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A single amino acid in EBNA-2 determines superior B lymphoblastoid cell line growth maintenance by Epstein-Barr virus type 1 EBNA-2

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ABSTRACT

Sequence differences in the EBNA-2 protein mediate the superior ability of type 1 EBV to transform human B cells into lymphoblastoid cell lines compared to type 2 EBV. Here we show that changing a single amino acid (S442D) from Serine in type 2 EBNA-2 to the Aspartate found in type 1 EBNA-2 confers a type 1 growth phenotype in an LCL growth maintenance assay. This amino acid lies in the transactivation domain of EBNA-2 and the S442D change increases activity in a transactivation domain assay. The superior growth properties of type 1 EBNA-2 correlate with greater induction of EBV LMP-1 and about 10 cell genes, including CXCR7. In ChIP assays, type 1 EBNA-2 is shown to associate more strongly with EBNA-2 binding sites near the LMP-1 and CXCR7 genes. Unbiased motif searching of the EBNA-2 binding regions of the differentially regulated cell genes identifies an EICE motif that closely corresponds to the sequences known to mediate EBNA-2 regulation of the LMP-1 promoter. It appears that the superior induction by type 1 EBNA-2 of the cell genes contributing to cell growth is due to their being regulated differently from most EBNA-2 responsive genes and in a similar way to the LMP-1 gene.

IMPORTANCE

The EBNA-2 transcription factor plays a key role in B cell transformation by EBV and defines the two EBV types. Here we identify a single amino acid (Ser in type 1, Asp in type 2) of EBNA-2 that determines the superior ability of type 1 EBNA-2 to induce a key group of cell genes and the EBV LMP-1 gene, which mediate the growth advantage of B cells infected with type 1 EBV. EBNA-2 binding sites in these cell genes have a sequence motif similar to the sequence known mediate regulation of the EBV LMP-1 promoter. Further detailed

analysis of transactivation and promoter binding provides new insight into the physiological regulation of cell genes by EBNA-2.

INTRODUCTION

Epstein-Barr virus (EBV) is the main cause of infectious mononucleosis in western countries and also contributes to several types of human cancer. Some of these diseases vary in incidence dramatically in different parts of the world and it is possible that natural variation in EBV contributes to differences in disease incidence. It has recently been proposed that differences in the properties of an EBV strain isolated from a Chinese nasopharyngeal carcinoma might contribute to its role in that disease (1). Mutations in the EBNA-3B gene linked to diffuse large B cell lymphoma also support the idea that variation in EBV sequence may be linked to human disease (2).

Type 1 and type 2 strains are the main natural functional variation known in Epstein-Barr virus (3, 4). The types are defined by sequence differences in the EBNA-2 gene but there are also linked sequence changes in the EBNA-3 family of genes (3). Although both virus types activate human B cells upon infection, type 1 strains are much better than type 2 strains at transforming human B cells into proliferating lymphoblastoid cell lines (LCLs) (5). Type 1 is the main EBV type prevalent all over the world, but type 2 EBV is found frequently in sub-Saharan Africa, where it can be equally abundant as type 1 EBV. At present the biological significance of the two types of EBV is not understood and there is no specific link of these types to human disease. However, the difference in growth transformation remains the clearest example of functional variation of EBV. Most genes of EBV have a low level of natural sequence variation (less than 5% at the amino acid level) but type 1 and type 2 EBNA-2 are only 56% identical at the amino acid level. Replacement of the EBNA-2 gene in

type 2 EBV with type 1 EBNA-2 confers a type 1 transformation efficiency on the type 2 strain (6), showing that EBNA-2 is the functionally important gene for the growth transformation phenotype.

EBNA-2 is a transcription factor for viral and cell genes transcribed by RNA polymerase II but does not bind DNA directly. EBNA-2 forms a complex with cell DNA binding proteins, which target it to promoters. The best characterised of these cell proteins is RBP-Jκ (also known as CSL) but PU.1 (7, 8), ATF/CRE (9) and EBF1 binding sites (10) have also been shown to mediate EBNA-2 function at certain promoters. Early studies of EBNA-2 function focussed on viral promoters or artificial promoters where the binding sites are close to the transcription start site (TSS) but recent ChIP-sequencing studies in human B cell lines (10, 11) have shown that EBNA-2 binding sites at cellular gene targets are predominantly located far away from gene TSSs, at distances up to 100 kb up- or downstream.

To investigate why type 1 EBV is more efficient at promoting LCL growth, we previously made chimaeras of the type 1 and type 2 EBNA-2 genes and tested them for the ability to maintain growth of an LCL conditionally dependent on transfected EBNA-2. The results (12) showed that sequences from the C-terminal part of type 1 EBNA-2 were sufficient to confer the maintenance of LCL growth when swapped into type 2 EBNA-2. This part of type 1 EBNA-2 contains the Arginine-Glycine (RG) repeat, Conserved Region 7 (CR7) and transactivation domain (TAD). We also identified cell and viral genes that are differentially regulated by the EBNA-2 types (13). The EBV LMP-1 gene is induced more rapidly and strongly by type 1 EBNA-2 (13) and LMP-1 is known to be required for growth transformation by EBV. About 300 cell genes are induced directly by type 1 EBNA-2 (14) but only about 10 of these are regulated differentially, all induced more strongly by type 1

EBNA-2. The most differentially regulated gene is CXCR7, which was shown to be essential for proliferation of LCLs (13).

In this paper we map the part of type 1 EBNA-2 responsible for the superior growth maintenance properties more precisely. Remarkably, changing a single amino acid (S442D) from Serine in type 2 EBNA-2 to the Aspartate found in type 1 EBNA-2 confers a type 1 growth phenotype in the LCL growth maintenance assay. This amino acid is located in the TAD of type 1 EBNA-2 and confers stronger transactivation function and increased binding to some sites at differentially regulated genes. We identify a sequence element that is enriched in the EBNA-2 binding regions of the LMP-1 promoter and cell promoters which are differentially regulated by the EBNA-2 types but not in EBNA-2 binding sites of genes that are regulated equally by type 1 and type 2 EBNA-2. This motif includes both a PU.1 (Spi-1) and IRF binding sequence. It closely resembles the ETS-IRF composite element (EICE) in the IgL λ gene enhancer and a similar sequence that mediates PU.1 activation of the LMP-1 promoter. This element may therefore confer the differential effects of type 1 and type 2 EBNA-2 on both LMP-1 and cell gene activation.

MATERIALS AND METHODS

Cell culture: EREB2.5 cells (15) contain an ER-EBNA-2 fusion protein regulated by β-estradiol and were maintained as described previously (12). Daudi is an EBV-positive BL cell line (16) and BJAB is an EBV negative B lymphoma line (17). Daudi: pHEBoMT- EBNA-2 cell lines were grown in RPMI 1640 medium (Gibco, UK) supplemented with 10% (v/v) heat-inactivated foetal bovine serum (FBS) and 0.3 mg/ml hygromycin B (Roche, Germany). HEK 293 cells were cultured in Dulbecco's Modified Eagle medium (DMEM, Gibco) supplemented with 10% FBS and antibiotics. Primary B cells were purified from buffy coat residues by negative

selection using RosetteSEP Human B cell Enrichment cocktail (Stemcell Technologies, Canada) and infected with B95-8 EBV as described previously (12).

Plasmids and cloning: The QuikChange II Site-directed mutagenesis kit (Stratagene, UK) was used to introduce SD431-432HN, EEP434-436PEA, F438I, S442D, D442S or G460Y site-directed mutations into type 1 EBNA-2, type 2 EBNA-2 or chimaera 6 EBNA-2 (E2C6) in pBS-EBNA-2 plasmids (12). The modified EBNA-2 sequences were then transferred into the p294 plasmid as described previously (12).

