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Gene Array Identification of Epstein Barr Virus-Regulated Cellular Genes in EBV-Converted Burkitt Lymphoma Cell Lines

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SUMMARY: Epstein Barr virus (EBV) is associated with various B-cell neoplasms such as post-transplant lymphoproliferative disease or Burkitt lymphoma. B-lymphocyte reprogramming by EBV involves the control of numerous cellular genes. To identify such EBV-deregulated genes, we have compared the gene expression profile of EBV-negative Burkitt lymphoma cell lines (BL) (BL2, BL30, BL70) with their EBV-converted counterpart (BL2-B95, BL30-B95, BL70-B95) by cDNA array. Statistical analysis of the results was made using Ward's cluster analysis method. Results showed that the expression of up to 26% of the 1176 cellular genes analyzed may be modified in EBV-converted BL cells. Within this set of genes, a subset of genes markedly regulated in EBV-converted BL cells was defined as those for which expression in EBV+ cells was increased or decreased more than 2-fold. Expression of various genes was modulated in agreement with their previously reported regulation by EBV or by transcription factors activated by EBV. Numerous genes were newly identified as modulated in EBV-converted BL cells. Some of these results were verified by both semiquantitative RT-PCR and Western blotting, and were consistent with functional studies. Functional classification of EBV-regulated genes gave a comprehensive picture of cellular reprogramming by EBV in BL , by pointing out cellular modules such as cell cycle, apoptosis, and signal transduction pathways, including BCR and TNF receptor family and interferon pathways. Furthermore, and perhaps most importantly, cDNA array results point to three families of transcription factors, Rel/NF-κB, STAT1, and Ets-related proteins Spi-B, Elf-1, and Ets-1 as putative cellular targets of EBV. (*Lab Invest 2002, 82:1463–1479*).

Epstein-Barr virus (EBV) is an ubiquitous human herpes virus associated with a number of human malignancies including immunodeficiency-related lymphoproliferative diseases, Burkitt's lymphoma (BL), Hodgkin's lymphoma, and nasopharyngeal carcinoma (for review, see Cohen, 2000). In vitro, EBV-infected BL cell lines have been extensively studied as a model of EBV latency or for searching for cellular targets of the virus (see, for example, Henderson et al, 1991; Henriquez et al, 1999). Immunoblastic and plasmacytoid differentiation of EBV-infected BL cell was observed years ago (Gregory et al, 1990; Rowe et al, 1987), corresponding to group III of BL cell lines and showing that EBV is able to reprogram BL cells.

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EBV latent proteins mimic and/or reroute cellular transduction pathways (for review, see Thorley-Lawson and Babcock, 1999). Molecular interactions between cellular factors and EBNA2, LMP1, or LMP2A are examples of such rerouting. EBNA2 directly interacts with the cellular protein RBP-Jk. On the one hand, EBNA2/RBP-Jk binding with so-called EBNA2-response elements on the EBV episome transactivates both LMP1 and Wp/Cp latency III promoters of the other viral genes coding for latent proteins. On the other hand, targeting of RBP-Jk by EBNA2 is associated with differentiation blockade and cellular proliferation of quiescent B-cells infected by EBV (Hsieh et al, 1996; Strobl et al, 1997) through the control of some defined cellular genes, among them CD21/EBV receptor, activation marker CD23 (Calender et al, 1987; Cordier-Bussat et al, 1993), and *c-fgr* oncogene. EBNA2 could also decrease the cell proliferation rate of BL cells (Jochner et al 1996, Strobl et al 2000). LMP1 is able to induce both cell proliferation and protection against apoptosis. LMP1 acts as a constitutive activated receptor mimicking the B-cell/T-cell CD40-CD40L interaction and leading to the activation of the transcription factor NF-κB (Leibowicz, 1998; Mosialos et al, 1995), to JNK/p38 phosphorylation,

and to phosphorylation and nuclear translocation of STAT1. LMP2A is able to deliver a rescue signal to B cells and directly interacts with src-kinase lyn and with the kinase syk, both involved in the BCR (B cell receptor) signaling pathway (Caldwell et al, 2000). These examples highlight the fact that each EBV latent protein may control a part of the cellular host program, and suggest that knowledge of the whole set of cellular genes deregulated by EBV (ie, EBV-related transcriptome) would be very informative in the definition of cellular targets of the virus.

One cellular model developed to analyze EBV-cellular targets was designed by Calender et al (1987) and consists in the in vitro establishment of EBV-converted BL cells from EBV-negative BL cell lines. The major advantage of this cellular model is that it allows comparison of EBV-converted BL cells with their genetically identical EBV-negative counterpart. Therefore, genetically identical BL cell lines, infected or not by EBV, would allows one to analyze the whole cellular EBV-related transcriptome and to identify EBV cellular targets.

We have compared the gene expression profile of in vitro EBV-converted BL cell lines with their EBV-negative counterpart using cDNA array. Statistical analysis of cDNA results was performed using Ward's cluster analysis method. Functional classification of clustered genes allowed the identification of EBV-deregulated cellular modules such as proliferation, apoptosis, response to interferon, and signal transduction. Furthermore, three families of transcription factors, Rel/NF-κB, STAT1, and ETS proteins were identified as putative cellular targets of EBV in this model.

Results

Phenotypic Characterization and Functional Analysis of EBV-Converted BL Cells Compared with Their Genoidentical EBV-Negative Counterpart

Three different pairs of BL cell lines in vitro infected with EBV (BL2, BL30, BL70 versus BL2-B95.8, BL30-B95.8, BL70-B95.8) have been used in this study. This cellular model has been previously described (Calender et al, 1987). As expected, genomic amplification of the FR3-JH framework region of the immunoglobulin heavy chain gene showed the clonal origin of both EBV-negative and their EBV-converted BL counterpart. Amplification of the EBNA1 locus evidenced the absence of the EBV genome in EBV-negative BL cells whereas their EBV-converted counterpart exhibited the virus (Fig. 1). Characterization of FQp, Cp and Wp promoter usage and analysis of EBV latent protein expression showed a latency III stage (data not shown) of the three EBV-converted BL cell lines, in agreement with the initial description of the cellular model (Calender et al, 1990). In agreement with the latency III stage of EBV-converted BL cells, EBV-converted cells exhibited morphologic changes such as immunoblastic and plasmablastic differentiation with expression of LMP1 and expression of the activation marker CD23 and decrease of CD77 expression (Fig. 1).

Functional studies showed that EBV infection was associated with an increased cell doubling time of BL cells (Fig. 2A and Table 4). Analysis by flow cytometry showed that EBV was systematically associated with a decrease of cells in S phase and an increase of cells in G0/G1 phase (Fig. 2B and Table 4). Apoptosis, as assessed by flow-cytometry (SSC/FSC scatter-gram, sub-G1 peak, and Annexin V staining), was decreased in EBV-converted BL cells (Fig. 3 and Table 4). These results show that the presence of the EBV genome in BL cells is associated with stable phenotypic changes, decrease of cell proliferation rate, and inhibition of spontaneous apoptosis, raising the question of the cellular targets of the virus.

Clustering of Genes Differently Expressed in EBV-Converted BL Cells and EBV− BL Cell Lines

The labeled cDNA probes were synthesized and a set of 1176 human complementary sequences arrayed on a nylon membrane was used. Figure 4 shows one example of a typical hybridization result using this cDNA macro array technique. As described in "Materials and Methods," the statistical analysis used to identify EBV-regulated genes in this model was based on the log transformed EBV+/EBV− ratios of signal intensities after local smoothing and normalization, before clustering.

To answer the question of which genes are coregulated in EBV+ cells when compared with their EBV− counterpart, we performed a hierarchical clustering of the normalized log transformed EBV+/EBV− ratios using Ward's method. Theoretically, at least three groups of genes were expected: those that are up-regulated, those that are down-regulated, and those that are not affected by EBV. To obtain on the one hand clusters that would be large enough to allow further statistical analysis and, on the other hand, clusters that would clearly separate the three expected classes of genes assessed by cDNA array in EBV+ when compared with EBV− cell lines, we set a cut-off at eight clusters. The prediction was that eight clusters would be sufficient to describe the three expected classes of genes. In this tree of eight clusters, four branches could be identified (Fig. 5A).

