were seeded at a density of 2 x 10^5 cells/ml in the presence or absence of oestrogen and were counted at days 1, 2, 3 and 4 after seeding (b). Cell numbers represent viable (trypan blue excluding) cells.

Discussion

To study the role of EBNA2 in transcription regulation and transformation we have generated an inducible system in which EBNA2 function can be reversibly switched on and off. This was achieved by fusing EBNA2 to the hormone binding domain of the oestrogen receptor at either the N or the C terminus. Both constructs apparently can transactivate viral EBNA2-responsive promoters in a hormone-dependent fashion and exhibit a low degree of background promoter activation in the absence of hormone.

Both constructs were also able to substitute for wild-type EBNA2 in primary B cell transformation, provided that oestrogen was added to the culture medium.
Fig. 4. Growth arrest and transactivation of the TP1 promoter occur at identical oestrogen concentrations. ER/EBNA2 expressing BL41/K3 and BJAB/K3 cells were tested for growth (a) and transactivation of the TP1 promoter (b) at different oestrogen concentrations. Cells were transfected with TP1 promoter luciferase constructs and oestrogen was added immediately after transfection. In parallel, cells were cultured in media supplemented with oestrogen at various concentrations for 4 days and living cells were counted after exclusion of dead cells by trypan blue staining.

(Kempkes et al., 1995b). To further characterize the system we investigated whether the gene encoding the fusion protein is provided in trans thus complementing the EBNA2 defect of P3HR1 virus or is introduced into the P3HR1 virus genome by homologous recombination. Analysing eight individual conditionally transformed cell clones we have shown here that transformation was the result of a double infection of a cell with two virions, one carrying the P3HR1 virus genome, and the second the EBNA2–oestrogen receptor fusion gene. This does, of course, not rule out the possibility of homologous recombination as a rare event leading to integration of the EBNA2 fusion gene into the P3HR1 virus genome at the site of the P3HR1 virus deletion. It is at present impossible to decide whether the frequency of homologous recombination between the P3HR1 virus genome and the flanks derived from another viral strain (B95-8) was not sufficiently high to observe recombination, or whether virus carrying an EBNA2 fusion gene in cis but only two BamHI-W repeats will have a lower transforming potential than a mixed population of virions complementing each other during B cell transformation.

In addition to studying EBNA2–oestrogen receptor fusion proteins in the course of B cell transformation, ER/EBNA2 was stably introduced into various B cell lymphoma lines in which EBNA2 function could be switched on by the addition of oestrogen. This resulted in the surprising observation that functional EBNA2 induces proliferation arrest or severe suppression of proliferation. Growth arrest was consistently observed in all cell lines stably transfected with the fusion construct and in all individual cell clones which exhibited transactivating potential for the LMP- and TP1-promoters in the presence of oestrogen.

There are two possibilities to explain this finding. The first makes the assumption that the growth inhibiting function is a novel function of the fusion protein, which has been acquired by the protein–protein fusion process and is absent from EBNA2 wild-type protein. This is difficult to reconcile in view of the fact that the fusion protein has an activity and specificity for transactivation of viral and cellular genes that is very similar to that of wild-type EBNA2 (B. Kempkes, unpublished results) and that the fusion protein is able to complement the EBNA2 defect of P3HR1 virus in the most rigorous biological assay we have at hand, i.e. transformation of primary human B cells. We consider it extremely unlikely
that the protein–protein fusion process would have created a novel function (arrest of proliferation) without having a deleterious effect on the complex biochemical and biological activities of EBNA2 required in the process of B cell transformation.

The second possibility is that the growth retarding or inhibiting activity of the fusion protein is in fact a novel intrinsic function of EBNA2 which is dependent on the cellular and viral background. We propose that such an activity of EBNA2 exists but has not been noticed in the absence of an inducible system.