Inducible expression of EBNA-2 was obtained from the metallothionein promoter in the new pHEBoMT-EBNA-2 series of plasmids. The MT-EBNA-2 expression cassette was inserted into pHEBo (18) as a *Not*I fragment, having converted the *Bam*HI site of pHEBo to a *Not*I site using an oligonucleotide. The MT-EBNA-2 expression cassette was assembled using the metallothionein promoter from pMEP4 as an *Xho*I–*Hind*III fragment linked to the *Hind*III-*Bgl*II EBNA-2 gene from pSG5-EBNA-2. The type 2 and type 2 S442D alleles of EBNA-2 had also been introduced into pSG5-EBNA-2 by swapping an *Eco*RI-*Bgl*II fragment from corresponding pBS-E2 plasmids (12) after converting their original *Sac*I site to *Bgl*II.

GAL4-DBD EBNA-2 TAD fusions were obtained by PCR amplification of EBNA-2 TADs (amino acids 426-463 of type 1 EBNA-2) in the pBS-EBNA-2 series of plasmids using primers flanked by *Bam*HI and *Not*I restriction sites. These EBNA-2 TAD *Bam*HI-*Not*I fragments were cloned into pcDNA3.1-GAL4 DBD (Addgene, USA).

Transfections: The EREB2.5 growth assay was performed as described previously (12). For HEK 293 cell transfections, $3x10^5$ cells were transfected with 4 µg plasmid DNA in 6-well plates using Lipofectamine 2000 (Invitrogen, UK). For the TAD luciferase reporter assays,

$2x10^6$ BJAB cells were transfected using the Neon system (Invitrogen). A mixture of 500 ng pFR-Luc reporter plasmid (Agilent, UK), 10 ng pRL-CMV *Renilla* plasmid and 200 ng pcDNA3.1-GAL4 DBD fused to EBNA-2 TAD was used. Electroporation conditions were 1200 V, 20 ms pulse width and 2 pulses. Transfected cells were transferred into 2 ml RPMI 1640 medium supplemented with 10% FBS and antibiotics and assayed after 24 h.

To generate Daudi: pHEBoMT- EBNA-2 stable cell lines, $2x10^6$ cells were transfected with 6 µg pHEBoMT-EBNA-2 plasmid using the Neon transfection system (Invitrogen) set at 1400 V, 30 ms pulse width, 1 pulse. Transfected cells were selected with 300 µg/ml hygromycin B.

Immunoblotting antibodies: Cell samples were lysed and analysed by western immunoblotting as described previously (12). Detection of EBNA-2 was with PE2 (Dako, USA), β-actin with AC-74 (Sigma, UK), LMP-1 with CS. 1-4 (Dako) and GAL4-DBD with RK5C1 (Santa Cruz, USA) antibodies.

Luciferase assays: 24 hours after transfection, BJAB cells were lysed in 1X passive lysis buffer supplied in the Dual-Luciferase Reporter Assay kit (Promega, UK). Firefly luciferase and *Renilla* luciferase activity were measured for each cell lysate. *Renilla* luciferase activity was determined to normalize the values of firefly luciferase activity for transfection and extraction efficiency.

RNA extraction and qRT-PCR: Total cell RNA was extracted from treated cells using the RNeasy kit (Qiagen, UK). cDNA was prepared using the Protoscript M-MuLV First strand cDNA Synthesis kit (New England Biolabs, NEB, UK) and random primers. For CXCR7 RNA, qPCR was carried out with GCAGCCAGCAGAGCTCACAGTTG (Fw) and

TGGGCATGTTGGGACACATCACC (Rev) primers, with GAPDH as a normalizing control. mRNA levels were quantified using the $\Delta\Delta C_T$ method.

5′ Rapid amplification of cDNA ends (5′ RACE): EREB2.5 cells were washed twice and resuspended at $5x10^5$ cells/ml in RPMI 1640 medium supplemented with 10% FBS and antibiotics. Cells were treated with or without 1 μM β-estradiol (Sigma) for 4 days. RNA was extracted and 5′ RACE was then performed using the 5′ RACE System for Rapid Amplification of cDNA Ends (Invitrogen). The gene-specific primers for CXCR7 used were CAAGATGTAGCACTGCGTGTCATAGCC and AGAGCAGGACGCTTTTGTTGGGC. 5′ RACE products were cloned, sequenced and mapped on to the hg19 human genome sequence using BLAT.

Chromatin Immunoprecipitation: Chromatin immunoprecipitation-quantitative PCR (ChIP-qPCR) assays for EBNA-2 were carried out as described previously (19-21) using chromatin from Daudi: pHEBoMT- EBNA-2 cell lines. Cells at $5x10^5$ /ml were treated for 24 hours with 4-10 μM cadmium chloride ($CdCl_2$) before chromatin preparation. A non-specific IgG immunoprecipitation was used as a negative control in all ChIP experiments. ChIP-qPCR primers used in this study were: CXCR7 – for ChIP-seq MACS peak 16, Fw: AGATCGGTAGTTGGATGGGTTT and Rev: CTCATGGTTCTATGTCCTCACCA and for non-binding region, Fw: CACACGAAGGCTGGAGTAGT and Rev: AGCATGAGAGGAGTGTTGACC; CXCR7 ChIP-seq MACS peak 13, Fw: TGTGTGTGTCTGCCTGTGG and Rev: TGGCTGCAGACTTGCATTAT and for non-binding region, Fw: CCATCAGTGAATGGTGGTCA and Rev: TGCCGTGTAACATGGAAGG; LMP-1 – for ChIP binding region, Fw: AGAGGAGGAGAAGGAGAGCAA and Rev: CCTGAGGATGGAACACGAC and for non-binding region, Fw: GGACACGCTCCTTCTTGG and Rev: ACTGGCTGGATTCTACGCTACT; CCL3 – for ChIP binding region, Fw: GCTGGAGAGTTCATGCACAG and Rev: TTCTCCTGTGAGTGTGAAGAGG and

for non-binding region, Fw: GAGGTATGCTGATTGATTGTGAA and Rev: CTACCTTCTCAGCCAGATTATATGC.

Motif Searching: ChIP-sequencing data from Mutu III cells (11) was used to identify significant EBNA-2 binding sites (MACS <10-7) around nine previously identified differentially regulated cell gene loci and nine equally regulated gene loci (13). Any binding peak within the gene or upstream or downstream from the gene, but not within an adjacent gene was included in the analysis. DNA sequences from the EBNA-2 binding sites at differentially regulated cell genes plus the LMP-1 promoter region from -320 to +1 (a total of 58 sequences) were then subjected to unbiased analysis by MEME-ChIP for non-centrally enriched motifs of between 6 to 12 nts (22). DNA sequences from the binding sites at equally regulated cell genes (a total of 80 sequences) were analysed in parallel.

RESULTS

Fine mapping amino acid sequence that mediates superior growth properties of type 1 EBNA-2

Our previous chimaera mapping of the type 1 EBNA-2 sequences responsible for its superior growth promoting properties in EREB2.5 cells was extended, focussing on the transactivation domain (TAD). The loss of EREB2.5 growth maintenance with a type 2 TAD compared to a type 1 TAD (compare chimaera C6 and C7 in Fig 1A) showed that some type 1 amino acids in this region must be required. There are relatively few amino acid differences in this part of the protein so it was feasible to mutate each significantly different amino acid either individually or in groups (Fig 1A). These chimaeras were then tested as before (12) by transfection into EREB2.5 cells, measuring the ability to maintain cell growth in the absence

of β-estradiol (Fig 1B). The results showed that mutant S442D rescued the growth maintenance ability of chimaera C6 to become equivalent to that of C7. Other mutations tested did not rescue cell growth. This shows that S442D is the only significant amino acid difference in the TAD region for this assay. The EBNA-2 proteins were all expressed with the expected size on a Western blot (Fig 1C).

