Allele-specific repression of lymphotoxin- α by activated B cell factor-1

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Genetic variation at the human *LTA* locus, encoding lymphotoxin- α , is associated with susceptibility to myocardial infarction, asthma and other diseases. By detailed haplotypic analysis of the locus, we identified a single-nucleotide polymorphism (SNP) at *LTA*+80 as a main predictor of *LTA* protein production by human B cells. We found that activated B-cell factor-1 (ABF-1) binds to this site *in vitro* and suppresses reporter gene expression, but only in the presence of the *LTA*+80A allele. Using haplotype-specific chromatin immunoprecipitation, we confirmed that ABF-1 is preferentially recruited to the low-producer allele *in vivo*. These findings provide a molecular model of how *LTA* expression may be genetically regulated by allele-specific recruitment of the transcriptional repressor ABF-1.

Identifying human DNA polymorphisms that regulate gene expression is essential for understanding the genetic basis of common diseases but is technically challenging. Valuable insights into molecular mechanisms can be derived by *in vitro* analysis of protein-DNA binding and reporter gene expression, but we have limited tools to interrogate putative regulatory polymorphisms *in vivo*. Such analysis needs to consider natural chromatin structure and natural haplotypic combinations of polymorphisms. It also must be able to detect small differences in gene expression, which could be crucial for disease susceptibility if a physiologically important mediator is involved.

We attempted to address these issues for *LTA*, an important early mediator of immune and inflammatory responses¹. Regulation of *LTA* is finely calibrated to resist dangerous pathogens but not to cause inflammatory damage to the host, as seems to occur in bacterial sepsis² and

Figure 1 *LTA* protein production is associated with *LTA* haplotype. (a) Haplotype structure at the *LTA* locus. SNPs present at an allele frequency >0.1 in the panel of 26 LCLs are shown (minor allele frequencies given in brackets) together with haplotypes (haplotype frequencies shown at the head of the columns). Designations of SNPs refer to the nucleotide position relative to transcriptional start site. Clade A is uniquely defined by *LTA*+10A, *LTA*+252G and *LTA*+723A; clade B is uniquely defined by *LTA*+80A and *LTA*+368C; and clade C is uniquely defined by *LTA*+438(10) and *LTA*+495C. The frequencies of these main haplotype clades were 0.27, 0.46 and 0.27, respectively.

(b) Analysis of *LTA* production by haplotype pair. A scatter plot of *LTA* concentration in cell culture supernatant from the panel of LCLs is shown per 10^6 cells, plotted as a symbol representing each cell line together with the geometric mean (horizontal lines). A total of six replicate experiments were done. The cell lines heterozygous with respect to haplotypes A, B and C are shown categorized at a given time point according to the pair of haplotypes present in that cell line (A+B, A+C or B+C). At 24 h after stimulation, cells bearing haplotypes B+C or B+A were associated with significantly lower *LTA* concentrations than those bearing haplotypes A+C in a paired *t*-test (two-tailed; 226.7 versus 377.2, $P=0.001$ and 260.5 versus 377.2, $P=0.031$ for comparison of haplotypes B+C with A+C and of haplotypes B+A with A+C, respectively), whereas B+C versus B+A showed no difference (226.7 versus 260.5, $P=0.12$). Similarly, after 48 h, cells bearing haplotypes B+C or B+A were associated with significantly lower *LTA* concentrations than those bearing haplotypes A+C in a paired *t*-test (two-tailed; 371.3 versus 557.0, $P=0.001$ and 407.7 versus 557.0, $P=0.007$ for comparison of haplotypes B+C with A+C and of haplotypes B+A with A+C, respectively), whereas B+C versus B+A showed no difference (371.3 versus 407.7, $P=0.25$).

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cerebral malaria³. An intronic SNP at *LTA*+252 is associated with susceptibility to myocardial infarction⁴, asthma^{5,6}, bacterial sepsis^{7,8}, cancer^{9,10} and autoimmune diseases^{11,12}. Haplotypes bearing the *LTA*+252G allele show relatively high levels of reporter gene expression⁴ and *LTA* protein production by peripheral blood mononuclear cells in some^{13,14} but not all^{15,16} studies. An investigation of allele-specific quantification of transcripts and phosphorylated polymerase II loading was consistent with high *LTA* expression by haplotypes carrying the *LTA*+252G allele¹⁷. Inspection of the data suggests that the main effect did not reside with the *LTA*+252G allele itself, however, but rather in some other difference between the most common haplotype, which did not include the *LTA*+252G allele, and the haplotype containing this allele.

To investigate the relationship between genotypic variation at *LTA* and phenotype, we first determined the sequence diversity at the locus by cloning and sequencing the gene and its flanking regions in a panel of lymphoblastoid cell lines (LCLs). We previously reported an analysis of *LTA* haplotypes in five individuals who were heterozygous with respect to *LTA*+252 (ref. 17). We now sequenced 52 chromosomes derived from unrelated individuals of European descent. We found that 12 polymorphic sites were present at >10% allele frequency, including 11 SNPs and 1 tandem-repeat polymorphism. This variation resolved into three main haplotype clades spanning *LTA* (Fig. 1a).

We examined the same panel of 26 LCLs before and after stimulation with a mitogen (phorbol myristate acetate (PMA) plus iono-

mycin) that strongly induces *LTA* in this cellular model. In six replicate stimulation experiments, we determined *LTA* concentration in cell culture supernatants by ELISA 0, 24 and 48 h after stimulation and normalized this concentration to cell numbers at each time point. When we analyzed SNPs individually, *LTA*–294T, *LTA*–293A, *LTA*+10A, *LTA*+80A, *LTA*+252G, *LTA*+368C, *LTA*+723A, *TNF*–863A and *TNF*–308A all showed significant associations with *LTA* production after stimulation and, to a lesser extent, in the resting state (Supplementary Table 1 online). This does not mean that all these SNPs are functional, as the high linkage disequilibrium at *LTA* distributes associations across the locus.

