EBNA1 regulates cellular gene expression by binding cellular promoters

Allon Canaan[a,b,1], Izhak Haviv[c], Alexander E. Urban[a,d], Vincent P. Schulz[e], Steve Hartman[d], Zhengdong Zhang[f], Dean Palejev[a], Albert B. Deisseroth[g], Jill Lacy[b], Michael Snyder[d], Mark Gerstein[f], and Sherman M. Weissman[a,b,1]

Departments of [a]Genetics, [b]Yale Cancer Center, [d]Molecular Cellular and Developmental Biology, [f]Molecular Biophysics and Biochemistry, and [e]Pediatrics, Yale University School of Medicine, New Haven, CT 06520; [c]Peter MacCallum Cancer Institute, East Melbourne, Victoria 3002 Australia; and [g]Sidney Kimmel Cancer Center, 10835 Altman Row, San Diego, CA 92121

Contributed by Sherman M. Weissman, October 28, 2009 (sent for review July 21, 2009)

Epstein–Barr virus (EBV) is associated with several types of lymphomas and epithelial tumors including Burkitt's lymphoma (BL), HIV-associated lymphoma, posttransplant lymphoproliferative disorder, and nasopharyngeal carcinoma. EBV nuclear antigen 1 (EBNA1) is expressed in all EBV associated tumors and is required for latency and transformation. EBNA1 initiates latent viral replication in B cells, maintains the viral genome copy number, and regulates transcription of other EBV-encoded latent genes. These activities are mediated through the ability of EBNA1 to bind viral-DNA. To further elucidate the role of EBNA1 in the host cell, we have examined the effect of EBNA1 on cellular gene expression by microarray analysis using the B cell BJAB and the epithelial 293 cell lines transfected with EBNA1. Analysis of the data revealed distinct profiles of cellular gene changes in BJAB and 293 cell lines. Subsequently, chromatin immune-precipitation revealed a direct binding of EBNA1 to cellular promoters. We have correlated EBNA1 bound promoters with changes in gene expression. Sequence analysis of the 100 promoters most enriched revealed a DNA motif that differs from the EBNA1 binding site in the EBV genome.

Epstein Barr virus (EBV) is known to latently infect human hosts and to immortalize host cells. EBV is associated with several lymphoid and epithelial cancers including Burkitt's lymphoma (BL) and nasopharyngeal carcinoma (NPC) (1). Primary EBV infection is believed to take place in the oral cavity, where EBV infects epithelial cells, spreads to lymphoid tissue, and infects B-lymphocytes (2). A portion of these B lymphocytes undergo immortalization by EBV, which leaves the infected host with a reservoir of latently infected B lymphocyte clones. Although normal immunocompetent hosts rarely develop EBV-associated lymphomas, in the setting of congenital or acquired immunodeficiency, these EBV immortalized B cells may give rise to lymphoproliferative disorders or lymphomas. The property of EBV that directly links the virus to these lymphomas is its ability to establish latent infection and immortalize lymphocytes to continuously proliferating lymphoblastoid cell lines (LCLs) (3, 4). Although the molecular events involved in B cell immortalization are not fully understood, the process involves recruitment of cellular genes by EBV to mediate transforming events (5–8). The mechanism of EBV-mediated carcinogenesis in epithelial cells is less clear, however, the process involves activation of cellular genes, as well (9). In addition, specific EBV^{+} malignancies are associated with distinct patterns of viral gene expression (latency type I, II, or III and in some cases lytic genes, as well), EBNA1 is consistently expressed in all EBV cancers (10). EBNA1 localizes to the nucleus and interacts with the chromosomes (11). EBNA1 forms a homodimer and binds to the EBV genome (12) at specific sequences located in the latent origin of replication (OriP) (13). As a DNA binding protein, EBNA1 cross-links the EBV episomal genome to the cellular chromosomes, thus providing a distribution mechanism and maintaining the constant copy number of EBV episomal genomes during cell divisions (11, 14). EBNA1 binds the dyad symmetry (DS) repeats at the OriP, and this interaction partially denatures the EBV DNA strands required for the EBV episomal replication (13, 15). In addition to episomal maintenance and replication, EBNA1 is a transcription factor that mediates the expression of other latent viral genes, including EBNA2 and the latent membrane protein 1 (LMP1) (16). EBNA1 executes its transcription activity through binding to the family of repeats (FR), a segment of the OriP, which functions as a transcription enhancer (16, 17). Given the multiple functions of EBNA1 in episomal maintenance, replication, and transcription, it represents a key molecule for maintenance of latent EBV infection. Furthermore, genetic analysis has shown that EBNA1 expression is an absolute requirement for B cell transformation (18). Interestingly, B cell-specific expression of EBNA1 in a transgenic mouse model resulted in the appearance of B cell lymphoma in some of these mice, suggesting that EBNA1 by itself might be oncogenic (7).

For the past three decades, many investigators have studied the impact of EBV or specific EBV-encoded genes on cellular gene expression using a variety of gene transfer and subtractive hybridization techniques. These studies have confirmed that EBV recruits cellular growth-regulatory and antiapoptotic genes to mediate transformation (8, 19, 20). Recently gene array technology enables us to follow changes in gene expression patterns of an extensive number of known or unknown genes.