How can the growth inhibiting effect of EBNA2 be explained? There are two possibilities, not mutually exclusive, which both relate to the fact that the EBNA2 expression level is rather high in the transfected cell lines. There is apparently no selection for low EBNA2 expression, if EBNA2 is expressed as a nonfunctional protein in the absence of hormone, and addition of hormone may lead to unphysiologically high EBNA2 levels. According to the first model, EBNA2 might stimulate proliferation at low concentration and inhibit proliferation when present at high concentration. For transcription factors, stimulation of transcription at low concentration and inhibition at high concentration is known as squelching. If inhibition of proliferation is due to squelching, we would expect to see a correlation between inhibition of proliferation and inhibition of transactivation in the stably transfected cell lines when EBNA2 is fully active in the presence of hormone. By titrating the oestrogen concentration it was impossible, however, to dissociate the proliferation inhibiting and the transactivating function of EBNA2. On the contrary, the concentration of oestrogen resulting in maximal inhibition of proliferation caused the highest degree of transactivation. We have therefore to consider mechanisms for inhibition of proliferation by EBNA2 other than inhibition of transactivation through squelching. Gel shift experiments with nuclear extracts of cells expressing conditional EBNA2 fusion protein revealed a depletion of complex III (both 11 bp repeats occupied by RBP-Jκ) (Meitinger et al., 1994) after addition of hormone that is not seen in extracts of lymphoblastoid and EBV positive Burkitt’s lymphoma (BL) cell lines (B. Kempkes, unpublished results). The amount of functional EBNA2 liberated upon addition of oestrogen may thus be high enough to sequester other factors whose availability might be required for continuous proliferation.

According to the second model, EBNA2 might interfere with a pathway which is activated in the human lymphoma cell lines and is driving proliferation of these cells. If so, activation of EBNA2 expression in EBV positive BL cells or infection of EBV negative BL cells with EBV should slow down proliferation to a degree reflecting the EBNA2 expression level of the cells. EBNA2 expression is apparently extremely finely tuned in vitro in proliferating BL cells, since the EBNA2 expression level does not seem to differ significantly in cells with 1 to 2 compared to 50 to 100 viral genome copies per cell (Abbot et al., 1990; Cordier et al., 1990). A growth retarding effect of B95-8 virus has in fact been observed after in vitro infection of the EBV negative BL cell line BL41, which has resulted in loss of the tumorigenic potential in a B95-8 virus converted BL41 cell clone (Torsteinsdottir et al., 1989). Although this effect has been ascribed to LMP1 protein (Cuomo et al., 1992), a contribution of EBNA2 to this phenomenon has not been excluded. It is also a general observation that group III BL cell lines proliferate significantly more slowly than EBV negative and EBV positive group I BL cell lines (Falk et al., 1993). Spontaneous loss of the EBNA2 open reading frame might even accelerate proliferation and might be selected for in vitro as observed in P3HR1 (Rabson et al., 1982) and Daudi cells (Klein et al., 1974). We thus propose that the growth retarding effect of wild-type EBNA2 for BL cells has in fact been observed earlier, but that it has not been possible to link this effect unambiguously to EBNA2 without the availability of an inducible system.

To discriminate between the two models outlined above, the gene encoding the conditional EBNA2 fusion protein will now be stably introduced into primary human B cells which have been transformed by EBV in vitro. Switching on EBNA2 in such cells should provide an answer to the question as to whether expression of high levels of EBNA2 is inhibiting proliferation only in established B cell lymphoma lines or also in primary B cells transformed by EBV.

In conclusion we have shown that EBNA2--oestrogen receptor fusion is involved in B cell transformation by complementing the EBNA2 defect of P3HR1 virus in trans. Stable expression of these constructs in human B cell lymphoma lines has unexpectedly revealed a proliferation inhibiting function of EBNA2. The dual system of positive and negative growth regulation by EBNA2 described here may help to further elucidate the biochemical mechanism of action of EBNA2.

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