Although C7 and C6 S442D both had similar activity in the EREB2.5 growth assay, they were quantitatively not as effective as type 1 EBNA-2 or the chimaera C2. The contribution of S442D was investigated further by making this single amino acid change in type 2 EBNA-2, without any other changes (Fig 2A). The reciprocal amino acid change of D442S was also made in type 1 EBNA-2. With this minimal disruption of the whole protein structure, the S442D point mutant converted type 2 EBNA-2 to be equally effective as type 1 EBNA-2 in the EREB2.5 growth assay (Fig 2B). The reciprocal point change of D442S abolished the activity of type 1 EBNA-2 in the EREB2.5 assay. Again, these EBNA-2 proteins were all expressed with the expected size on a Western blot (Fig 2C). The results show that the aspartate at 442 is essential for type 1 EBNA-2 function in the cell growth assay and the S442D point change is sufficient to convert type 2 EBNA-2 to be as effective as type 1 EBNA-2 in this assay.

Stronger activation domain function of type 1 EBNA-2 is mediated by aspartate 442

Since Asp 442 lies in the transactivation domain (TAD) of EBNA-2, we tested whether S442D might give a higher transactivation function. The TADs were fused to the Gal4 DNA-binding domain and tested for the ability to induce expression of a synthetic promoter containing Gal4 DNA binding sites in a transient transfection reporter assay in BJAB cells (Fig 3A). This system had been used for the original identification of the TAD of type 1 EBNA-2 (23). The activity of type 1 EBNA-2 TAD was about 2 fold higher than type 2 EBNA-2 TAD (Fig 3B).

S442D increased type 2 EBNA-2 transactivation activity to be close to that of type 1 and the reciprocal D442S mutation in type 1 EBNA-2 reduced the activity to be similar to that of type 2 EBNA-2 TAD (Fig 3B). The other mutations that did not increase LCL growth in Fig 1B also did not increase TAD activity (Fig 3B). Asparate 442 can therefore confer increased activity to the TAD of type 2 EBNA-2 and was unique among the amino acids tested in having this activity.

Although they were all expressed from equivalent plasmid structures, it was difficult to control for equal expression of the Gal4 fusion proteins in the transfected BJAB cells because the proteins were unstable and could not be detected by western blotting of the BJAB cell extracts. When the same plasmids were transfected into 293 cells (which have a much higher transfection efficiency) and extracts western blotted for Gal4, all were detected but only the stable Gal4 part of the fusion protein could be observed (Fig 3C). Most likely the TAD part of the fusion protein is degraded in the cell extracts leaving only the stable Gal4 DNA-binding domain.

We conclude that a slightly stronger activation domain function of type 1 EBNA-2 is mediated by aspartate 442. This may be a significant factor in the activation of specific genes by EBNA-2 during B cell transformation that results in the superior growth transforming properties of type 1 EBNA-2.

CXCR7 RNA is rapidly degraded upon EBV infection and then re-induced by EBNA-2

LMP-1 and CXCR7 are the genes that are most differentially regulated by type 1 and type 2 EBNA-2 (12) and the mechanism of their regulation is the focus of this study. However, it was noticeable in our previous analysis that uninfected B cells already contain CXCR7 mRNA

(12). A more detailed investigation of CXCR 7 mRNA levels during EBV infection has now shown an initial rapid drop followed by a re-induction. One day post infection with type 1 EBV (B95-8), the level is reduced (Fig 4A) but by 3 days post infection the level has increased to exceed that of non-infected cells. The initial degradation of CXCR7 RNA appears to result from signal transduction upon viral infection because UV-inactivated EBV caused a similar reduction at day 1 to untreated EBV (Fig 4A). The supernatant left after pelleting the EBV from the virus preparation by ultracentrifugation did not reduce CXCR7 levels so it appears to be the virus infection that caused the degradation in CXCR7 mRNA. The reduction in CXCR7 mRNA levels was even greater at 4 or 8 hours post infection (Fig 4B) indicating that the CXCR7 mRNA level 1 day post infection is already increasing again, consistent with the expression of EBNA-2 at this time (Fig 4C). Further evidence for regulation by signal transduction came from a rapid and dramatic reduction in CXCR7 levels in response to TPA treatment of the B cells without EBV (Fig 4A, B). Our previous analysis using BAC B95-8 EBV rather than the EBV induced from B95-8 cells used here showed a slightly more rapid re-induction of CXCR7 (12). This most likely reflects a different amount of infectious virus used in the experiments. We conclude that the difference in CXCR7 regulation by type 1 and type 2 EBV upon infection of primary B cells (12) seems to lie in a delayed and weaker re-induction of CXCR7 levels after infection by type 2 EBV.

Type 1 EBNA-2 binds at higher levels to LMP-1 and CXCR7 gene regulatory elements

To determine whether differences in binding of type 1 and type 2 EBNA-2 to gene regulatory elements also play a role in the increased activation of LMP-1 and CXCR7 by type 1 EBNA-2, we performed ChIP-qPCR analysis using newly generated Daudi cell lines stably transfected with plasmids inducibly expressing EBNA-2. Inducible expression of type 1, type 2 or type 2

S442D EBNA-2 was mediated via the cadmium-responsive metallothionein promoter and in these constructs the EBNA-2 poly proline repeat length was equalised in the type 1 and type 2 EBNA-2 alleles to generate EBNA-2 proteins of almost the same size. Following induction, the superior activation of the endogenous LMP-1 gene in the Daudi EBV genome by type 1 EBNA-2 is clearly apparent (Fig 5A). Consistent with its growth promoting properties in the EREB2.5 LCL assay, type 2 EBNA-2 with the S442D mutation induced LMP-1 as efficiently as type 1 EBNA-2 in this Daudi cell system (Fig 5A). The type 2 S442D mutant was also as effective as type 1 EBNA-2 at inducing CXCR7 mRNA (Fig 5B), compared to the weak induction by type 2 EBNA-2 in these cells (Fig 5B). In contrast to primary resting B cells, Daudi cells had a low endogenous level of CXCR7 mRNA prior to the induction by EBNA-2. Several different mRNAs have been reported for CXCR7 (Fig 6A) so we determined which of these mRNAs is induced by EBNA-2 using a 5' RACE assay with RNA from EREB2.5 cells (Fig 5C). Sequencing the single RACE product (Fig 5C) demonstrated that the promoter with a TSS at 237,478,380 on chromosome 2 in the human hg19 genome assembly (highlighted in Fig 6A) is the TSS induced by EBNA-2. It was this mRNA that was assayed in the qPCR experiment shown in Fig 5B and in Fig 4.

The sequences required for EBNA-2 activation of the LMP-1 promoter are close to the transcription start and have been mapped in detail previously (7-9, 24, 25). Recent ChIP-sequencing analyses of EBNA-2 binding sites in the Mutu III BL cell line and in an LCL (10, 11) have shown that most EBNA-2 binding sites in the human genome are located far away from gene TSSs at distances up to 100 kb up- or downstream. Using Mutu III ChIP-sequencing data, we identified seventeen peaks of EBNA-2 binding (MACS <10-7) upstream and downstream from the confirmed CXCR7 gene TSS (Fig 6A and Table 1).

ChIP-qPCR assays were carried out following 24 hours of $CdCl_2$ induction of EBNA-2 as time course experiments revealed (data not shown) that this was the optimum time to observe differential effects of type 1 and type 2 EBNA-2. Western blot analysis demonstrated that similar levels of type 1, type 2 and type 2 S442D EBNA-2 were precipitated by the EBNA-2 PE2 monoclonal antibody in ChIP samples (Fig 5D). ChIP-qPCR analysis detected significantly increased binding of type 1 EBNA-2 compared to type 2 EBNA-2 at two representative EBNA-2 binding sites (marked as MACS peaks 16 and 13 in Fig 6A) at the CXCR7 locus (Fig 6B, C) and at the LMP-1 promoter (Fig 6D). In contrast, binding of both type 1 and type 2 EBNA-2 to a site at the equally regulated CCL3 gene was equivalent, indicating gene specificity (Fig 6E). In these assays the binding of type 2 S442D EBNA-2 at both the LMP-1 promoter and the CXCR7 binding site peak 16 was stronger than type 2 EBNA-2 but lower than that of type 1 EBNA-2 (Fig 6B, D). Binding of type 2 EBNA-2 at CXCR7 peak 13 was not increased by the S442D mutation (Fig 6C).