In the present work, we used cDNA-microarray analysis to study differential cellular gene expression in the presence or absence of constitutively expressed EBNA1 in the BJAB BL and the 293 epithelial cell lines. Examination of the genes regulated in the presence of EBNA1 revealed little similarity between the two cell lines. These results suggest that the effect of EBNA1 on cellular gene expression is highly tissue specific. We have demonstrated that EBNA1 directly binds cellular promoters and induces changes in gene expression from some genes that are under these bounded promoters (21–23). Other studies confirmed our finding that EBNA1 regulates cell expression in NPC (9, 24). However, comparison with our data reveals very little similarity, which might be due to EBNA1 affecting cellular gene expression in a tissue specific manner. We have explored the ability of EBNA1 to bind to cellular promoters using Chromatin Immuno-precipitation hybridized to a human promoter array (ChIP-chip). We report here on a genome wide scale of EBNA1 interaction with the host promoters. Sequence analysis of the promoters that were engaged by EBNA1 has revealed a DNA motif. Furthermore, we have overlapped the ChIP-chip data with Exon array data to distinguish between sequence motifs in down-regulated gene promoter and in up-regulated gene promoters.

MICROBIOLOGY

Author contributions: A.C. and S.M.W. designed research; A.C. and I.H. performed research; A.E.U., S.H., A.B.D., M.S., and M.G. contributed new reagents/analytic tools; A.C., V.P.S., Z.Z., D.P., J.L., and S.M.W. analyzed data; and A.C. wrote the paper.

The authors declare no conflict of interest.

[1]To whom correspondence may be addressed. E-mail: allon.canaan@yale.edu or sherman.weissman@yale.edu.

This article contains supporting information online at www.pnas.org/cgi/content/full/0911676106/DCSupplemental.

Table 1. Genes up-regulated in the presence of EBNA1 in BJAB

Unigene	Chrm	Official gene symbol and name	Profile*
Cellular Growth			
Hs.77100	8	GTF2E2, General transcription factor IIE, polypeptide 2 (beta subunit, 34 kD)	3.253
Hs.518249	3	CNBP, CCHC-type zinc finger, nucleic acid binding protein	2.171
Hs.437075	7	CREB5, cAMP response element-binding protein 5	2.037
Hs.85155	14	ZFP36L1, Zinc finger protein 36, C3H type-like 1 (EGF-response factor 1)	3.001
Hs.326035	5	EGR1, Early growth response 1	2.086
Hs.534313	8	EGR3, Early growth response 3	2.231
Hs.83753	20	SNRPB, Small nuclear ribonucleoprotein polypeptides B and B1	2.441
Hs.711055	10	TIAL1, TIA1 cytotoxic granule-associated RNA-binding protein-like 1	6.204
Hs.75256	1	RGS1, Regulator of G-protein signalling 1	2.636
Cellular Migration			
Hs.75703	17	CCL4, Chemokine (C-C motif) ligand 4 (homologous to mouse Mip-1b)	13.759
Hs.514107	17	CCL3, Chemokine (C-C motif) ligand 3 (homologous to mouse Mip-1a)	8.582
Hs.143961	17	CCL18, Chemokine (C-C motif) ligand 18	3.412
Energy Supply			
Hs.490874	1	MTX1, Metaxin 1	3.220
Hs.558396	10	SCD, Stearoyl-CoA desaturase (delta-9-desaturase)	3.051
Hs.510078	6	SGK1, Serum/glucocorticoid regulated kinase 1	3.080
Miscellaneous			
Hs.304682	20	CST3, Cystatin C	2.555

*Results represent averaged gene expression changes from four pooled experiments as a ratio of scores for BJAB/EBNA1 over BJAB.

Results

Microarray Profiles for EBNA1-Positive and EBNA1-Negative 293 and BJAB Cell Lines. We have used the human B cell line and the epithelial cell line, BJAB and 293, respectively, which have been stably transfected to constitutively express EBNA1, to identify cellular genes that may be regulated by EBNA1. Using microarray analysis (see *SI Text*), the gene array profiles of the EBNA1-expressing BJAB/EBNA1 and 293/EBNA1 were compared to their EBNA1-negative counterpart cell lines, respectively. Analysis of the BJAB and 293 cell lines stably transfected with EBNA1 revealed cellular genes that are constitutively up-regulated or down-regulated in the presence of EBNA1. The array data are arranged in tables showing average ratio between scores from the EBNA1 transfected cell line over its counterpart cell line (Tables 1–4). Each gene appears with its HUGO name, its chromosomal location, and UniGene cluster number.

We have identified numerous genes that had different expression levels in BJAB and BJAB/EBNA1, as displayed in Table 1 (up-regulated) and Table 2 (down-regulated). These genes were classified into functionally related groups. Among the up-regulated genes, we have identified genes that are involved in interactions with nucleic acids, including transcriptional regulation. The protein products for most of these transcription factor genes possess "Zinc finger" domains and are associated with cellular growth. Other up-regulated genes function in cellular migration or energy usage. In Table 2 we have listed the group of the down-regulated genes. Within this we have identified genes that are involved in immunosurveillance and cellular growth.

Similar analysis was carried out for the 293/EBNA1 versus the 293 cell lines. However, examination of the microarray data revealed genes that were distinct from those identified in the BJAB cell lines. Among the genes that were up-regulated in 293, we identified genes involved in cellular growth, DNA stability, and immunity (Table 3). Among the genes that were down-regulated by EBNA1 in 293, we identified genes involved in cellular growth and cellular transport (Table 4).