We next calculated the mean of normalized log transformed EBV+/EBV− ratios for each cluster for the three BL pairs. A graphical representation of the clusters allowed identification of three groups comprising those for which the mean was above, below, and close to the zero line (Fig. 5B). Thus, as predicted, three groups of genes were identified, those that are up-regulated, down-regulated, or not affected by EBV. This graphical representation of the eight clusters allowed analysis of the clustering tree in terms of EBV-related gene regulation (Fig. 5C). The first branch contained clusters 4, 7, and 8 with 78 genes that were up-regulated in EBV-converted BL cells. The second branch contained a unique cluster (cluster 6) of 10 genes specifically down-regulated in the BL70 EBV

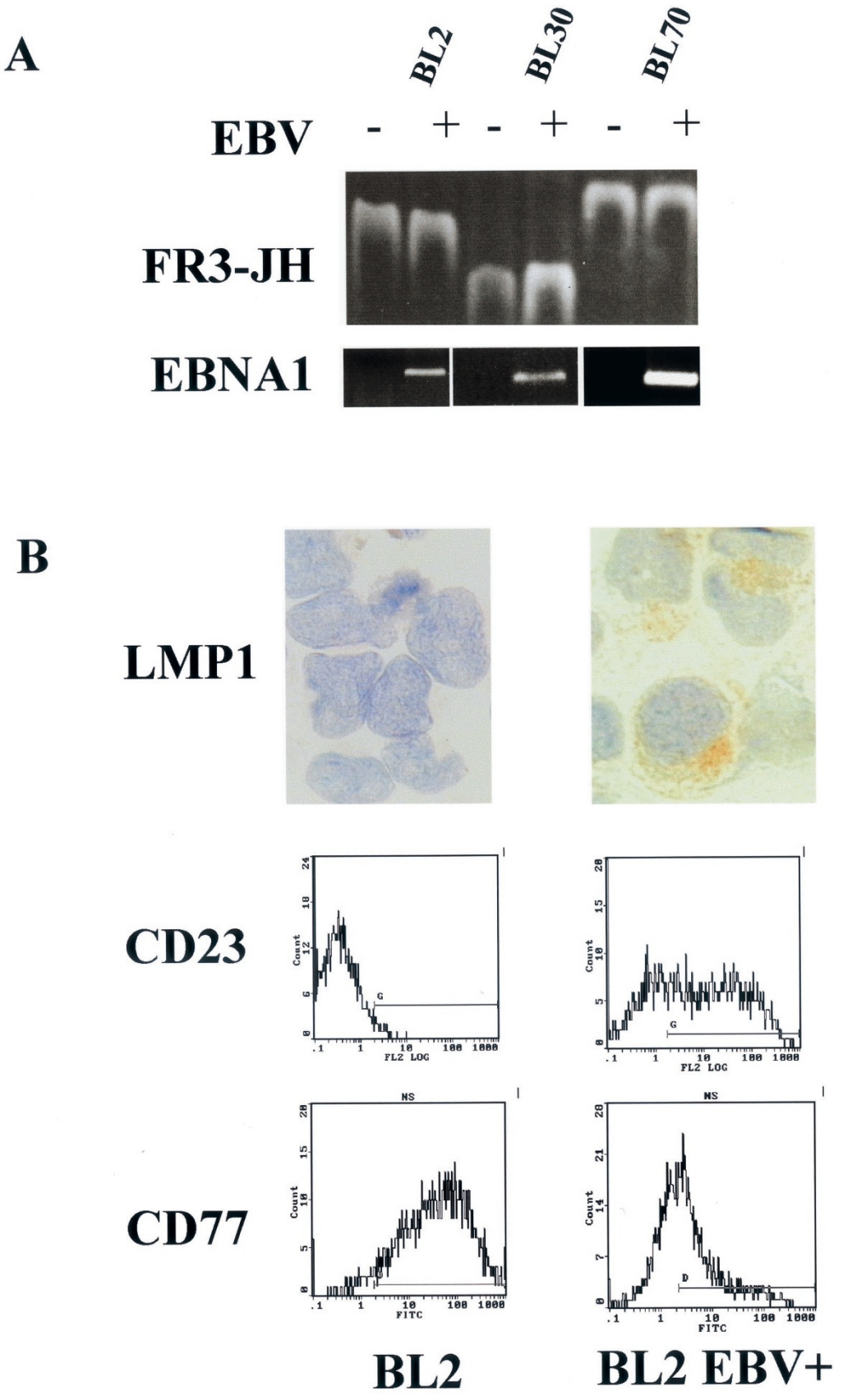

Figure 1.

Characterization of Burkitt's lymphoma (BL) cells and their Epstein Barr virus (EBV)-converted counterpart. A, Genomic amplification of the FR3-JH framework region and the EBNA1 locus of the BL pairs tested. B, LMP1, CD23, and CD77 expression in BL2 and BL2-B95.8 cells. CD23 and CD77 expression was assessed by flow cytometry on 5000 cells.

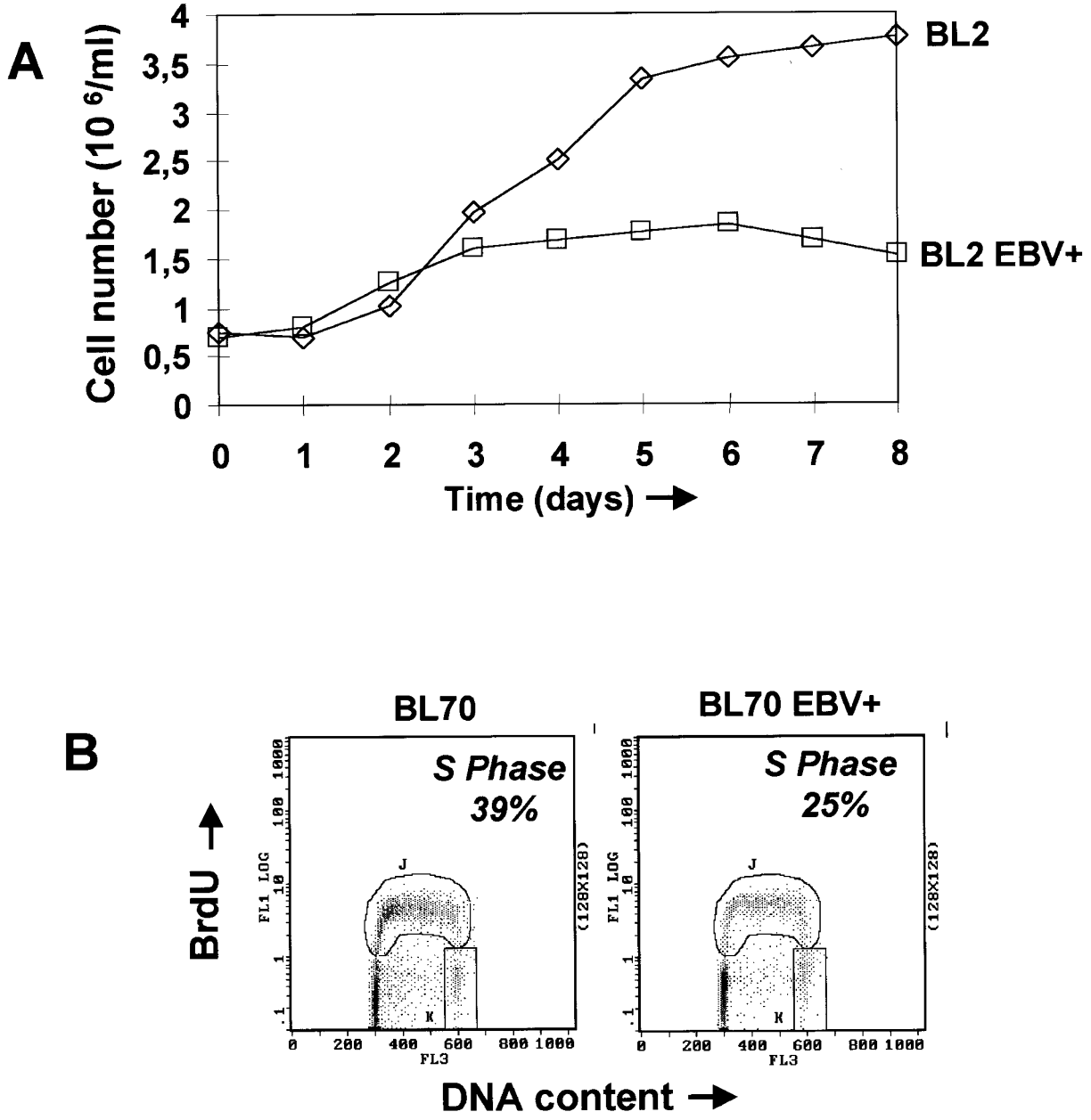

Figure 2.

Cell growth and S phase characteristics of BL cells and their EBV-converted counterparts. A, Cell growth curves of BL2 (*rhombus*) and BL2-B95.8 cells (*squares*). B, Analysis of BrdU incorporation in BL70 (left panel) and BL70-B95.8 cells (right panel). The percentage of cells having incorporated BrdU is indicated in each graph.

converted cell line. The third branch corresponded to clusters 2 and 5 containing 232 EBV down-regulated genes, and the fourth branch corresponded to clusters 1 and 3 containing the remaining 856 genes that were not regulated in EBV-converted BL cells. These results show that the eight gene clusters identified by Ward's method could be summarized in three main branches corresponding to the three groups of expected genes in the three BL pairs plus one particular branch specific to the BL70 pair.