Our data therefore suggest that reduced transactivation function and reduced gene-specific binding of type 2 EBNA-2 contribute to the impaired activation of specific viral and cell genes by type 2 EBNA-2. In combination, these effects likely result in the impaired growth transforming function of type 2 EBNA-2. The S442D mutation in type 2 EBNA-2 restores transactivation function and partially rescues the reduced gene-specific binding observed for type 2 EBNA-2 at some binding sites.

An ETS-IRF composite element (EICE) is enriched in EBNA-2 binding regions of genes that are differentially regulated by EBNA 2 types

EBNA-2 does not bind directly to DNA in a sequence-specific manner but accesses DNA sites by associating with a cellular partner protein. RBP-Jκ (CSL) is the best characterised of these,

313 although EBNA-2 has also been shown to interact with PU.1 (Spi-1) *in vitro* and pull down
314 the protein from extracts (7). In fact, in addition to an RBP-Jκ site, binding sites for PU.1 and
315 EBF1 in the LMP-1 promoter are also required for activation of LMP-1 expression by EBNA-2
316 (7, 10, 25).

317 To identify sequences that might characterise the EBNA-2 response elements of genes that
318 are more strongly induced by type 1 EBNA-2 compared to type 2 EBNA-2, we used MEME-
319 ChIP to perform unbiased motif searching on EBNA-2 binding peaks identified from ChIP-
320 sequencing experiments carried out in Mutu III BL cells (11). For this analysis we identified
321 significant EBNA-2 binding peaks around a set of nine previously identified (13) differentially
322 regulated genes (CXCR7, ADAMDEC1, IL1B, MARCKS, CCL3L3, HES1, ZAP70, FCRL2, IL4) and
323 nine equally regulated genes (GCET2 (GCSAM), CD69, BATF, FCRL3, CCR7, HEY1, CCL3,
324 RUNX3, DTX1). The LMP-1 promoter region from +1 to -320 was also included in the analysis
325 of differentially regulated gene regulatory elements. This analysis returned only one
326 significantly-enriched motif in the EBNA-2 binding sites from differentially regulated genes
327 (Fig 7) and no enriched motifs for the equally regulated gene binding sites. Remarkably, this
328 unbiased analysis identified a consensus motif that occurs 31 times in the set of binding
329 sites analysed from differentially regulated genes, including one occurrence in the LMP-1
330 promoter (Table 1). In fact, the motif found in the LMP-1 promoter is the site previously
331 shown to bind PU.1 and a factor previously termed LBF4 (7) (Table 1). This element
332 represents an overlapping binding site for ETS (e.g. PU.1/Spi-1) and IRF transcription factors
333 (ETS-IRF composite element; EICE), the best known example of which is located in the IgL λ
334 enhancer (5′-AAAAGGAAGTGAAACCA-3′) (26). The GGAA motif represents a core ETS
335 binding site and is combined with an overlapping IRF site (AAxxGAAA) site. The enriched

motif identified in our analysis closely resembles the IgL λ enhancer site (Fig 7). The remaining 30 examples of the enriched motif in the differentially regulated cell gene EBNA-2 binding regions distributed as follows: MARCKS (11), FCRL2 (4), ADAMDEC1 (1), CCL3L3 (1), HES1 (2), ZAP70 (1), CXCR7 (10). 10 motifs were identified at the CXCR7 locus distributed across seven of the EBNA-2 binding peaks identified by ChIP-sequencing analysis (Fig 6A and Table 1), including the CXCR7 MACS peaks 13 and 16 that were analysed in the ChIP experiment shown in Fig 6B and C. These data therefore indicate that a reduced ability of type 2 EBNA-2 to activate gene expression via this motif may be a key factor that leads to the reduced transforming ability of type 2 EBV.

DISCUSSION

The results in this paper indicate that EBNA-2 regulation of the differentially regulated cell promoters is likely to be similar to that of LMP-1 and involve regulation through a motif that includes a binding site for ETS family members such as PU.1 and IRF proteins. A combination of higher transactivation domain activity mediated by D442 in type 1 EBNA-2, the greater binding of type 1 EBNA-2 at specific gene regulatory elements and interaction with cell factors that may mediate increased binding is likely to result in the increased expression of these genes and superior growth of cells containing type 1 EBNA-2.

In future work we hope to identify the biochemical composition of the complexes that form at these EBNA-2 binding sites and also mutate the sites to demonstrate directly that they mediate the gene regulation by EBNA-2. Since acidic amino acids are frequently present in TADs (27), it is perhaps not surprising that D442 should increase TAD activity but a physiological phospho-mimetic mechanism for the S442D change seems unlikely because the serine in type 2 EBNA-2 does not lie in any known consensus phosphorylation site

protein sequence (28). At present it may seem surprising that an amino acid change in the transactivation domain can mediate gene specific effects and increase the level of EBNA-2 bound in the ChIP assays shown in Fig 6B and D. However, the domains of function of EBNA-2 at these sites may differ from those known from EBNA-2 interaction with RBP-Jk and additional protein interactions are likely to contribute to the levels of binding observed functions at promoters in the cell or viral chromosome.

Although PU.1 is able to bind the LMP-1 PU.1 site (7), there is no direct evidence that PU.1 (Spi-1) is the cell factor which mediates EBNA-2 binding at cell genes with similar motifs to that present in the LMP-1 promoter. It is possible that EBNA-2 interacts directly with PU.1 since a GST- type 1 EBNA-2 fragment (amino acids 310-376) binds to *in vitro* translated mouse PU.1 and was also able to deplete PU.1 from a human B cell extract (7) but we saw no requirement for the 310-376 region from type 1 EBNA-2 in our chimaera growth assays. The role of D442 in type 1 EBNA-2 would therefore not appear to be indirectly contributing to an interaction with PU.1. It is, however, possible that this residue contributes to the binding of type 1 EBNA-2 to another factor that can also activate gene expression via this site such as an IRF protein that also binds the composite element or another member of the ETS family.

Some protein interactions of EBNA-2 have already been mapped to the TAD region but these do not seem to be to the exact region on which we have focussed. Amino acids 448-471 were found to be sufficient to mediate interaction between type 1 EBNA-2 and the Tfb1/p62 subunit of TFIIH (29) and W458 of B95-8 type 1 EBNA-2 (equivalent to W454 of the W91 isolate (30)) was required for interaction with p300 and PCAF (31). More likely, the presence of the additional acidic amino acid in the TAD of type 1 EBNA-2 could contribute to

the characteristic relatively non-specific protein-protein interaction function of TADs and thereby stabilise the assembly of transcription complexes with coactivators such as histone acetyl transferases and the transcription machinery. The specific involvement of the EICE motif we have identified could reflect a particular need for the binding and assembly of a stable ETS/IRF complex at the LMP-1 and cell genes that are activated less well by type 2 EBNA-2 lacking this residue. PU.1 is known to be an important pioneer factor in opening up chromatin sites (32), so there may be specific features of the chromatin around these genes that would explain a higher dependency on the factors that associate with EICEs.