Examination of the chromosomal locations for the affected genes in BJAB and 293 cell lines revealed little similarity. Interestingly, this microarray analysis revealed only one gene that was regulated in the presence of EBNA1 in both 293 and BJAB cell lines. We have found that expression of the MYC oncogene was decreased 2-fold in both 293 and BJAB cell lines in the presence of EBNA1. This is an unexpected finding because MYC regulation has not been attributed previously to the expression of EBNA1 (6).

Table 2. Genes down-regulated in the presence of EBNA1 in BJAB

Unigene	Chrm	Official gene symbol and name	Profile*
Cellular Growth			
Hs.202453	8	MYC, V-myc avian myelocytomatosis viral oncogene homolog	0.490
Hs.654408	4	NFKB1, Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)	0.434
Hs.163924	4	NR3C2, Nuclear receptor subfamily 3, group C, member 2	0.417
Hs.132858	4	RAP1GDS1, RAP1 GTP-GDP dissociation stimulator 1	0.432
Hs.75573	4	CENPE, Centromere protein E (312 kD)	0.468
Hs.125503	4	MAPK10, Mitogen-activated protein kinase 10	0.446
Immunity			
Hs.524760	12	OAS1, 2′,5′-oligoadenylate synthetase 1 (40–46 kDa)	0.487
Hs.458485	1	ISG15, Interferon-stimulated protein, 15 kDa	0.359
Hs.389724	1	IFI44L, Interferon induced protein 44-like	0.374
Hs.480938	4	LRBA, LPS-responsive vesicle trafficking, beach and anchor	0.392
Hs.82116	3	MYD88, Myeloid differentiation primary response gene (88)	0.426
Miscellaneous			
Hs.523332	10	OAT, Ornithine aminotransferase (gyrate atrophy)	0.448
Hs.1908	10	PRG1, Proteoglycan 1, secretory granule	0.310

*Results represent averaged gene expression changes from four pooled experiments as a ratio of scores for BJAB/EBNA1 over BJAB.

Table 3. Genes up-regulated in the presence of EBNA1 in 293

Unigene	Chrm	Official gene symbol and name	Profile*
Cellular Growth			
Hs.98428	17	HOXB6, Homeo box B6	2.383
Hs.523852	11	CCND1, Cyclin D1 (PRAD1: parathyroid adenomatosis 1)	2.386
Hs.56145	X	TMSNB, Thymosin, beta, identified in neuroblastoma cells	2.175
Hs.522584	X	TMSB4X, Thymosin, beta 4	4.307
DNA Stability			
Hs.477879	11	H2AFX, H2A histone family, member X	2.295
Hs.530461	1	HIST2H2AA3, histone cluster 2	2.101
Hs.46423	6	H4FG, H4 histone family, member G	4.602
Hs.28491	X	SSAT, Spermidine/spermine N1-acetyltransferase	3.847
Immunity			
Hs.522074	X	DSIPI, Delta sleep inducing peptide, immunoreactor	4.013
Hs.114286	12	CD9, CD9 antigen (p24)	2.241
Hs.702002	17	NPTX1, Neuronal pentraxin I	2.246
Miscellaneous			
Hs.26630	16	ABCA3, ATP-binding cassette, sub-family A, member 3	2.013
Hs.513490	16	ALDOA, Aldolase A, fructose-bisphosphate	2.679

*Results represent averaged gene expression changes from two pooled experiments as a ratio of scores for 293/EBNA1 over 293.

Quantitative Real-Time PCR Validates Microarray Results. As described in *Materials and Methods*, to generate reliable microarray results, we have sorted the initial microarray data to generate the final lists of EBNA1-affected genes for both BJAB and 293 cell lines. To verify these gene lists, we examined several of the genes by QRT-PCR. QRT-PCR is an amplification based process that is more sensitive and quantitative than the microarray analysis, which depends on a rate limiting step of hybridization to a solid matrix. Because QRT-PCR improves our ability to detect changes in gene expression, we examined expression of selected genes in both cell lines, although these genes were initially detected by microarray in one cell line only.

We selected 10 genes from either the BJAB or the 293 genes' lists and included genes that were up-regulated or down-regulated by EBNA1. In addition, we used QRT-PCR to verify that EBNA1 is expressed only in the EBNA1^{+} samples. As an internal control we examined the MAPK1 gene, which according to the microarray data, was not affected by EBNA1, and thus should generate equal amounts of PCR product regardless of EBNA1 presence. Table S1 summarizes the results from the QRT-PCR analysis as proportion of ratios. A ratio for each gene expressed in EBNA1-positive cell line to its EBNA1-negative cell line is normalized to the ratio of MAPK1 expression in EBNA1-positive cell line and EBNA1-negative cell line. Thus, scores <1 indicate down-regulation and >1 indicate up-regulation of gene expression. Interestingly, the MYC gene was down-regulated in the presence of EBNA1 in both cell lines, confirming the microarray analysis. Moreover, altered expression of the 10 selected genes from the microarray analysis was confirmed by QRT-PCR (indicated by arrows in Table S1). In addition, the QRT-PCR revealed altered expression of some genes in the other cell line, as well (e.g., H4FG, OAS1, CENPE, etc.). Seven of the selected genes demonstrated similar regulation in both cell lines, suggesting an EBNA1-specific effect. Three genes demonstrated opposite regulation in BJAB and 293 cell lines, suggesting tissue specific effects.

To conclude, the QRT-PCR analysis confirmed the expression of the genes initially found by microarray analysis. Moreover, the higher resolution of the QRT-PCR revealed gene expression changes in both of the cell lines even where microarray analysis failed to detect them.