Clustering analysis showed that 310 genes were putatively regulated by EBV in the three BL cell lines tested. We chose to restrict the analysis to genes that were putatively markedly regulated by EBV. Within the category of EBV-regulated genes, we defined as markedly EBV-regulated genes those for which the normalized log transformed EBV+/EBV− ratio was below −0.7 or above 0.7 for at least two of the three BL pairs (corresponding approximately to 2-fold increase or decrease, a commonly used threshold (Bertucci et al, 1999; Chang and Laimins, 2000) and was of the same sign for the third one. Twenty-one genes were therefore identified as markedly increased and 26 genes as markedly decreased in EBV-converted BL cells in these cells (Tables 2 and 3). Within this set of genes, SHP-1, p21, Rb, c-myc, IRF1, SOCS3, TNFR I+II, HLA DR, HLA class I alpha chain, and superoxide dismutase, were known to be regulated by EBV in

various cellular system or were known to be target genes of transcription factors activated by EBV such as NF-κB and STAT1 (see below) (Auernhammer et al, 1999; Delibrias et al, 1997; Girdlestone et al, 1993; Jochner et al, 1996; Lin et al, 2000; Marks-Konczalik et al, 1998; Wang et al, 1990; Wang and Yu-Lee, 1996; Yen et al, 1991; Zhang et al, 1994).

Cellular Functions Deregulated in EBV-Converted BL Cell Lines

Numerous genes, such as PCNA, Replication Factor C, PARP, DNA ligase I, HMG-I(Y), E2F5, E4BP4 (also called NF-IL3), IL-15 receptor, Syk, Lyn, Tec, CD30L, RP-105, and Ets proteins, were not previously reported as EBV-regulated (Tables 2 and 3). These genes were classified according to their known involvement in different cellular functions. This functional classification highlights the cell cycle, apoptosis, signal transduction pathways of BCR and TNF receptor family and interferon response as being modulated by EBV and points to three families of transcription factor, STAT1, NF-κB, and ETS-related protein (Tables 2 and 3).

EBV-Regulation of Cell Cycle in EBV-Converted BL Cells

Regarding the regulation of the cell cycle, expression of cell cycle inhibitors p21 and Rb was increased in EBV-converted BL cells and expression of cdc25B, a cell cycle inducer, was decreased. Expression of molecules involved in DNA replication, such as PCNA, Replication Factor C, PARP, and DNA ligase I, was decreased in EBV-converted BL-cells. Expression of

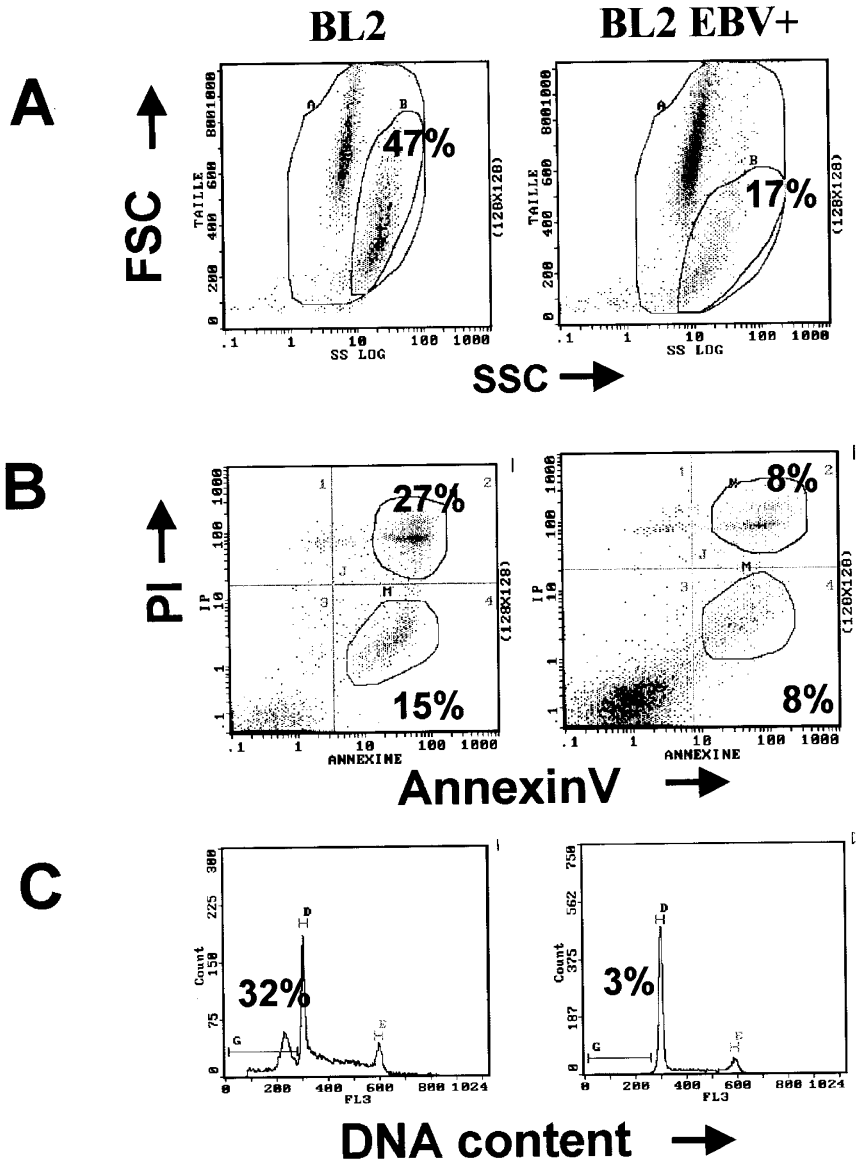

Figure 3.

Flow cytometry analysis of spontaneous apoptosis of BL2 (left panels) and BL2-B95.8 (right panels) cells. A, SSC/FCS dot plot. B, Annexin V/propidium iodide dot plot staining. C, DNA content measurement after propidium iodide staining of permeabilized cells. Percentage of apoptotic cells is indicated in each graph.

factors controlling the transcription of proliferating genes such as HMG-I(Y), E2F5, c-myc was also decreased. Increase of p21 mRNA and decrease of c-myc expression was confirmed by semiquantitative RT-PCR and Western blotting (Fig. 6, A and B). Nonphosphorylated Rb protein was also increased by western blot in EBV-converted BL cells. Both down-regulation of c-myc expression in BL cells and p21 up-regulation by EBNA2 latent protein have been previously reported (Jochner et al, 1996; Lin et al, 2000; Ruf et al, 1999). Expression of TRAP1/Hsp75 gene was also decreased, in agreement with cDNA results on c-myc target genes (Coller et al, 2000). Increase in expression of genes coding for cell cycle inhibitors and decrease of proliferating-associated genes would suggest that the proliferation rate of EBV-converted BL-cells is decreased. These results gave a framework of hypothesis explaining why, as shown in Figure 2, the proliferation rate of EBV-converted BL cells was decreased.

EBV-Regulation of Apoptosis in EBV-Converted BL Cells

We showed that apoptosis is decreased in EBV-converted cells (Fig. 3). Expression of c-myc was decreased in EBV converted-BL cells (Table 3 and Fig. 6). In addition to its role in the cell proliferation, c-myc may induce apoptosis if the cell cycle is down-regulated (Askew et al, 1991; Evan et al, 1992). Ruf et al (1999) have demonstrated that suppression of apoptosis by EBV in Akata BL cells was associated with down-regulation of c-myc expression. Expression of both tyrosine-protein kinase Tec and transcription factor E4BP4 (NF-IL3) was increased. Regulation of the latter gene was confirmed by semiquantitative RT-PCR (Fig. 6). On one hand, Tec phosphorylation is associated with activation of the Erk MAPK kinases (Tomlinson et al, 1999), and on the other hand, activation of E4BP4 (NF-IL3) is mediated by these Erk kinases, leading to protection against apoptosis (Kuribara et al, 1999). Expression of the heterochromatin homolog 1 (HP1) gene, associated with apoptosis in BL cell lines (Brockstedt et al, 1998), was decreased. This functional analysis of the cDNA results led to the conclusion that EBV protects BL cells by increasing and decreasing expression of apoptosis inhibitors and proapoptotic factors respectively.