In this paper we have tested the function of EBNA-2 alleles in the context of type 2 EBNA-LP and EBNA-3 genes. We previously considered whether there could be a type-specific cooperation with EBNA-LP but could find no evidence for that (12). Since EBNA-2 and EBNA-3 family proteins are frequently found at the same chromosomal locations in BL ChIP-sequencing and can compete for binding sites (11), it remains to be determined whether type-specific EBNA-3 effects could also contribute to the virus phenotype. In the future it will be important also to test the S442D mutation in a recombinant EBV to determine whether it can reconstitute the process of B cell transformation but we have already shown the effects of the S442D change in two different assays in EREB2.5 cells and in Daudi cells, modulating expression of LMP-1 and CXCR7 as was seen with type-specific virus infection (12). It would also be interesting to mutate the EICE motifs we have identified in some of the cell gene promoters to test directly whether they mediate the differential regulation of gene expression by type 1 and type 2 EBNA-2 observed. This might be done using new CRISPR or TALEN techniques (33) but the fact that a large number of EBNA-2 binding sites have been identified at many of the target gene loci and the strong possibility of

405 redundancy in these elements would make this a lengthy and technically challenging
406 undertaking.

407 It is interesting that a single amino acid in EBNA-2 determines the growth maintenance
408 phenotype when there are so many amino acid differences between type 1 and type 2
409 EBNA-2. From an evolutionary point of view, once the DNA sequence is sufficiently different
410 to prevent homologous recombination, the variants tend to persist in the population so it is
411 not necessary for all the amino acid differences to be subject to selection to maintain the
412 characteristic type 1 and type 2 EBNA-2 sequences. The biological significance of type 1 and
413 type 2 EBV is still not understood; some possibilities are that their origin might relate to the
414 evolutionary history of EBV, to MHC selection or to the very high levels of other infectious
415 diseases prevalent in sub-Saharan Africa. There is some evidence that type 1 EBV is better at
416 transforming resting B cells than activated B cells (34) so it is possible that a difference in
417 EBNA-2 could allow persistence of type 2 in populations with a chronically-activated
418 immune system. Type 2 EBV has also very recently been reported to be able to infect T cells
419 (R. Rochford, personal communication) so some of the sequence differences may relate to
420 other phenotypes that are not apparent in the B cell system we have studied. However, the
421 difference in B cell growth transformation efficiency remains the clearest example of
422 functional variation in Epstein-Barr virus types.

423 In this paper we have shown that a combination of higher transactivation domain activity
424 mediated by D442, the greater binding of type 1 EBNA-2 at differentially regulated gene loci
425 and a potential influence on cell factors that interact with ETS-IRF composite elements that
426 may mediate the greater binding is likely to determine the increased expression of these
427 genes and superior growth of cells infected with viruses containing type 1 EBNA-2.

Acknowledgements

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Table 1

31 sites found by MEME in the EBNA-2 binding regions of genes differentially regulated by type 1 and type 2 EBNA-2 that contribute to the enriched consensus motif.

Gene (peak)[a]	Strand[b]	*p*-value[c]	Sites	
MARCKS (peak 1)	+	1.44E-07	TGGAGTCTCT	TTTCACTTCCTC TTGTCAAACC
MARCKS (peak 15)	+	6.66E-07	TCCTTCCCCT	TTTTTCTTCCTC TATCTACCTA
MARCKS (peak 14)	+	1.61E-06	GGCCTGCCTT	TCTTACTTTCTC AGCAATTTAA
LMP1[d]	-	2.35E-06	AACACACGCT	TTCTACTTCCCC TTTCTACGCT
MARCKS (peak 15)	+	2.35E-06	CGTTCTACTT	TTTCACTTCCTG TTTTGTTCCT
MARCKS (peak 6)	-	2.35E-06	CAATTTCAAA	TTCTACTTCCTT
FCRL2 (peak 6)	-	2.35E-06	AGTGGCACTA	TTCTACTTCCTT T
CXCR7 (peak 12)	-	1.17E-05	CATTCTCAGC	TTTCGCTTTCTT AAGAAAAGAA
MARCKS (peak 3)	+	1.61E-05	TCAGTTCTAT	TTCTGCTTTCTT AGTGTCTTCA
ADAMDEC1 (peak 1)[e]	-	1.75E-05	TGAGCCTATG	TCTCGCTTCCTG CTGGGGTTTA
HES1 (peak 2)	-	1.75E-05	GGGTGGGATC	TCATACTTCCTC CCTGAGCTAG
MARCKS (peak 16)	-	2.73E-05	TCTCAAACAG	TTTTATTTTCTG CTCATTGTAG
CCL3L3 (peak 1)	-	3.08E-05	GACAACAATA	TCTCACGTCCTC TCACTGTGGA
MARCKS (peak 15)	+	3.08E-05		TCCTCCTTCCCC TTTTTTCTTC
HES1 (peak 6)	-	3.57E-05		TTTTTCTTTCCG CTTTGGTCTT
FCRL2 (peak 5)	-	3.92E-05	CTTCATTCTC	TTTCTTTTCCTT TAGCAAGAGT
FCRL2 (peak 1)	-	4.31E-05	GCCCTGATTG	TTCTGCTTTCAC ATACACCTGC
MARCKS (peak 4)	-	4.68E-05	TCCATGTCCT	TTCAGCTTCCCC ACAAATGGCC
CXCR7 (peak 12)	-	5.76E-05	A	TTTTTCATTCTC AGCTTTCGCT
MARCKS (peak 3)	-	6.19E-05	AATAACTGTC	TCTACCTTTCTC GAATTGTCTA
CXCR7 (peak 1)	+	6.19E-05	TAACACCCCC	TCCCATTTCCCC CAGTAACGAA
CXCR7 (peak 12)	+	6.73E-05	ATTCGGGGTT	TTTTCTTTTCTT AAGAAAGCGA
CXCR7 (peak 4)	+	7.39E-05	ATTGGG	TTTGCCTTTCTC TAGTTCTCTA
CXCR7 (peak 16)	-	8.49E-05	GCTCTCATGG	TTCTATGTCCTC ACCATTCCA
CXCR7 (peak 12)	-	9.21E-05	CTTTCTCTAC	TTTCACTTCATT GTAAA
ZAP70 (peak 1)	-	1.16E-04	ACCAACACAG	TTTCATTTCCAG ATTTCTTTCC
CXCR7 (peak 13)	-	1.67E-04	AACCCACATC	TCTCTCTCCCCC GTGCCACCCC
FCRL2 (peak 7)	-	1.78E-04	GGTTCTTGCT	TTTTTCTTCTAC TACAACTGAT
MARCKS (peak 2)	+	1.90E-04	GTGTTTCATT	TCTAACATTCTC CTGACCTGAG
CXCR7 (peak 8)	+	2.52E-04	GGAAACAATG	TTATACTTCTCC TTTTCAG
CXCR7 (peak 7)	+	4.52E-04	CGATGCGATG	TTTTGCTCCCGT ACCAGAATTT

[a] EBNA-2 binding sites identified by ChIP-sequencing analysis in the Mutu III BL line with MACS <10-7 annotated by peak number across the gene locus.

[b] + indicates the motif is found in the DNA sequence provided and – indicates the motif is found in the reverse complement of the sequence.

[c] The *p*-value of a site is computed from the match score of the site with the position specific scoring matrix for the enriched motif.

[d] The LMP1 promoter sequence from +1 to -320 was also included in the sequence set.

[e] ADAMDEC1 peak 1 did not meet the MACS significance cut-off but was included based on ChIP-qPCR evidence of EBNA-2 binding.