Chromatin Immuno-Precipitation Analysis on Hu-Promoter Array Reveals Wide Genome Engagement of Cellular Promoter by EBNA1. EBNA1 is a DNA binding protein, which was well documented to bind EBV DNA sequences and orchestrate EBV gene expression by engaging EBV promoters. After the microarray studies we wondered whether we can find a direct interaction of EBNA1 with cellular promoters that may contribute to the changes in gene expression patterns, which we found. Indeed, ChIP-chip analysis clearly showed that certain cellular promoter regions were bound to EBNA1 (Table S2 shows a selected list of these promoters). By performing QRT-PCR analysis on the ChIP DNA samples that were used for the ChIP-chip, we have confirmed most of our findings. Out of the 26 promoters that we have tested, a majority of 20 promoters, (shown in bold) were successfully confirmed by QRT-PCR, while six (shown in italic) were not. Considering the technical differences in comparing ChIP-chip analysis to ChIP-QRT-PCR analysis, we have overall verified at least 77% of the promoters bound by EBNA1 to be true positive scores. Focusing on the top high scored promoters on the hit list, we have verified 19 out of the first 20 promoters (95%). A closer examination of two representatives from Table S2 is shown in Fig. S1. Melting curve analysis and agarose gel fractionation are shown for the promoters of CHUK and MUC1, for three independent repetitions. Both

Table 4. Genes down-regulated in the presence of EBNA1 in 293

Unigene	Chrm	Official gene symbol and name	Profile*
Cellular Growth			
Hs.202453	8	MYC, V-myc avian myelocytomatosis viral oncogene homolog	0.457
Hs.9999	19	EMP3, Epithelial membrane protein 3	0.494
Metabolic Stress			
Hs.523836	11	GSTP1, Glutathione S-transferase pi	0.488
Hs.481918	5	SLC1A3, Solute carrier family 1 (glial high affinity glutamate transporter), member 3	0.473
Miscellaneous			
Hs.388841	7	ZPBP, Zona pellucida binding protein	0.429
Hs.592187	22	Homo sapiens mRNA, cDNA DKFZp434A115	0.366

*Results represent averaged gene expression changes from two pooled experiments as a ratio of scores for 293/EBNA1 over 293.

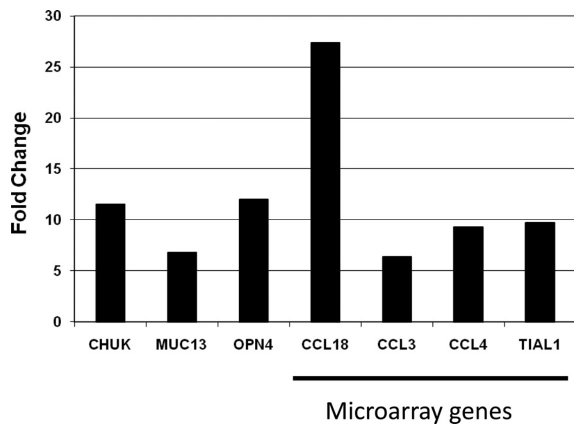


Fig. 1. QR-PCR analysis of ChIP DNA samples demonstrates sequence enrichment for selected promoter genes. The three gene promoters on the left were depicted from Table S2 (marked by *). The four enriched promoters on the right represent genes that were found to be up-regulated by EBNA1 (Table 1). QR-PCR data were process according to the equation: Fold Change = $2^{-(\text{experimental CT- reference CT})-(\text{control CT- reference CT})}$. Cycle threshold (CT) values were obtained from the PCR quantification sigmoid curves. The beta-Actin gene was used as the reference gene for the analysis of each depicted gene. EBNA1 expressing and nonexpressing ChIP samples are represented as ''experimental'' and ''control,'' respectively.

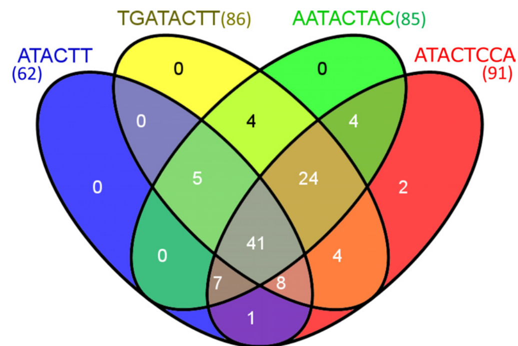


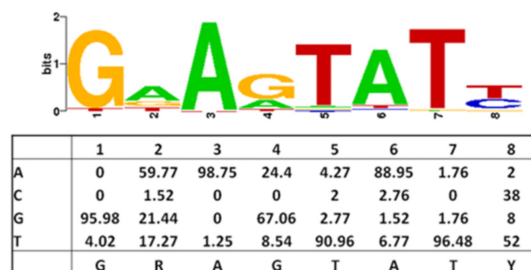
Fig. 2. Venn diagram analysis of the 100 most enriched ChIP-chip sequences for the presence of the four EBNA1binding sequences demonstrates the presence of more than one of these sequences in the EBNA1 bound promoters. The DNA sequences from the 50 regions with the greatest fold enrichment for each chipset were analyzed using the Weeder motif detection software to identify overrepresented motifs using default normal scan parameters except that both strands were examined with allowance for more than one motif occurrence per sequence. Two motifs were detected using the first slide of the Promoter array, and three motifs were detected using the second slide. Four of the five motifs shared a common ATACT sequence, and these were clustered together using the STAMP program to generate a consensus binding site. The four similar motifs were also used to scan the combined set of the 100 sequences with the greatest fold enrichment using the POSSUM software with default parameters except that the score threshold was set to 4. Numbers in brackets represents the number of promoters, of the 100 tested, which contain that sequence.