Modulation of Genes Involved in BCR Signaling

BCR signal transduction is inhibited in EBV-infected B-cells (Caldwell et al, 1998) and LMP2A EBV latent protein interacts directly with the kinases Syk and Lyn (Caldwell et al, 2000; Miller et al, 1995). Expression of SHP-1, a phosphatase that attenuates the BCR signal transduction (Muraille et al, 2000), was up-regulated in EBV-converted BL cells. This was confirmed by RT-PCR and Western blotting (Fig. 6). Genes coding for tyrosine kinases Syk, Lck, and Bruton's tyrosine kinase (BTK) were down-regulated in EBV-converted BL cells. This suggests that, in addition to direct interactions between LMP2A and cellular kinases, EBV may regulate the BCR signaling through transcription regulation of some defined cellular kinases and phosphatases.

Regulation of ETS-Related Proteins

Expression of three genes coding for Ets-related transcription factor, Spi B, ELF-1, and ETS-1, were markedly down-regulated in EBV-converted BL cells. Expression of Ets-related gene ETV-6 (also called TEL) was increased (even when the normalized log transformed EBV+/EBV− ratio was slightly lower than 0.7). Down-regulation of three Ets genes (Spi B, ELF-1, and ETS-1) involved in activation of gene transcription suggests that regulation of Ets-dependent genes could be important for EBV survival.

Modulation of Genes Involved in NF-κB and STAT1 Activation

Four genes, interferon response factor 1 (IRF1), interferon induced 56-kd protein (IFI 56), interferon in-

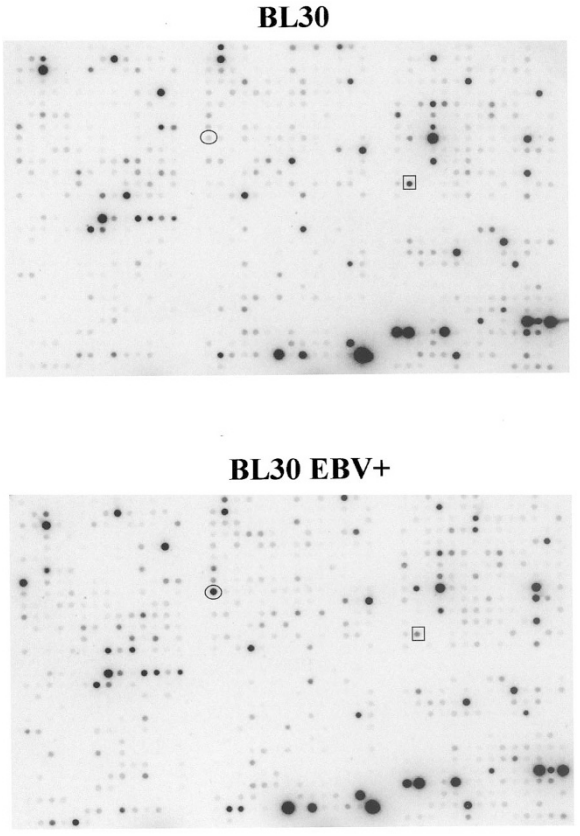

Figure 4.
BL30 EBV+ cells and BL30 EBV− counterpart cells comparative hybridization using cDNA macroarray. α^{32}P cDNA labeled probes were synthesized in parallel from 10 μg of total RNA of BL30-B95.8 cells and from their EBV− counterpart, and were hybridized simultaneously on two identical nylon membranes on which 1176 different cDNAs are spotted. Quantification of the hybridization signal was performed strictly in parallel using the same phosphor screen. The original image was a 16-bit TIF image corresponding to a 40-μm scanning step of the phosphor screen. The software automatically adjusted the contrast. Some of these genes have an apparent increased expression (in *circle* O PTP1C) in EBV-converted BL cells; others have an apparent decreased expression (in *square* SpiB) in EBV-converted BL cells. Background signal depended on the local hybridization intensity.

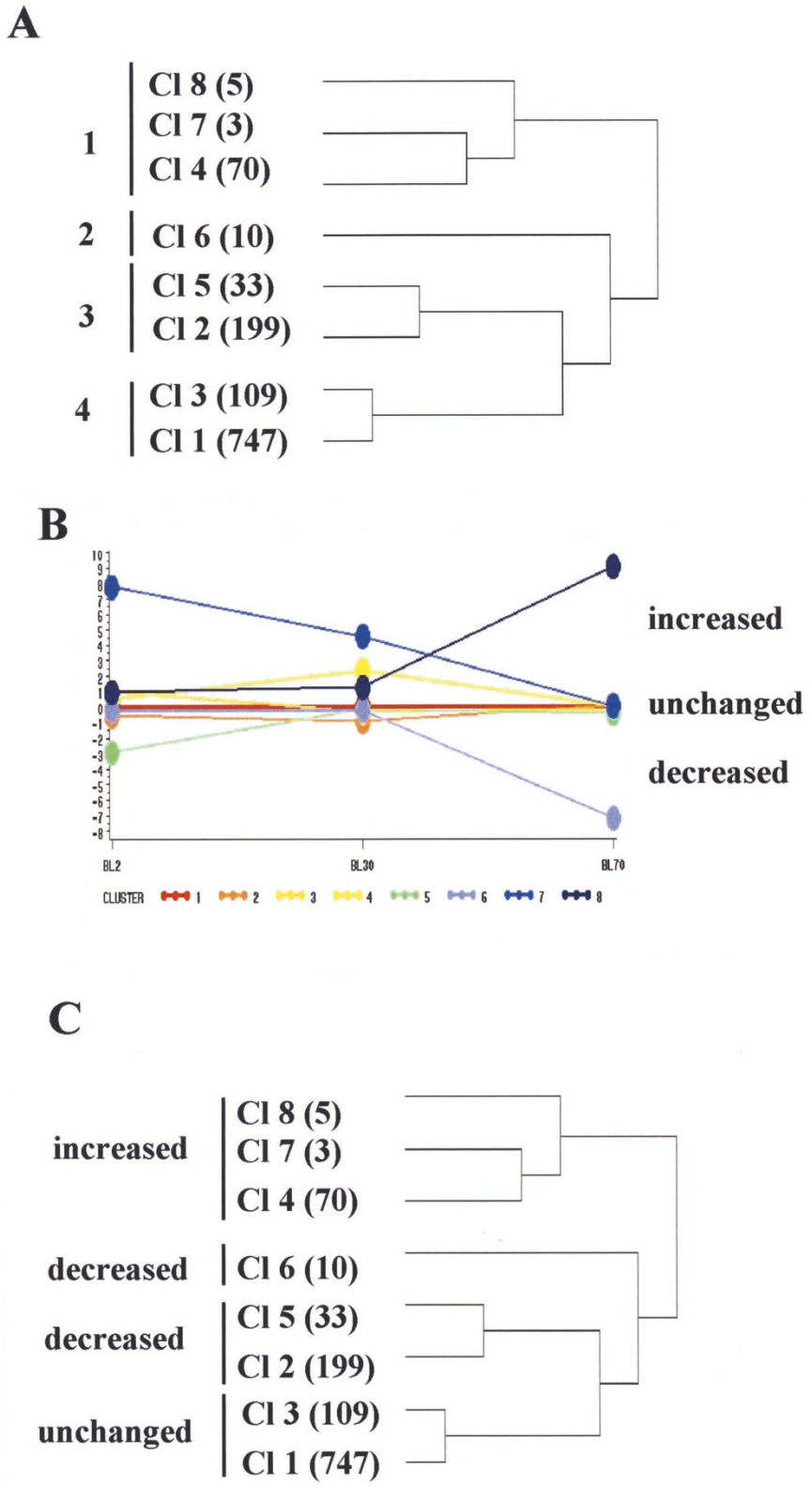

Figure 5.

Dendrogram of the eight clusters after Ward's hierarchical clustering of normalized EBV+/EBV− ratios for the three BL pairs and representation of the means of the normalized EBV+/EBV− ratios for each BL pair for interpretation. A, EBV+/EBV− intensity ratios of the three different BL pairs have been log transformed and a local smoothing was calculated before normalization. Ward's hierarchical cluster analysis method was applied to classify genes with similar EBV+/EBV− normalized intensity ratios in the three BL pairs. A cut-off at eight different clusters was set. Four branches are well individualized. The number of genes in each cluster is indicated in parentheses. B, The eight clusters are represented with a different color and a number (1 to 8). The different spots correspond to the mean of the normalized intensity ratios for each cluster of each BL pairs. C, Analysis of the dendrogram presented in A according to the mean of the normalized intensity ratios for each cluster of each BL pairs. Clusters 4, 7, and 8 contain 78 genes for which expression was increased in the three EBV+ BL cells compared with their EBV− counterpart. Clusters 2 and 5 contain 232 genes with a decreased expression in EBV-converted BL cells. Clusters 1 and 3 contain 856 genes for which expression was not affected in EBV-converted BL cells. Cluster 6 contains 10 genes with a decreased expression in EBV-converted BL70 cells only.