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567

568 **Figure Legends**

569

570 **Figure 1. S442D in the EBNA-2 chimaera 6 background complements the deficiency of type**
571 **2 EBNA-2 in the EREB2.5 growth assay. (A)** Growth phenotype of EBNA-2 chimaeras in the
572 EREB2.5 growth assay from previous results (12) are shown for reference. The positions of
573 the RBP-Jκ association, RG, CR7, NLS and TAD domains of EBNA-2 are indicated. The protein
574 sequence of the TAD is shown below for type 1 and type 2 EBNA-2. The five mutations
575 indicated were made separately in the EBNA-2 chimaera C6. **(B)** Live cell counts for EREB2.5
576 growth assay with S442D EBNA-2 C6 mutant compared to chimaeras C6 and C7. An average
577 of data from at least 4 experiments, each with duplicate transfections is shown; error bars
578 indicate standard deviations. The other mutants tested SD431-432HN, EEP434-436PEA,
579 F438I and G460Y in the C6 EBNA-2 gave no cell growth in this assay (data not shown). **(C)**
580 Western blot of protein extracts from EREB2.5 cells transfected with T1, T2, C6, C7 and C6
581 mutant EBNA-2 proteins. Cells were harvested 5 days after transfection and proteins
582 extracted by RIPA lysis. Type 1 EBNA-2 migrates at 85 kDa, type 2 at 75 kDa. β-actin was
583 monitored as a loading control.

584 **Figure 2. S442D in type 2 EBNA-2 is sufficient to sustain LCL proliferation in the EREB2.5**
585 **assay. (A)** Cartoon similar to Fig 1A showing structures of T2 S442D and T1 D442S. **(B)** Cell
586 counts as in Fig 1 for EREB2.5 cell growth assay of indicated plasmids expressing wild-type

T1 (black), T2 (white), T1 D442S (light dots), T2 S442D (diagonal stripes), C6 S442D (grey) and empty vector (v, dark dots) 1-4 weeks after transfection. **(C)** Western blot analysis of protein extracts from EREB2.5 cells transfected with indicated EBNA-2 expression plasmids as described in Fig 1.

Figure 3. Transactivation domain reporter assay for EBNA-2 mutants. (A) Representation of transactivation domain assay. A firefly luciferase reporter gene can be activated by the GAL4 DNA binding domain (DBD)-EBNA-2 TAD fusion protein being tested**.** GAL4 DBD binds to GAL4 binding sites in the luciferase reporter plasmid promoter. **(B)** Transactivation domain assay of EBNA-2 TAD and mutants in BJAB cells assayed 24 hours after transfection. TAD and luciferase activity is given relative to the empty vector (GAL4) after normalizing for transfection efficiency with a co-transfected *Renilla* luciferase plasmid. Two independently derived T2 S442D TAD plasmids used in this assay are shown. Results are depicted as the mean ± standard deviations from 3 independent experiments. **(C)** Western blot of HEK 293 cells transfected with pcDNA3.1-GAL4 DBD plasmids expressing the different TAD fusions. Protein extracts were analysed by western blotting using the GAL4 DBD antibody and β-actin was used as a loading control.

Figure 4. CXCR7 mRNA is rapidly degraded upon EBV infection and is then re-induced by EBNA-2. (A) Primary B cells were infected with type 1 B95-8 EBV or with UV-inactivated B95-8 EBV (UV B95-8). Separate primary B cell samples were exposed to supernatant remaining after pelleting the EBV virus by ultracentrifugation (SUP) or were treated with TPA (TPA). RNA was extracted after 1 or 3 days, cDNA was prepared and qPCR was performed for CXCR7. CXCR7 mRNA level normalized to GAPDH is shown relative to the mock infection day 0 value. Error bars represent standard deviations from at least 3

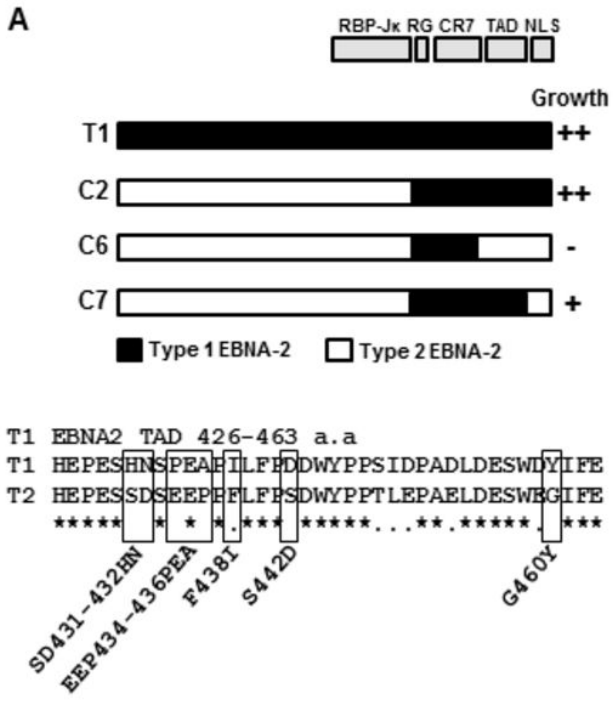
independent experiments. **(B)** Primary B cells were infected with B95-8 EBV or treated with TPA as in part A but analysed for CXCR7 mRNA after 4 or 8 hours. **(C)** Protein extracts from the experiment shown in part A were tested for EBNA-2 by immunoblotting (with β-actin as a loading control). We showed previously that EBNA-2 protein expression is first detected between 12 and 16 hours after infection (35).

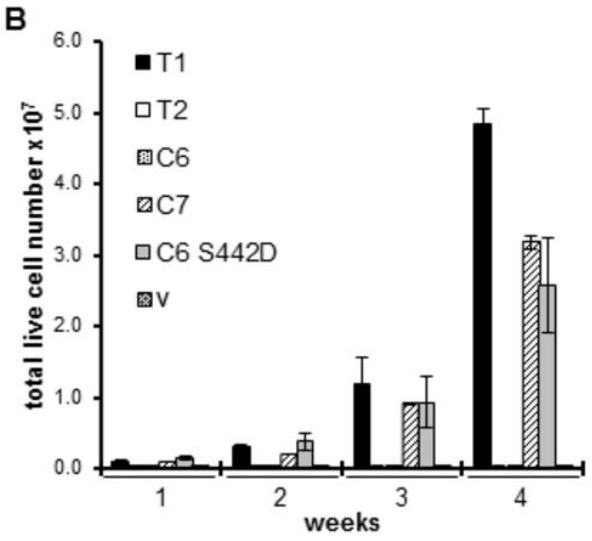
Figure 5. Differential induction of LMP-1 and CXCR7 in Daudi cells with inducible type 1, type 2 or type 2 S442D EBNA-2. (A) Daudi cell lines stably transfected with pHEBoMT plasmids expressing type 1 (T1), type 2 (T2) or type 2 S442D (T2 S442D) EBNA-2 were induced (+) with cadmium chloride ($CdCl_2$) for 24 hours or left uninduced (-). Protein samples were analysed by immunoblotting for EBNA-2, LMP-1 and β-actin (as a loading control). The EBNA-2 poly proline repeat region has been equalised in these EBNA-2 plasmids so the EBNA-2 proteins are all about 75 kDa in these cell lines. **(B)** The T1, T2 and T2 S442D cell lines were treated with $CdCl_2$ and total cell RNA was extracted after 12, 24 and 36 hours. RNA was converted to cDNA and analysed by qPCR to quantify CXCR7 mRNA levels. The histograms show the CXCR7/GAPDH mRNA ratio normalized to the T1 36 hour value set at 100. Error bars represent standard deviations from at least 3 experiments. **(C)** 5′ RACE was performed using EREB2.5 cells treated (+) or untreated (-) with β-estradiol in the culture medium. Water (H_2O) was used as a negative control for the PCR reaction in the experiment. The single RACE product migrated at about 300 bp on an agarose gel stained with ethidium bromide. **(D)** Immunoblotting control for ChIP experiment shown in Fig 6. Daudi cell lines were induced (+) with $CdCl_2$ for 24 hours or left uninduced (-). Nuclear extracts were prepared and EBNA-2 ChIP was performed using the PE2 antibody. An unrelated mouse monoclonal IgG antibody was used as a negative control in the ChIP. ChIP

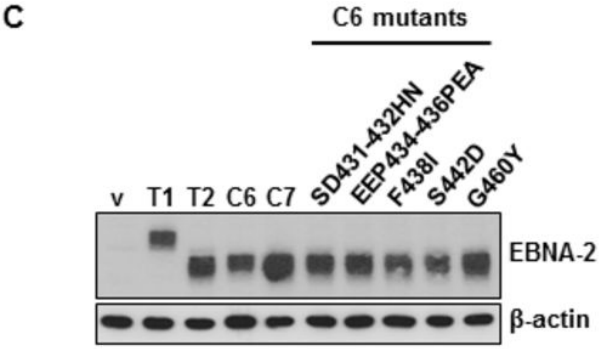
samples were then immunoblotted for EBNA-2 (PE2 antibody) to check for approximately equal precipitation of EBNA-2.