melting curves represent specific amplicon identical to EBNA1 positive or negative cell lines. However, these amplicons are enriched in the EBNA1 positive cell lines, as shown by the peak level on the melting curves and by the band intensity on agarose gel. Subsequently, we examined the fold change enrichment for several promoters including CHUK, MUC13, and OPN4 from Table S2 and few promoters of genes from Table 1 (Fig. 1). Nevertheless, not all of the genes that were affected by EBNA1 showed an enrichment change by QRT-PCR for their promoters. Hence, the change in the expression of these genes might be mediated by other genes that were affected by EBNA1, or EBNA1 was bound to a remote sequence that may affect the requirement of transcription factors to these promoters.

Fig. S2 provides the integrated genomic view (IGB) of the four promoters on the left side of Fig. 1 with three other selected promoters, among them was the β actin promoter, which was used to validate enrichment measured by QRT-PCR. The latter three promoters have shown no signal in IGB and had no enrichment change as was measured by both ChIP-chip and QRT-PCR.

Analysis of DNA Sequences from Cellular Promoter Bound to EBNA1 Reveals a Motif. To examine whether EBNA1 has a specific DNA binding sequence within the engaged cellular promoters, we have examined the 50 strongest hits from the four ChIP-chip array data. Weeder analysis has identified consensus sequences for each array separately and then through multiple alignments using STAMP we have generated a common match eight base pairs sequence. This consensus sequence has no exact match to any of the currently recognized transcription factors' motives (Fig. 2). Interestingly, this motif differs from the EBNA1 recognition sequence documented for its interactions with the DNA of the EBV genome. DNA sequence alignment analysis of the sequence to the viral recognition site confirms the two sequences to have no similarity (13). Subsequently, we have wondered how many promoters contain any of the four consensus sequences that were identified and how many promoters had more than one of the sequences in their promoters. The 100 strongest hits of the arrays were subjected to this examination and the results are presented as a Venn diagram of the four motifs (Fig. 3). We have found that each motif was found in 60–90 of the 100 promoters. In addition, most of these promoters had more than one type of the identified sequences and 41 promoters had all four consensus sequences.

Still, the identification of the motif did not provide us with information regarding the direction in gene expression regulation, influenced by EBNA1. To identify EBNA1 binding sequences, which are associated with down-regulation or with the up-regulation of cellular genes, we had to relate the ChIP-chip data to the microarray data and to group the EBNA1 bounded sequences in up-regulated or down-regulated separated groups. However, our cDNA microarray array was not well annotated and covered only one third of the currently known genes. Therefore, we have repeated the microarray analysis using the advanced Human Exon array platform, which covers all currently annotated genes. Whole transcript extracts were obtained from three biological replicates of BJAB and BJAB/EBNA1cell lines. These extracts were hybridized to the GeneChip Human Exon 1.0 ST Array (Affymetrix). A gene list of cellular genes that had at least 2-fold changes between BJAB to BJAB/EBNA1 was generated (Partek Genomic). This gene list was separated into two lists of up-regulated and down-regulated genes. An overlap between each of the gene lists and the ChIP-chip data list have identified 37 promoters for up-regulated genes that



	1	2	3	4	5	6	7	8
A	0	59.77	98.75	24.4	4.27	88.95	1.76	2
C	0	1.52	0	0	2	2.76	0	38
G	95.98	21.44	0	67.06	2.77	1.52	1.76	8
T	4.02	17.27	1.25	8.54	90.96	6.77	96.48	52
	G	R	A	G	T	A	T	Y

Fig. 3. A consensuses sequence motif for EBNA1 binding to cellular promoters. Weeder motif detection was performed on 50 DNA sequences, which had the greatest fold enrichment in the ChIP-chip studies. The four similar sequences that were identified were subjected to multiple alignments using STAMP to generate the position weight matrix.

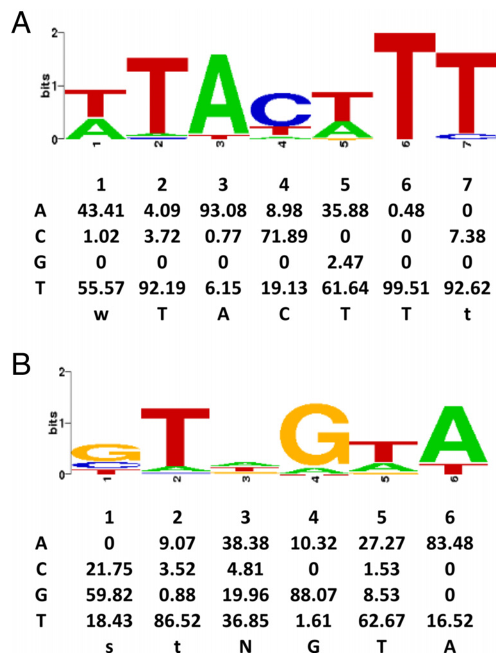


Fig. 4. STAMP Analysis of Sequences Obtained by WEEDER for Promoters of Genes Regulated by EBNA1. The list of promoters bound to EBNA1 was screened against up-regulated (*A*) and down-regulated (*B*) gene lists identified by the Exon array analysis. The sorted promoter lists were analyzed separately for the appearance of consensus motives.

were bound by EBNA1 and 39 promoters for down-regulated genes. Separated sequence analysis for the two groups of promoters was repeated and two sequence motives were identified (Fig. 4).