Table 1. Primer Sequences and Conditions Used for Semiquantitative RT-PCR and PCR

Genes	Primers (5′–3′)	Denaturing temperature (°C)	Annealing temperature (°C)	Extension temperature (°C)	Number of cycles
PKR	L: GCTGGAGTGCAATGACACAG R: GAAAATTTGGGGGTGGTTTT	94	60	72	26
STAT1	L: AGTGGTACGAACTTCAGCAGC R: TGATCATAGACATCTGGATTGG	94	50	72	26
SHP1	L: GACCCCATGGTCGGGCCAG R: CACCCGAGAGGTGGAGAAAGGC	94	58	72	26
p21	L: GGAAGACCATGTGGACCTGT R: CCCAGCACTCTTAGGAACCTC	94	60	72	26
c-myc	L: CCAGCAGCGACTCTGAGG R: GTTGTGTGTTCGCCTCTTGA	94	60	72	26
IL15Rα	L: TCCCAGCTCAAACAACACAG R: AGGAGAGACACAGCGCTCA	94	60	72	26
E4BP4	L: CCCATCCATTCTCCAGTTGA R: GTGCTCCGATTTGAGAGACC	94	60	72	26
GAPDH	L: CAGGGAAGCTCAAGGGAGAT R: GAGATGGGGACAGGACCATA	94	60	72	26
β-globin	L: CAACTTCATCCACGTTCACC R: ACACAACTGTGTTCACTAGC	94	60	72	35

All templates were initially denatured for 10 minutes at 94° C, each step was 45 seconds long and the final extension temperature was at 72° C for 10 minutes.

duced 78-kd protein (IFI-78 or MXA), and STAT-induced STAT inhibitor 3 (SOCS-3), were markedly EBV-up-regulated genes with a normalized log transformed EBV+/EBV− ratio above 0.7. These genes are known to be involved in the interferon response. Among these four genes, IRF1 and SOCS-3 are directly regulated by the transcription factor STAT1 (Paul et al, 2000; Wang and Yu-Lee, 1996). The cell cycle inhibitor p21 is also a target gene for STAT1 (Chin et al, 1996) and was increased in EBV-converted BL cells (see above). The interferon inducible RNA-dependent protein kinase (PKR) regulated by IRF-1 (Beretta et al, 1996), and STAT1 itself were clustered with EBV up-regulated genes, but with a normalized log transformed EBV+/EBV− ratio below 0.7. These results were confirmed for STAT1 and PKR by semiquantitative RT-PCR and Western blotting (Fig. 6). Gel retardation experiments showed clearly an increase of STAT1 binding activity in EBV-converted BL cells (Fig. 7).

The alpha chain of IL-15 receptor is another molecule involved in STAT1 activation through JAK3 phosphorylation (Yu et al, 1998). cDNA array results showed that this gene was markedly up-regulated in EBV-converted BL cells. Semiquantitative RT-PCR results were in agreement with these results. The alpha chain of the IL-15 receptor may also be involved in both NF-κB activation and protection against apoptosis through TRAF2 binding (Bulfone-Pau et al, 1999; McDonald et al, 1998).

Expression of NFKB1 gene coding for both p105 NF-κB precursor/inhibitor and p50 NF-κB subunit was decreased. Three genes, TNF-R I+II, CD30L, and RP-105, coding for membrane receptor involved in TRAF molecule binding, NF-κB activation, and protection against apoptosis, were markedly up-regulated in EBV-converted BL-cells. The gene coding for superoxide dismutase (SOD) and those involved in MHC class II expression such as MHC class II alpha chain and MHC class II invariant chain are known to be regulated by NF-κB (Marks-Konczalik et al, 1998) and were markedly up-regulated in EBV-converted BL cells. These results show an increase in expression of genes coding for both NF-κB activators and NF-κB target genes. Gel retardation experiments showed clearly an increase of NF-κB binding activity in EBV-converted BL 2 cells (Fig. 7).

Discussion

We used the cDNA array technology to identify genes putatively regulated by EBV in BL cells. The cellular model consisted of three EBV-negative BL cell lines (BL2, BL30, BL70) and their genoidentical EBV-converted counterpart (BL2-B95.8, BL30-B95.8, BL70-B95.8) expressing the whole range of latent viral proteins. Analysis of the results allowed us to establish an EBV-related expression profile and characterize cell functions modified by the virus.

One critical step in the analysis of cDNA results is the statistical analysis. To identify genes regulated by EBV, we chose to analyze the EBV+/EBV− signal intensity ratios for each gene. As described in other reports (Eisen et al, 1998, for example), we used the log transformed value of EBV+/EBV− signal ratios. Analysis of cDNA results may include a calculation step consisting of equalizing the signal intensities of housekeeping genes (Eickhoff et al, 1999). However, this calculation method may sometimes induce artifact results as discussed by Bhatia et al (1994) and Savonet et al (1997) and does not take into account the local background due to signal diffusion when radiolabeled probes are used. To attenuate the local background and to increase the signal contrast we

Table 2. Genbank Accession Number, Mean of EBV+/EBV− Ratio, and Name of the Genes Markedly Increased by EBV, Classified According to their Known Cellular Functions

Increased expression (21 genes)		Mean of EBV+/EBV− ratio for the 3 BL pairs
Cell proliferation		
Cell cycle		
M15400	Retinoblastoma-associated protein (RB1); PP110; P105-RB	2.98
D84212	Aurora-related kinase 1 (ARK1)	3.67
U09579	Cyclin-dependent kinase inhibitor 1 (CDKN1A); WAF1, p21	7.49
Translation		
M27364	Elongation factor 1 α (EF1 α)	3.68
Protein processing		
Y08614	CRM1 protein	2.68
Inhibition of apoptosis		
X64318	E4BP4	1.89
U31628	Interleukin-15 receptor α subunit precursor	$1.13*10^1$
Interferon response		
X14454	Interferon regulatory factor 1 (IRF1)	3.54
X03557	Interferon-induced 56-kd protein (IFI-56K)	$1.2*10^1$
M33882	Interferon-regulated resistance GTP-binding protein MXA	6.32
AB004904	STAT-induced STAT inhibitor 3	5.89
Signal transduction		
Receptors		
U07707	Epidermal growth factor receptor substrate 15 (EPS15)	2.98
M32315	Tumor necrosis factor receptor (TNFR) + (TNFR2)	3.81
L09753	CD30 ligand (CD30L); CD153 antigen	3
M27543	Guanine nucleotide-binding protein G(K) α 3 subunit (GNA3)	4.79
Kinases		
D29767	Tyrosine-protein kinase tec	2.85
Tyrosine phosphatase		
X62055	Protein-tyrosine phosphatase 1C (PTP1C) SHP1	$1.45*10^2$
Cell adhesion		
D83597	RP105	$1.46*10^1$
Immune system		
K01171	HLA class II histocompatibility antigen α chain	$1.33*10^1$
X00497	HLA-DR antigen-associated invariant subunit	$6.36*10^1$
U58514	Chitinase precursor	$2.84*10^2$

Genes listed are defined as markedly increased in EBV-converted BL cells (ie, for which the log transformed EBV+/EBV− ratio was higher than 0.7 for at least 2 of the 3 BL pairs and was higher than 0 for the third one).

calculated the local intensity of the signal of one gene relative to its neighbors and the different experiments were normalized before using Ward's cluster analysis method (Everitt, 1974; Ward, 1963). It is noteworthy that Ward's cluster method allowed us to identify a group of 10 genes for which expression was decreased only in EBV-converted BL70 cells, suggesting a cell line effect for these genes. The three other groups of genes corresponded to the three classes of expected genes. Eight hundred fifty-six genes were recognized as being expressed at similar levels in both EBV+ and EBV− BL cells, 232 were down-regulated, and 78 were up-regulated in the three EBV-converted BL cell lines. Thus, 26% of the 1176 genes tested may be regulated by EBV, which indicates that this virus deeply modifies the genetic program of its infected host. To identify cellular function markedly regulated by EBV, we selected, among the 310 putatively EBV-regulated genes recognized by cluster analysis, those for which the absolute value of the normalized log transformed EBV+/EBV− signal ratio was higher than 0.7, corresponding to at least 2-fold increase or decrease. The use of this criterion allowed us to identify 21 genes with a marked increased expression and 26 genes with a marked decreased expression in EBV+ BL cells.