Figure 6. EBNA-2 ChIP binding at CXCR7, LMP-1 and CCL3 promoter elements. (A) ChIP-sequencing (ChIP-seq) data for type 1 EBNA-2 binding sites at the CXCR7 gene locus in an LCL (GM12878) and in the Mutu III BL cell line using methods described previously (11). The 5' RACE experiment showed the middle CXCR7 isoform (highlighted) to be the CXCR7 RNA induced by EBNA-2. ChIP-seq data showing EBNA-2 binding at the CXCR7 gene locus in the GM12878 LCL and in Mutu III BL cells. The y-axis displays sequence reads per million background subtracted reads. Significant peaks of EBNA-2 binding (MACS<10-7) in Mutu III cells are indicated by filled boxes (numbered as 1-17 from left to right). The MACS peak locations of the motifs identified by MEME-ChIP that contribute to the EICE consensus (Table 1) are marked with asterisks (*). **(B - E)** ChIP-qPCR was carried out on nuclei from cells shown in Fig 5D using primers specific to regions known to be bound and not bound by T1 EBNA-2 in ChIP-seq shown in part A. **(B)** CXCR7 peak 16, **(C)** CXCR7 peak 13, **(D)** LMP-1 and **(E)** CCL3 gene loci. qPCR values for EBNA-2 ChIP (black) and IgG control (white) are given, relative to ChIP input (%). Results are shown as the mean ± standard deviations from 3 independent ChIP experiments. p-values: n.s=not significant, **<0.01, ***<0.005.

Figure 7. The enriched motif generated by MEME-ChIP analysis of EBNA-2 binding sites at nine differentially regulated cell genes and the EBV LMP-1 promoter shown in standard form (left) and as the reverse complement (right). The overlapping PU.1 and IRF motifs that constitute an EICE are indicated. Details of the matching sites are shown in Table 1.







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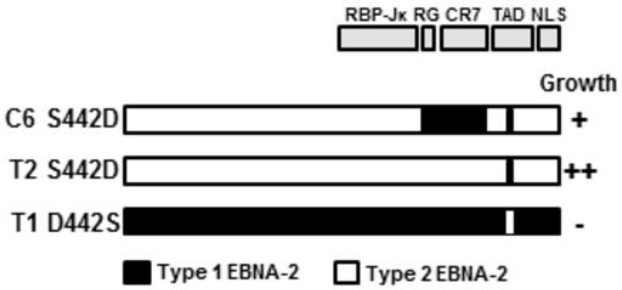

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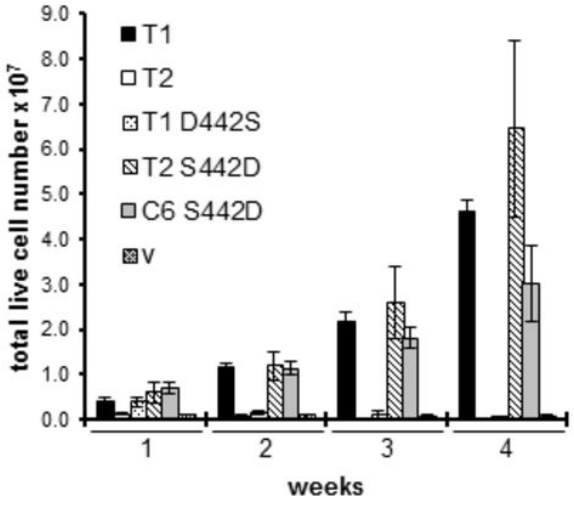

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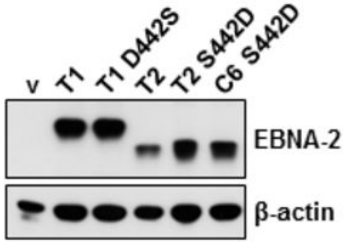