Discussion

The development of EBV-associated lymphoma is related to B cell transformation after latent infection by EBV (3, 4). In contrast, the role of EBV in EBV-associated NPC is less clear. All EBV-associated tumors express EBNA1, one of several EBV genes that might have oncogenic properties, as demonstrated by the appearance of spontaneous lymphoma in an EBNA1 transgenic mouse model (7), and by the absolute requirement for EBNA1 expression to enforce cellular immortalization (18). Because EBNA1 is a known transcriptional factor that is critical for the expression of other EBV latent genes, we suggested that EBNA1 may regulate or alters cellular gene expression, as well (21, 22).

Using cDNA microarrays we compared cellular gene expression profiles in EBNA1-positive and EBNA1-negative B cell and epithelial-derived cell lines. We have shown that EBNA1 expression impacts on the cellular gene expression profiles. EBNA1 regulates cellular genes from different chromosomes and from different locations on each chromosome. The difference in gene expression profiles in 293 and BJAB demonstrated a tissue-specific effect of EBNA1 on cellular gene expression. These microarray results were verified by QRT-PCR analysis of several regulated genes. In addition, the QRT-PCR analysis potentially revealed EBNA1-specific gene expression effects, as well. We have divided the affected genes into up-regulated and down-regulated genes and subdivided them into functionally related groups. Our attempts to functionally classify the genes revealed that various genes associated with cellular growth are regulated in BJAB and 293 cell lines (Tables 1–4). In addition, some of these genes have never before been reported to be regulated by EBV. We have conducted a bioinformation survey to speculate on the role and molecular characteristics of these genes.

An examination of the BJAB up-regulated growth related sub division indicates that it consists of genes involved in cell proliferation. Interestingly, most of these genes are known to directly interact with nucleic acids and their majority possesses a "Zinc finger" motif: CNBP, CREB5, ZFP36L1, EGR1, EGR3. Nevertheless, the growth regulatory effect of EBNA1 in BJAB cells may mediate a balance between reducing the apoptotic threshold while supporting cellular proliferation (Tables 1 and 2). In addition to the growth regulating genes, we identified immunity-related genes in BJAB that were down-regulated. OAS1, ISG15, and IFI44L were reported to be a part of antiviral cellular defense system, which inhibits viral expansion (29, 30) (Table 2). EBNA1 also up-regulates BJAB genes that are involved in cellular migration (Table 1). Interestingly, the chemokine CCL18 was reported to direct B cells into lymph nodes (31).

In comparison with BJAB, the impact of EBNA1 on 293 results in expression changes in different genes. Like in BJAB there is a reduction of MYC, but there is also induction of other genes associated with growth: CCND1, TMSNB, and TMSB4X (Table 3). Other up-regulated genes in 293 cells are associated with DNA stabilization and immunity (Table 3). Interestingly, H2AFX, a DNA stabilizing gene, has been reported to reduce DNA translocations (32), common in EBV related lymphomas but not in NPC. There are observations of chromosomal abnormalities in NPC, which are not translocations but rather chromosomal deletions detected on multiple chromosomes (33). As for the induction of genes related to immunity, this usually reflects cellular defense mechanisms to intercept infection. However, the recruitment of the immune system to the site of infection might also benefit EBV by providing an opportunity for the virus to encounter and infect B cells. For example, CD9 is known to enhance cellular adhesion and membrane fusion that promotes viral infection (34) and its level was found here to be elevated (Table 3).

The current working hypothesis for EBV infection suggests that epithelial cells from the oral cavity are the primary site of infection (2). EBV infection of epithelial cells is a transient state from which EBV expands into local B cells. It is through this infection that immortal B cell clones are generated and maintained at latency type I, with the potential to develop into lymphomas. Our current findings indicate that there is a difference between the BJAB and 293 cell lines in their response to the expression of EBNA1. More up-regulation of genes related to an increase in cellular growth and reduction of apoptosis was found in BJAB/EBNA1, in comparison to 293/EBNA1. Genes related to immunity were up-regulated in 293/EBNA1, while in BJAB/EBNA1 gene associated with immunity were down-regulated. Further studies are necessary to elucidate the reason(s) for these differences in gene expression. Needless to say that it is premature to suggest that these genes have an impact on EBV infection shifting from epithelial cell into B cell, or that B cell is a preferred host for EBV than epithelial cell. The effect of EBNA1 stable expression on cellular gene expression in carcinoma cell lines was examined by Wood et al. (9). Intriguingly, the genes reported to be regulated in that report did not appear in our lists. Assuming minor technical differences, this may support our hypothesis that EBNA1 affects cellular gene expression in a tissue or cell specific manner.