From a technical point of view, it is interesting to note that various genes, such as p21, Rb, c-myc, IRF1, IFI 56, IFI78, SOCS3, TNFR I+II, HLA DR, HLA class I alpha chain, and superoxide dismutase, are known to be regulated by EBV or to be the targets of transcription factors activated by EBV. This, in our view, is part of the validation of the technique. On the other hand, some genes known to be up-regulated by EBV in both LCLs and BL cell lines such as c-fgr of bcl-2 were not detected as regulated by EBV at the mRNA level after cluster analysis of cDNA array results. In the case of c-fgr, the hybridization signal was rather intense in both EBV-negative and EBV-converted BL cells, without obvious difference by

Table 3. Genbank Accession Number, Mean of EBV+/EBV− Ratio and Name of the Genes Markedly Decreased in EBV-Converted BL Cells, Classified According to their Known Cellular Functions

Decreased expression (26 genes)		Mean of EBV+/EBV− ratio for the 3 BL pairs
Cell proliferation		
Cell cycle		
M81750	CDC25B; CDC25HU2; M-phase inducer phosphatase 2	$2*10^{-1}$
Oncogenes		
U01147	Active breakpoint cluster region-related protein	$3.5*10^{-1}$
Z29083	5T4 oncofetal antigen precursor	$3.86*10^{-1}$
Replication		
X06617	Histone H4	$2.75*10^{-1}$
M36067	DNA ligase I	$2.61*10^{-1}$
M23619	High mobility group protein (HMG-I)	$5.89*10^{-2}$
M15796	Proliferating cyclic nuclear antigen (PCNA);	$5.21*10^{-2}$
M87339	Activator 1 37-kd subunit; replication factor C 37-kd subunit (RFC)	$1.84*10^{-1}$
U79718	Endonuclease III homolog 1 (HNTH1) (OCTS3)	$7.16*10^{-2}$
M18112	Poly(ADP-ribose) polymerase (PARP; PPOL)	$7.86*10^{-2}$
Transcription		
J04101	Erythroblastosis virus oncogene homolog 1 (ETS-1);p54	$2.56*10^{-1}$
M82882	Ets-related transcription factor E74-like factor 1 (ELF1)	$2.55*10^{-1}$
X66079	Transcription factor Spi-B	$2.11*10^{-1}$
U15642	Transcription factor E2F5	$4.97*10^{-1}$
M58603	Nuclear factor κB DNA binding subunit (NF-κB; NFKB)	$1.87*10^{-1}$
V00568	c-myc oncogene	$8*10^{-2}$
Signal transduction		
Receptors		
U12595	Tumor necrosis factor type 1 receptor associated protein (TRAP1)	$1.39*10^{-1}$
GDP/GTP		
X78817	ρ-GAP hematopoietic protein C1 (RGC1)	$6.16*10^{-2}$
Kinases		
L29216	CDC-like kinase 2 (CLK2)	$4.5*10^{-1}$
L05148	70-kd ζ associated protein (ZAP70)	$2.2*10^{-1}$
U10087	Bruton's tyrosine kinase BTK	$2.73*10^{-1}$
V07236	Proto-oncogene tyrosine-protein kinase 1ck; p56-1ck	$3.4*10^{-1}$
Tyrosine phosphatase		
Y13936	Protein phosphatase 2C γ	$4.13*10^{-1}$
Stress response		
K00065	Cytosolic superoxide dismutase 1 (SOD1)	$1.81*10^{-1}$
M19645	78-kd glucose regulated protein precursor (GRP 78)	$4.15*10^{-1}$
Apoptosis		
L07515	Heterochromatin protein homolog 1 (HP1)	$2.75*10^{-1}$

Genes listed are defined as markedly decreased in EBV-converted BL cells (ie, for which the log transformed EBV+/EBV− ratio was below −0.7 for at least 2 of the 3 BL pairs and was below 0 for the third one).

Table 4. Cell Cycle and Apoptosis in BL Cell Lines

	BL2	BL2 B95	BL30	BL30 B95	BL70	BL70 B95
Cell cycle						
Doubling time (hours)	36	90	48	73	42	75
G0/G1 phase[a]	38.4	82.3	37.8	51.5	27.7	62.4
S phase[a]	51.3	10	52.6	24.4	38.7	25.7
Apoptosis						
FS/SS scatter gram[b]	36	10	29	16	48	19
Annexin V[b]	30	13	28	9	36	10
Sub-G1 peak[b]	21.5	4.4	20.4	5.9	30.4	3.6

[a] Percentage of cells.
[b] Percentage of apoptotic cells.

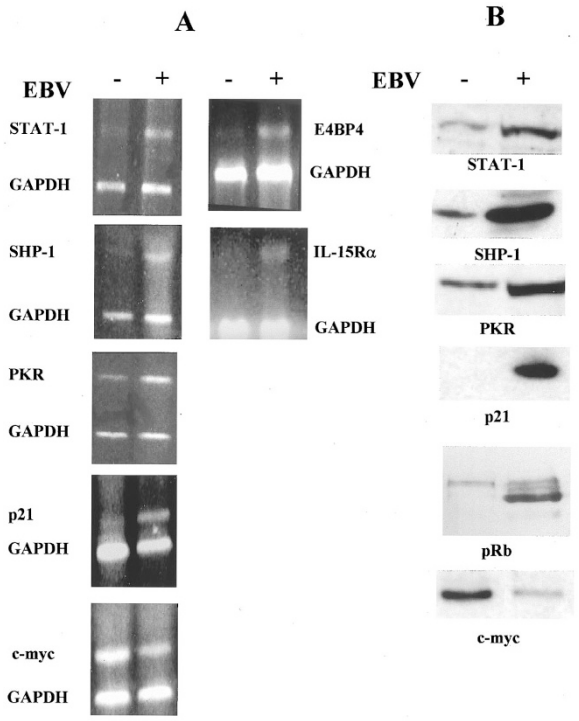

Figure 6.

Semiquantitative RT-PCR and Western blotting in BL2 cells. A, Messenger RNA levels of PKR, STAT1, SHP1, p21, IL15Rα, and E4BP4 and c-myc were compared with the mRNA levels of GAPDH after a 26-cycle amplification. B, Differential expression of PKR, STAT1, SHP1, p21, Rb, and c-myc proteins were also verified by Western blotting from total protein extracts of EBV− and EBV-converted BL cells. Note that levels of the hypophosphorylated Rb protein were increased in EBV positive cells (lower band) when compared with EBV positive cells (upper band)

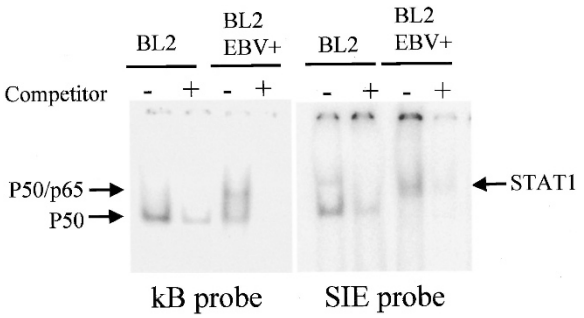

Figure 7.

NF-κB and STAT1 binding activity in BL2 cells and their EBV-converted counterpart. Twenty micrograms of nuclear proteins were incubated with the radiolabeled kB (A) or SIE (B) probe. Competition experiments were performed in presence (+) or absence (−) of 50-fold excess of the unlabeled competitor. Identification of the complexes binding to the probes was made by supershift experiments (not shown).

visual analysis. This may reflect the fact that the signal produced by the phospho-screen imager was saturated. Conversely, bcl-2 mRNA was not or hardly detected after hybridization of cDNA array nylon membrane. Bcl-2 protein expression is known to be increased by EBV latent protein LMP1 protein in B-cells (Rowe et al, 1994). The lack of detection of EBV-related Bcl-2 gene up-regulation by cDNA array may therefore reflect the lack of sensitivity of the cDNA array method in this case.

Another aspect of the validation of the cDNA array technique concerns the cellular function regulated by EBV in BL cells. The entire set of cDNA results suggests that EBV both down-regulates cell proliferation of BL cells and protects them against apoptosis. These results fit fairly well with our in vitro functional analysis of cell cycle and apoptosis. Indeed, we showed an increase of cell doubling time with a decrease of BrdU incorporation and a marked decrease of spontaneous apoptosis in EBV-converted BL cells. This clearly indicates that cDNA array may lead to the identification of functional cellular modules regulated by EBV. Moreover, as discussed by Zhu and Zhang (2000), cDNA array point to the genes participating in a common cellular function.