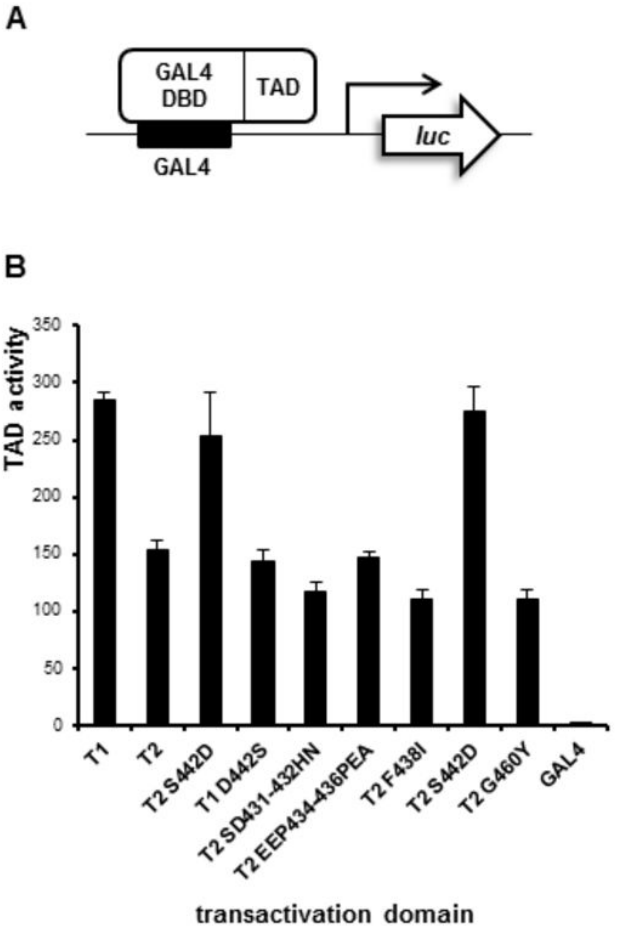

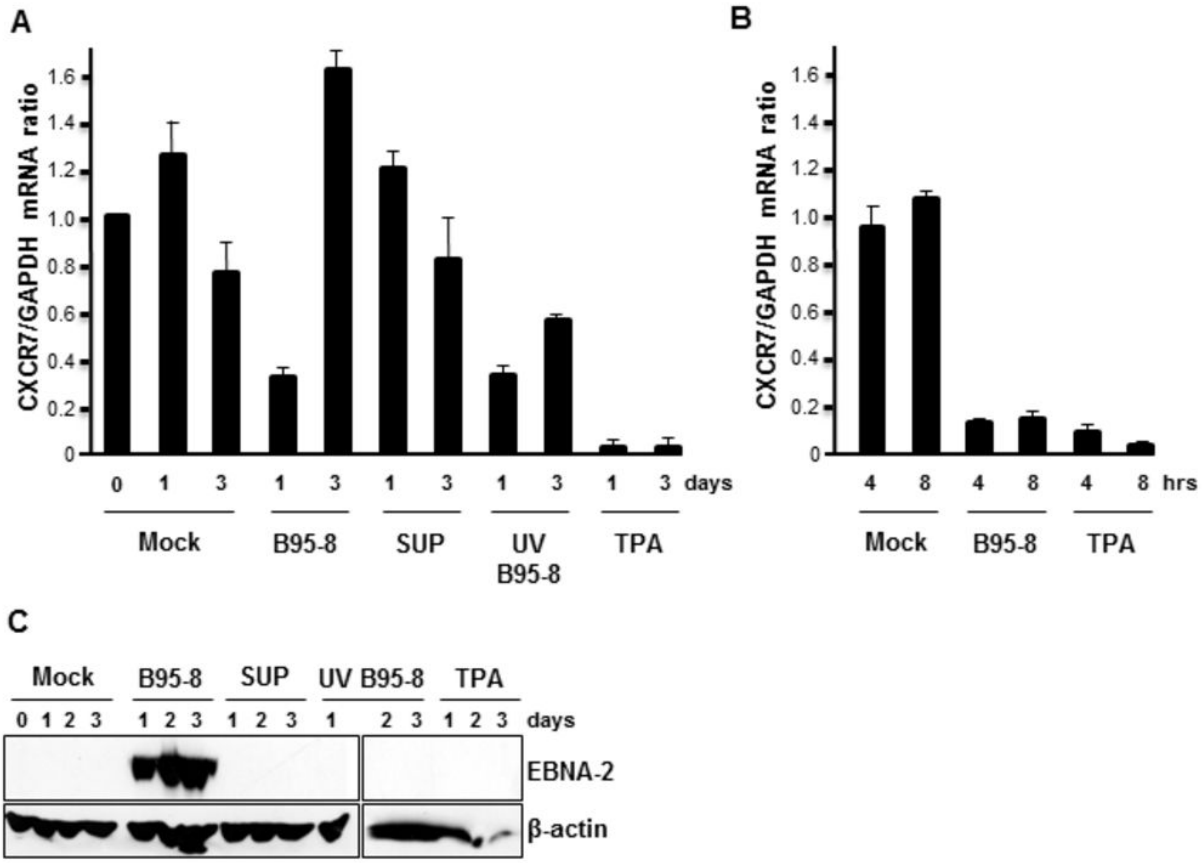

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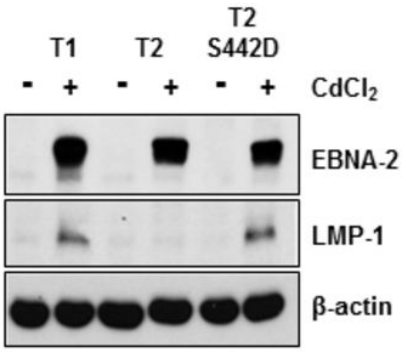
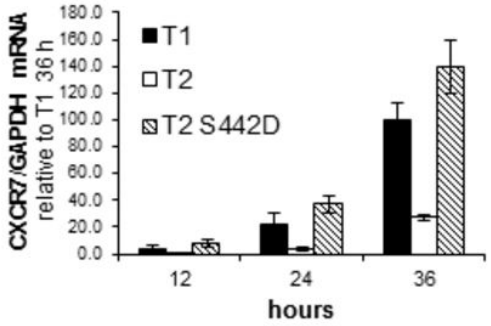


B



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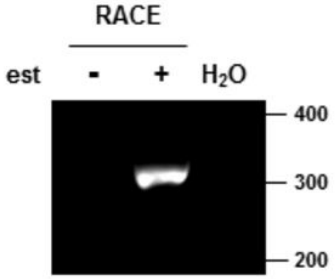

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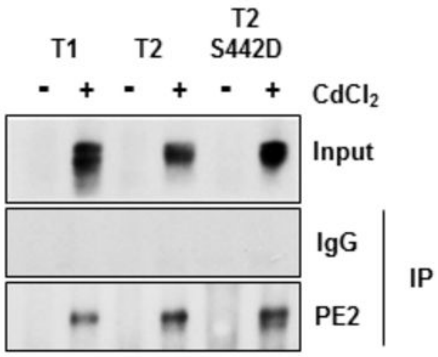

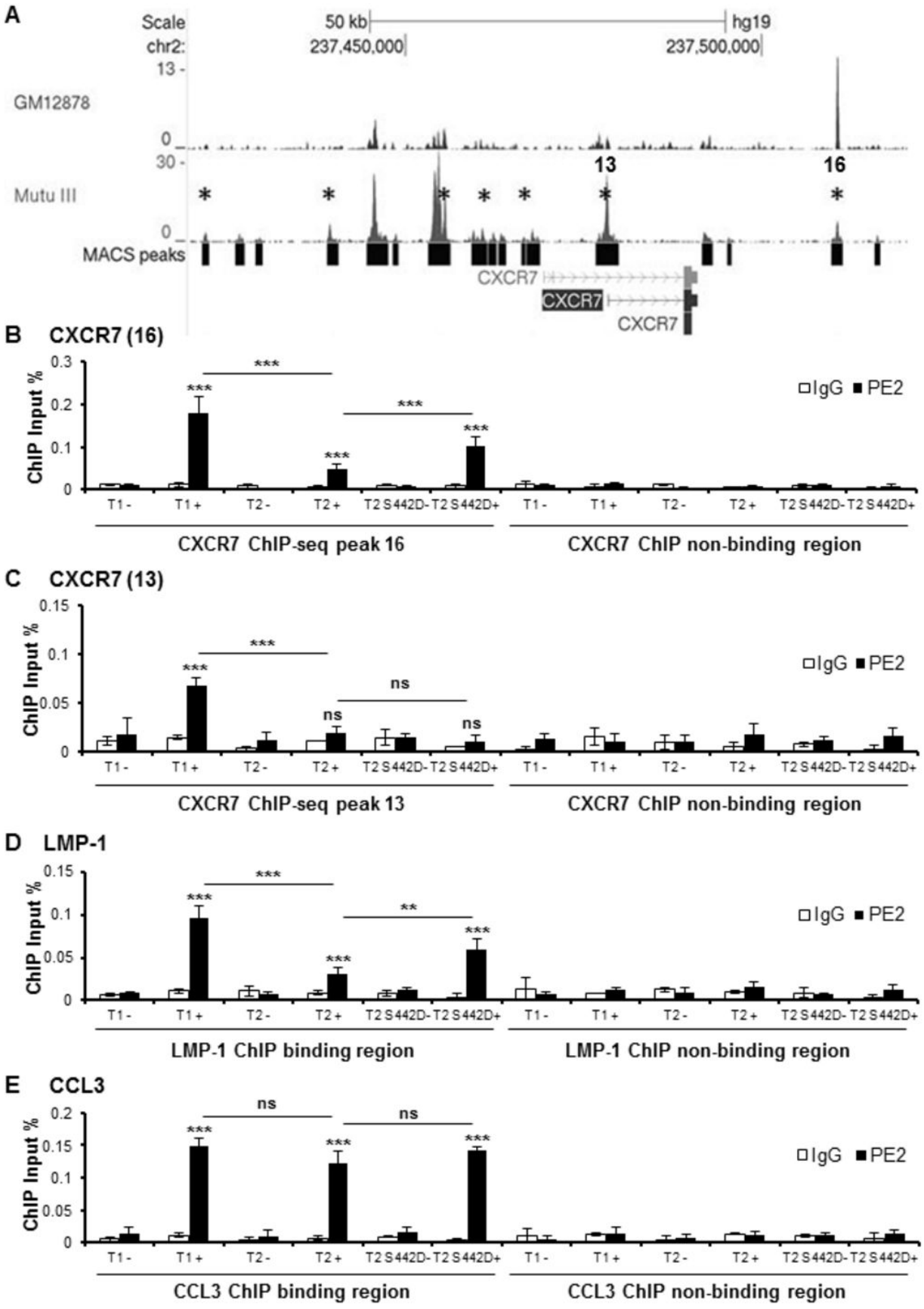

Enriched Motif (e-value 6.9e-003)

Reverse complement