The ability of EBNA1 to affect cellular gene expression could be possibly achieved by two routes. The indirect route, in which, cellular gene expression changes are the result of network interactions of EBNA1 with cellular proteins, which may regulate gene expression. The other option is the direct affect route, in which EBNA1 exerts its viral transcription ability to engage cellular promoters too. Although the first route of activity was extensively studied, the latter has only recently been suggested to exist (23, 24, 35). The rational to look for EBNA1 interaction with the cellular DNA was based on the following evidences: EBNA1 has the ability to bind DNA directly; EBNA1 is a transcriptional factor with a specific recognition sequence in the EBV genome; during latency

EBV is maintained in a low copy number in the nucleus of the host, thus, EBNA1 encounters in the nucleus mainly cellular DNA molecules. These assumptions have been proven realistic by ChIP-chip analysis, which provided us with numerous cellular promoter sequences being bound by EBNA1 (Table S2). However, DNA binding by itself does not confer direct regulation of gene expression by EBNA1. It was the finding of enrichment of cellular promoter sequences in the ChIP DNA samples for genes that their mRNA level was regulated by EBNA1 (Fig. 1) as was found by the microarray analysis (Table 1). Our studies (21, 22) and other studies (9, 24) have demonstrated the interference of EBNA1 with cellular genetics. Although other studies focused on the AP-1pathway in NPC (24) our studies examined the whole cellular genome interaction with EBNA1, primarily in Burkitt's lymphoma background. In addition, our motif sequence analysis was not based on the assumption that EBNA1 cellular DNA binding should be a derivative of the EBNA1 binding site located at the OriP region in the EBV genome (35).

The identification of EBNA1 binding motives (Figs. 2–4) in cellular promoters, which differ from the EBV sequence bound by EBNA1 represents an interesting evolutionary paradigm. During its parasitic evolution EBV had to coevolve with its human hosts, and to adapt changes that will increase its survival fitness. Numerous viruses and other parasites had developed a silent modus operandi to evade immune-surveillance. EBV infects almost all human populations and resides latent in its host, primarily in a portion of B lymphocytes. In this type of latency (type I) EBNA1 is the only protein expressed from the EBV genome. EBNA1 was previously regarded as solely a key element in EBV genetics (gene expression, genome replication, and maintenance). The ability of EBNA1 to tamper and modify gene expression of the infected host is an example of high level of pathogen adaption to its host. Although EBNA1 cellular activities might be beneficial for EBV infection, still EBNA1 has to be available for the direct interaction with the EBV genome. This might be problematic to EBV survival because most of the DNA molecules, which EBNA1 encounter in the nucleus are not of EBV origin but cellular ones. This problem could be possibly solved in three ways: higher affinity of EBNA1 toward the EBV DNA sequences than toward the cellular DNA sequences, use of multiple EBNA1 binding sequences in key EBV elements (like the numerous EBNA1 binding domain in the OriP), or use of both of the solutions together, which results in high avidity toward the EBV genome.

Materials and Methods

ChIP-Chip Hybridization to Human Promoter Array. ChIP was performed as was described in ref. 26. To obtain the EBNA1-DNA complexes we have purchased the anti EBNA1 antibody OT1x (Cyto-Barr), which was described to obtain ChIP of EBNA1 complexes with the OriP of the Epstein–Barr virus (27). Two replicates of ChIP with their controls (EBNA1 nonexpressing) samples were labeled and hybridized to a human promoter tiling chip set (Nimblegen Systems). The two-chip promoter set was based on the HG17 May 2004 human genome sequence assembly and covers approximately 4,200 bases upstream and 700 bases downstream of approximately 3,500 transcripts with oligos tiled approximately every 100 bases. The two replicate datasets were processed together using the Tilescope software (28) to identify genomic regions that were bound by EBNA1. Default parameters with iterative peak identification were used except that the window size was 100.

ACKNOWLEDGMENTS. This work was supported by the Lymphoma Research Foundation of America, Engelberg Foundation, Yale Cancer Center, and William H. Prusoff Foundation. Bioinformatics analysis was supported by a National Institutes of Health Yale Center of Excellence in Molecular Hematology core Grant P30 DK072442–03.