EBV-protection of BL cells against apoptosis is well known (Gregory et al, 1991). Regarding apoptosis, cDNA array results point to the antiapoptotic E4BP4 (NF-IL3) transcription factor and the proapoptotic heterochromatin homolog 1, the expression of which were respectively increased and decreased. The tyrosine kinase Tec was over-expressed in EBV-converted BL cells. Tec belongs to the BCR transduction pathway downstream of lyn activation and could compensate the absence of the Bruton's tyrosine kinase (Kitanaka et al, 1998). Tec activation may lead to MAPK activation (Tomlinson et al, 1999) and MAPK phosphorylation induces activation of the E4BP4 (NF-IL3) transcription factor (Kuribara et al, 1999). E4BP4 (NF-IL3) may also be activated after IL-4 treatment and EBV has been reported to induce IL-4 expression (Ohnishi et al, 1997; Tang et al, 1993).

The fact that the proliferation rate of EBV-converted BL cells was decreased may be surprising. Indeed, EBV induces proliferation and immortalization of quiescent B-cells in vitro (Thorley-Lawson et al, 1999), EBNA2 protein has a proliferative effect on EBV-infected B-cells (Kempkes et al, 1995). EBNA2 induces LMP1 expression, which plays a major role in B-cell transformation. This may point to the importance of the cellular model. Indeed, our data were obtained from a Burkitt lymphoma cell background, which is different from the one from nontransformed quiescent B-cells. Indeed, EBNA2 protein may decrease the proliferation rate of BL cells for example (Jochner et al, 1996; Strobl et al, 2000). In fact, decrease of the proliferation rate of EBV-infected BL cells has been evoked by Magrath et al (1980; Table 4) and was reported in LMP1-expressing EBV-converted BL cells by Torsteinsdottir et al (1989) and Cuomo et al (1992).

Regarding the cell cycle, our functional data and cDNA results fit fairly well. These results are consistent with over-expression of p21 protein and hypophosphorylation of Rb protein in EBV-converted BL cells, as well as decrease of c-myc protein levels in EBV-converted BL. Decrease of the proliferation rate of EBV-converted BL cells may involve a network of both proliferating genes and genes coding for cell cycle inhibitors. Indeed, expression of cell cycle inhibitors Rb and p21 were increased and expression of the

cellular activators as well as genes involved in DNA replication, such as cdc25B, c-myc, HMG-I(Y), E2F-5, PCNA, PARP, DNA ligase I, replication factor C, and TRAP1/Hsp75 (Rb-associated protein), were decreased. Regarding the putative pathways connecting these genes, it is known that the cell cycle inhibitor p21 inhibits phosphorylation of CDK-cyclin complexes (Lam and La, 1994). Nonphosphorylated Rb blocks E2F transcription factors, which control transcription of genes involved in DNA replication genes such as PCNA (Niculescu et al, 1998). Lin et al (2000) showed that transfection of EBNA2 increases p21 expression and retard cell growth. Moreover, EBNA2 down-regulates c-myc expression at the transcriptional level, probably through the control of regulatory elements of the translocated immunoglobulin gene (Jochner et al, 1996; Strobl et al, 2000). Both TRAP1 and HMG-I(Y) have been reported as c-myc target genes (Coller et al, 2000; Wood et al, 2000) and were found to be down-regulated in our experiments.

The cDNA array technique may allow identification of transcription factors controlling a defined set of genes. This can be achieved (i) directly by measuring the mRNA level of the transcription factor itself or (ii) indirectly by measuring mRNA levels of genes coding for upstream activators as well as for downstream target genes. Identification of ETS-related proteins corresponds to the former method. Regarding the latter method, our results point to the role of both STAT1 and NF-κB in the biology of EBV.

A down-regulation of Spi-B, ELF-1, and ETS-1 genes was found in EBV-converted BL cells. The immunoglobulin heavy chain is down-regulated in EBV-infected B-cells and in EBV-converted BL cells (data not shown). It is noteworthy that Ets-like lymphoid-specific elements have been identified in the IgH 3' enhancer region of immunoglobulin heavy chain (Grant et al, 1992). AP1/Elf-1 complexes may be bound to these motives. Together with our results, these observations raise the question of the role of Ets protein in the regulation of immunoglobulin expression in EBV-converted BL cells.

Six interferon response genes were up-regulated in EBV-converted BL cells: IFI 56, MXA, IRF-1, SOCS-3, p21, and PKR. Four of them, IRF-1, SOCS-3, p21, and PKR, are putatively regulated by STAT1 (Beretta et al, 1996; Chin et al, 1996; Paul et al, 2000; Wang and Yu-Lee, 1996). These results point to STAT1 as a transcription factor activated by EBV. Our gel retardation assay experiments confirmed this prediction by showing a STAT1 DNA binding activity in EBV-converted BL cells, whereas such activity was much weaker in their EBV-negative counterparts. Activation of STAT1 by LMP1 transfection through JAK3 phosphorylation has been previously reported (Gires et al, 1999).

Among the cellular genes related to STAT1 activation, we found that expression of the alpha chain of IL-15 receptor mRNA was also increased in EBV-converted BL cells. IL-15 response leads not only to natural killer lymphocyte activation but is also able to promote B-cell proliferation and differentiation (Armitage et al, 1995). IL-15 is also involved in an autocrine loop increasing cell proliferation in B cell chronic leukemia and in myeloma (Soderberg et al, 1997; Tinhofer et al, 2000). Like LMP1, activation of IL-15 receptor leads to activation of both STAT1 and NF-κB.

EBV-converted BL cell lines showed a marked increase in the expression of other cellular receptors, such as TNFRI+II, RP105, and CD30L, which are able to activate NF-κB in the presence of their respective ligands. Induction of TNF receptors expression by EBV has been previously reported (Durkop et al, 1999). EBV infection of B-cells induces TNFα secretion (Williamson et al, 1983) and TNFα gene is a NF-κB target gene (Collart et al, 1990; Shakhov et al, 1990). Loss of NF-κB in LCLs may be associated with a decrease of TNF synthesis and a synergy between LMP1 and TNFα has been suggested regarding both NF-κB activation and B-cell proliferation (Asso Bonnet et al, 1998).

An apparently paradoxical result was the decrease of NFKB1 mRNA levels in EBV-converted BL cells. NFKB1 gene codes for both the p105 precursor and p50 and is a NF-κB target gene (Cogswell et al, 1993; Rice et al, 1992). Furthermore, we found by gel retardation assay that NF-κB activation was constitutive in EBV-converted BL cells. In fact, transcription levels of genes coding for NFKB1 proteins does not allow us to make prediction on the transcription activity of Rel/NF-κB proteins. The carboxy-terminus end of p105 harbors sequence homologies with I-κB molecules, and indeed p105 inhibits the nuclear translocation of Rel/NF-κB complexes (Hatada et al, 1993; May and Ghosh, 1997; Naumann et al, 1993). Moreover, homodimers of p50 may inhibit transcription through κB sites in vivo (Israel et al, 1989; Kang et al, 1992). Nothing is known regarding possible compensations by the other Rel/NF-κB proteins in the BL model. Decrease of NFKB1 gene expression encoding the p105 protein in EBV-converted BL despite NF-κB activation, raises the question of NFKB1 gene regulation because it is an NF-κB target gene. Loss of NF-κB activity in EBV-infected B-cells has been reported to be associated with decrease of p105 protein levels (Feuillard et al, 2000). This suggests additional mechanisms of NFKB1 gene regulation in EBV-converted BL cells. It is noteworthy that proteins of the ETS family (including Ets-1 and Elf-1) may regulate NFKB1 promoter (Lambert et al, 1997) and that, in our experiment, both Ets-1 and Elf-1 transcription levels are decreased in EBV-converted BL cells.

In conclusion, a number of genes were identified as putatively EBV-regulated in BL cells. Known mechanisms combined with results of our experimental cDNA array data allows us to put forward a mechanistic framework concerning the pathways deregulated by EBV. Definition of such a framework will certainly be helpful in designing a new therapeutic strategy targeting the key transcription factors.

Materials and Methods

Cell Culture

The BL2, BL30, and BL70 cell lines and their EBV-converted counterpart (BL2-B95.8, BL30-B95.8, and BL70-B95.8; Calender et al, 1987) were established and infected by EBV in vitro at the International Agency for Research on Cancer (IARC, Lyon, France). Cells were grown 1 to 2 $\times$ 10^6 cells/ ml in RPMI 1640 (Eurobio, Les Ulis, France) supplemented with 10% decomplemented FCS (Dutcher, Brumath, France), 100 U/ml penicillin, 10 μg/ml streptomycin, 2 mM L-glutamine. The cells were grown at 37° C in a humidified 5% CO_2 atmosphere.

RNA Extraction and Preparation

For each cell line, 50 million cells were used for RNA extraction with the Trizol reagent (Gibco BRL Life Technology, Cergy Pontoise, France) at 1 ml for 10 million cells according to the recommendations of the manufacturer. Total RNA was then treated with DNAse I as recommended by the supplier of cDNA array membranes (Clontech Laboratories, Palo Alto, California). Absence of residual DNA was assessed by verifying the absence of PCR amplification of the beta-globin gene (see Table 1 for sequences of the primers).

cDNA Array

We used a set of 1176 human complementary sequences arrayed on a nylon membrane (Human Cancer Array 1.2, Clontech Laboratories). The complete list of complementary sequences and controls is available on the Web site of the supplier (http://www.clontech.com). Radiolabeled cDNA probes were synthesized by reverse transcription from 10 μg of total RNA using manufacturer's protocol with α^{32}P-dATP (Amersham, Pharmacia Biotech Europe, Saclay, France). After overnight hybridization, stringent washings were performed as recommended. Hybridized membranes were exposed to a phosphor screen and quantification of the radioactive signal was performed using a phosphor system imager (Cyclone Packard Instrument, Meriden, Connecticut). Numerical values were stored using Excel software. Labeling of cDNA probes, hybridization of cDNA nylon arrays, and quantification of the signal were performed in parallel for both EBV-converted BL cells and their EBV-negative counterpart.

Data Analysis

Analysis of gene expression differences between BL EBV− cells and their EBV+ counterpart (BL pair) was based on EBV+/EBV− ratios of signal intensities. All numerical values of signal intensities were log transformed and a local smoothing was calculated using the local mean, defined as the mean of the log transformed signal intensity of the nine neighbors for each gene. The difference between the log transformed signal intensity of each gene and the local mean was called the relative local log transformed signal intensity. The local log transformed EBV+/EBV− ratio was obtained for each gene by using the difference of the relative local log transformed signal intensities between EBV+ and EBV− cells for each gene. Distribution of relative local log transformed EBV+/EBV− ratios was gaussian with comparable levels of global signal intensities between both EBV+ and EBV− conditions, allowing normalization between each BL pair. To achieve this procedure, we followed these successive calculation steps:

If we define *Xg* as the log (signal intensity for a gene *g* in EBV− cells), *Yg* as the log (signal intensity of the same gene *g* in EBV+ cells), *XG* as the sum of log transformed intensities of nine neighbors of gene *g* in EBV−, and *YG* as the sum of log transformed intensities of nine neighbors of gene *g* in EBV+, then the relative local log transformed signal intensity for gene *g* in EBV− cells will be $XLg = Xg - XG/9$, the relative local log transformed signal intensity for gene *g* in EBV+ cells will be $YLg = Yg - YG/9$, and the relative local log transformed EBV+/EBV− ratio will be $Vg = Ylg - XLg$.

Finally, normalized *Vg* for a gene *g* was defined as $VNg = (Vg - MV)/EV$, where *MV* is the mean of *Vg* for the 1176 genes and *EV* is the corresponding standard deviation.

This allowed representing each gene by a vector defined with the VNg values of each experiment (corresponding to the three BL pairs analyzed) as coordinates. These vectors were used to establish a hierarchical clustering of genes using Ward's cluster analysis method, which is based on Euclidean distance, using SAS software (SAS Corp., Gregy-sur-Yerres, France). A cut-off at 8 clusters was set up to define groups for which levels of gene expression vary similarly in the same way in the EBV+ cells of the three BL couples. Technical validity of the method was checked by visual analysis of the membranes for each identified gene.

PCR and RT-PCR

DNA was extracted from 10 to 20 million cells using the phenol-chloroform method after K proteinase digestion. The third framework region was amplified using the consensus FR3 (5'ACA CGG CCT TGT ATT ACT GT 3') and JH primers (5' ACC TGA GGA GAC GGT GAC C 3') (Aubin et al, 1995) to assess the clonal relationships between EBV+ BL cell lines and their EBV− counterparts. Forty cycles (94° C 20 seconds, 57° C 30 seconds, 72° C 30 seconds) were performed. The PCR buffer contained $MgCl_2$ 1.5 mM, KCl 50 mM, and Tris-HCl 10 mM at pH 8.3. PCR products were size fractionated on 10% polyacrylamide gel. Amplification of the EBNA1 locus was performed using the two following primers (5'CCT GTA GGG GAA GCC GAT3' and 5' CAA TGG TGT AAG ACG ACA TT3') (Camilleri et al, 1997). PCR products were visualized after ethidium bromide staining.

Total RNA was extracted according to the procedure defined for cDNA array (see above). Reverse

transcription was performed with 3 μg of heat-denatured total RNA. Primers used for PCR were designed to hybridize at 60° C. The primers used are listed in Table 1. The PCR buffer contained $MgCl_2$ 2.5 mM, KCl 50 mM, and Tris-HCl 10 mM at pH 8.3. Each PCR was performed with 1/6 of the reverse transcription mixture containing the cDNAs. GAPDH-specific primers were systematically added to coamplified GAPDH cDNA, to normalize the PCR signal. The number of cycles ranged from 22 to 30 (usually 25) to optimize the signal ratio between the genes tested and GAPDH. PCR products were separated on 1% agarose gel in the presence of ethidium bromide.

Western-Blotting

Total protein extracts were from 5 million cells resuspended in 100 μl of the lysis buffer containing 0.01% bromophenol blue, 50 mM Tris HCl pH 6.8, 2% SDS, 20% glycerol, and 5% β-mercapto-ethanol. Lysed cells were frozen at −80° C and sonicated. Twenty microliters of boiled extracts were resolved on a 10% denaturating polyacrylamide gel at 300V for 3 hours and electrophoretically transferred onto a nylon membrane (Immobilon, Millipore, Bedford, Massachusetts). After 2 hours of dry milk blockade, first antibody was added in TBS (150 mM NaCl, 50 mM Tris-HCl pH 7.4), 0.1% Tween 20, 1% dry milk overnight. Antibodies were used against p21 (rabbit polyclonal Sc-397, Santa Cruz, San Diego, California) at 1/500, SHP1 (rabbit polyclonal from Cell Signaling, Beverly, Massachusetts) at 1/500, myc-Tag (clone 9E10 from Upstate Biotechnology) at 1/500, STAT1α (rabbit polyclonal from Cell Signaling) at 1/1000, PKR (MAB 7110 a generous gift of A. Hovanessian, Institut Pasteur, Paris, France) at 1/300. Rb Western blots were performed using a kit according instructions of the manufacturer (Cell Signaling). After washing in TBS 0.1% Tween 20, the second antibody was added goat anti-mouse at 1/5000 or goat anti-rabbit at 1/5000 for 1 hour. Revelation was performed after 2 hours washing in TBS 0.1% Tween 20, 1% dry milk by chemiluminescence reaction (Lumiglo; Amersham, Orsay, France) and autoradiography.

Protein Extracts and Electromobility Shift Assay

Nuclear and cytosolic proteins were extracted in parallel from cells as previously described (Feuillard et al, 1991). The κB oligonucleotide sequence was 5' AGTTGAGGGGACTTTCCCAGG 3'. The sequence of the SIE probe was 5' CATTTCCCGTAATC 3'. Gel shift experiments were performed with 20 μg of nuclear proteins as previously described (Feuillard et al, 1991). Competition experiments were performed with 50-fold excess of the corresponding nonradioactive double-strand oligonucleotide.

Immunolabeling

Immunolabeling was performed on acetone-fixed cells after cytocentrifugation (50,000 cells/slide) and on cell pellet sections. Anti-CD23 antibody (clone MHM6, from Dako, Glostrup, Denmark) was used diluted at 1/20. Anti-CD77 antibody (clone 38–13, a kind gift of Dr. J. Wiels, Institut Gustave Roussy, Villejuif, France) was used diluted at 1/20. Immunolabeling for flow-cytometry was performed as previously described (Feuillard et al, 1995) using an EPICS XL 4C Coulter apparatus. For immunocytochemistry, anti-LMP1 antibody (clone CS1–4 from DAKO, Glostrup, Denmark) was used diluted at 1/20. A standard avidin-biotin-peroxidase technique was performed according to the manufacturer's procedure (Vectastain kit, Burlingame, California).

Proliferation, Cell Cycle, and Apoptosis

To assess the cell proliferation rate, 20 million cells were suspended in 30 ml of standard medium (660,000 cells/ml). Cell concentration was determined from an aliquot of 300 μl of the cell suspension. Doubling time was measured during the exponential growth phase. Cell cycle analysis and detection of apoptosis was performed by flow-cytometry as described (Asso Bonnet et al, 1998) on the basis of BrdU incorporation into DNA and staining with propidium iodide. Fluorescence analysis of chromatin condensation and fragmentation was performed after cytocentrifugation of 50,000 cells and DNA staining with the Hoechst 33258 diluted at 20 μg/ml in PBS. Staining of cells with FITC-conjugated Annexin V (BD Biosciences, Erembodegen, Belgium) was performed according to the manufacturer's protocol.

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