1. Thompson MP, Kurzrock R (2004) Epstein–Barr virus and cancer. *Clin Cancer Res* 10:803–821.
2. Borza CM, Hutt-Fletcher LM (2002) Alternate replication in B cells and epithelial cells switches tropism of Epstein–Barr virus. *Nature Med* 8:594–599.
3. Dalens M, Zech L, Klein G (1975) Origin of lymphoid lines established from mixed cultures of cord-blood lymphocytes and explants from infectious mononucleosis, Burkitt lymphoma and healthy donors. *Int J Cancer* 16:1008–1014.
4. Zech L, Haglund U, Nilsson K, Klein G (1976) Characteristic chromosomal abnormalities in biopsies and lymphoid-cell lines from patients with Burkitt and non-Burkitt lymphomas. *Int J Cancer* 17:47–56.
5. Kube D, et al. (1999) Expression of Epstein–Barr virus nuclear antigen 1 is associated with enhanced expression of CD25 in the Hodgkin cell line L428. *J Virol* 73:1630–1636.
6. Kaiser C, Laux G, Eick D, Jochner N, Bornkamm GW, Kempkes B (1999) The proto-oncogene c-myc is a direct target gene of Epstein–Barr virus nuclear antigen 2. *J Virol* 73:4481–4484.
7. Wilson JB, Bell JL, Levine AJ (1996) Expression of Epstein–Barr virus nuclear antigen-1 induces B cell neoplasia in transgenic mice. *EMBO J* 15:3117–3126.
8. Nunez G, et al. (1989) Growth- and tumor-promoting effects of deregulated BCL2 in human B-lymphoblastoid cells. *Proc Natl Acad Sci USA* 86:4589–4593.
9. Wood VH, et al. (2007) Epstein–Barr virus-encoded EBNA1 regulates cellular gene transcription and modulates the STAT1 and TGFbeta signaling pathways. *Oncogene* 26:4135–4147.
10. Bornkamm, GW (2008) Epstein–Barr virus and the pathogenesis of Burkitt's lymphoma: More questions than answers. *Int J Cancer* 124:1745–1755.
11. Shire K, Ceccarelli DF, Avolio-Hunter TM, Frappier L (1999) EBP2, a human protein that interacts with sequences of the Epstein–Barr virus nuclear antigen 1 important for plasmid maintenance. *J Virol* 73:2587–2595.
12. Middleton T, Sugden B (1994) Retention of plasmid DNA in mammalian cells is enhanced by binding of the Epstein–Barr virus replication protein EBNA1. *J Virol* 68:4067–4071.
13. Ambinder RF, Shah WA, Rawlins DR, Hayward GS, Hayward SD (1990) Definition of the sequence requirements for binding of the EBNA-1 protein to its palindromic target sites in Epstein–Barr virus DNA. *J Virol* 64:2369–2379.
14. Rawlins DR, Milman G, Hayward SD, Hayward GS (1985) Sequence-specific DNA binding of the Epstein–Barr virus nuclear antigen (EBNA-1) to clustered sites in the plasmid maintenance region. *Cell* 42:859–868.
15. Reisman D, Yates J, Sugden B (1985) A putative origin of replication of plasmids derived from Epstein–Barr virus is composed of two cis-acting components. *Mol Cell Bio* 5:1822–1832.
16. Gahn TA, Sugden B (1995) An EBNA-1-dependent enhancer acts from a distance of 10 kilobase pairs to increase expression of the Epstein–Barr virus LMP gene. *J Virol* 69:2633–2636.
17. Reisman D, Sugden B (1986) trans activation of an Epstein–Barr viral transcriptional enhancer by the Epstein–Barr viral nuclear antigen 1. *Mol Cell Bio* 6:3838–3846.
18. Lee MA, Diamond ME, Yates JL (1999) Genetic evidence that EBNA-1 is needed for efficient, stable latent infection by Epstein–Barr virus. *J Virol* 73:2974–2982.
19. Gregory CD, et al. (1991) Activation of Epstein–Barr virus latent genes protects human B cells from death by apoptosis. *Nature* 349:612–614.
20. Schena M, et al. (1992) Growth- and differentiation-associated expression of bcl-2 in B-chronic lymphocytic leukemia cells. *Blood* 79:2981–2989.
21. Canaan A, Haviv I, Bowtell D, Lacy J (2002) *The Tenth Biennial Conference of the International Association for Research on Epstein–Barr Virus and Associated Diseases.* (Cairns, Australia).
22. Canaan A, Haviv I, Bowtell D, Lacy, J (2004) *Sixth International Conference on Malignancies in AIDS and Other Immunodeficiencies: Basic, Epidemiologic and Clinical Research.* (National Cancer Institute Bethesda, MD).
23. Canaan A, Urban EA, Weissman SM (2008) *American Society for Microbiology: Manipulation of Nuclear Processes by DNA Viruses.* (Charleston, SC).
24. O'Neil JD, et al. (2008) Epstein–Barr virus-encoded EBNA1 modulates the AP-1 transcription factor pathway in nasopharyngeal carcinoma cells and enhances angiogenesis in vitro. *J Gen Virol* 89:2833–2842.
25. Kong CF, Bowtell D (2002) Genomewide gene expression analysis using cDNA microarrays. *Methods Mol Med* 68:195–204.
26. Hartman SE, et al. (2005) Global changes in STAT target selection and transcription regulation upon interferon treatments. *Genes Dev* 19:2953–2968.
27. Chaudhuri B, Xu H, Todorov I, Dutta A, Yates JL (2001) Human DNA replication initiation factors, ORC and MCM, associate with oriP of Epstein–Barr virus. *Proc Natl Acad Sci USA* 98:10085–10089.
28. Zhang ZD, et al. (2007) Tilescope: Online analysis pipeline for high-density tiling microarray data. *Genome Biol* 8:R81.
29. Hassel BA, Zhou A, Sotomayor C, Maran A, Silverman RH (1993) A dominant negative mutant of 2–5A-dependent RNase suppresses antiproliferative and antiviral effects of interferon. *EMBO J* 12:3297–3304.
30. Meraro D, Gleit-Kielmanowicz M, Hauser H, Levi BZ (2002) IFN-stimulated gene 15 is synergistically activated through interactions between the myelocyte/lymphocyte-specific transcription factors, PU. 1, IFN regulatory factor-8/IFN consensus sequence binding protein, and IFN regulatory factor-4: Characterization of a new subtype of IFN-stimulated response element. *J Immunol* 168:6224–6231.
31. Hieshima K, et al. (1997) A novel human CC chemokine PARC that is most homologous to macrophage-inflammatory protein-1 alpha/LD78 alpha and chemotactic for T lymphocytes, but not for monocytes. *J Immunol* 159:1140–1149.
32. Celeste A, et al. (2002) Genomic instability in mice lacking histone H2AX. *Science* 296:922–927.
33. Hui AB, Lo KW, Teo PM, To KF, Huang DP (2002) Genome wide detection of oncogene amplifications in nasopharyngeal carcinoma by array based comparative genomic hybridization. *Int J Oncol* 20:467–473.
34. Tachibana I, Hemler ME (1999) Role of transmembrane 4 superfamily (TM4SF) proteins CD9 and CD81 in muscle cell fusion and myotube maintenance. *J Cell Biol* 146:893–904.
35. Dresang LR, Vereide DT, Sugden B (2009) Identifying sites bound by Epstein–Barr virus nuclear antigen 1 (EBNA1) in the human genome: Defining a position-weighted matrix to predict sites bound by EBNA1 in viral genomes. *J Virol* 83:2930–2940.