As a starting point in dissecting functionality, we carried out step-wise linear regression analysis using either all SNPs with >10% allele frequency or the three main haplotype clades as predictive variables for induced *LTA* protein production (dependent variable). Analysis of individual SNPs identified *LTA*+368C and *LTA*+80A (which in this data set always occur together) as the only significant predictive variables of *LTA* protein production (regression coefficient at 48 h after mitogen stimulation (b) = 143.9 and standard error (s.e.) = 61, P = 0.027). These two SNPs uniquely define haplotype clade B, which was the only significant predictor when we entered the three haplotype clades as predictive variables of induced *LTA* production (at 24 h after mitogen stimulation, b = 129.6 and s.e. = 61.8, P = 0.048; at 48 h after mitogen stimulation, b = 176.8 and s.e. = 65.2, P = 0.013). Cells carrying haplotype clade B thus produce, on average, less *LTA* than other cells (Fig. 1b), consistent with our previous association of this clade with low polymerase II loading and transcript abundance¹⁷.

To explore possible molecular explanations, we investigated the haplotype-defining SNPs for effects on protein-DNA interactions *in vitro* using electrophoretic mobility shift assays (EMSAs). In addition to *LTA*+368C and *LTA*+80A, we studied *LTA*–293A, which defines a large subset of clade B. When we incubated nuclear extracts from unstimulated and mitogen-stimulated LCLs with oligonucleotide probes spanning these SNPs, we observed allele-specific binding with probes spanning *LTA*+80 (Fig. 2a). A slowly migrating inducible complex bound to the C allele but not the A allele (complex I), whereas two constitutive complexes bound only to the A allele (complexes II and III). Competition EMSA experiments showed the specificity of allelic binding for these complexes (Fig. 2b). In contrast, *LTA*–293 showed no allele-specific differences in protein-DNA binding, and *LTA*+368 showed a very slowly migrating constitutive complex that seemed to be specific for the C allele and was not investigated further (Fig. 2a).

Several lines of evidence suggested that the allele-specific effects of the polymorphism at *LTA*+80 might involve the basic helix-loop-helix

Figure 2 Protein-DNA binding modulated by SNPs found on the functional haplotype. (a) EMSAs using nuclear extracts prepared from unstimulated (open circle) or mitogen-stimulated (filled circle) LCLs incubated with radioactively labeled probes spanning the sites of each of the SNPs in the two allelic forms. In this and all subsequent EMSAs, the location of the wells is indicated by an asterisk. (b) Competition EMSAs using radioactively labeled probes with either C (lanes 1–8) or A (lanes 9–16) at the *LTA*+80 SNP in a binding reaction with nuclear extract prepared from unstimulated LCLs. Complex I was specifically competed by 10⁻⁶ molar excess of unlabeled *LTA*+80C competitor probe, whereas unlabeled *LTA*+80A probe competed only at 100⁻⁶ molar excess. Complexes II and III were abolished by 100⁻⁶ molar excess of *LTA*+80A, in contrast to unlabeled competitor *LTA*+80C, which competed only weakly for binding. Molar excess of unlabeled probe matching an unrelated consensus binding site for Oct-1 did not compete for binding by complexes I, II or III, in contrast to a number of faster migrating complexes, which seem to be non-specific.

Figure 3 *In vitro* recruitment of E-box binding proteins at *LTA*+80A.

(a) Competition EMSAs using either a radioactively labeled probe matching *LTA*+80A or a consensus E-box binding element (E5) with nuclear extract prepared from unstimulated LCLs in the presence of 10- and 100-molar excess of unlabeled competitor probes as shown. (b) Supershift EMSAs using radioactively labeled probe spanning *LTA*+80A with nuclear extract prepared from unstimulated LCLs incubated in the presence of antibodies as indicated. Unstimulated nuclear extracts were used in these experiments as complexes II and III were present constitutively.

(bHLH) family of transcription factors. First, the sequence spanning the SNP at +80 shows homology with the E2-box consensus motif bound by class A bHLH proteins (CAGCTG)¹⁸ with a 1-bp mismatch (underlined) in the presence of an A at *LTA*+80 (CAGCAG), or a 2-bp mismatch with a C at *LTA*+80 (CCGCAG). When a probe matching a classical E-box binding element from the immunoglobulin heavy-chain (IgH) intronic enhancer¹⁹, E5, was radioactively labeled and used in an EMSA with nuclear extracts from unstimulated LCLs, we observed two protein-DNA complexes that comigrated with complexes II and III observed with *LTA*+80A (Fig. 3a). The comigrating complexes binding to the radioactively labeled E5 probe were competed away by molar excess of unlabeled probe matching *LTA*+80A but only weakly by *LTA*+80C (Fig. 3a).

Antibody studies of the allele-specific complexes II and III identified on EMSA as binding to the sequence spanning *LTA*+80A provided preliminary evidence that at least two E-box proteins were present (Fig. 3b). Complex II was supershifted on EMSA using nuclear extracts from LCLs in the presence of antibodies to ABF-1. ABF-1 is a class II bHLH family member recently identified in LCLs that is specifically expressed in lymphoid tissue²⁰. We found no evidence of supershift with antibodies to the usual heterodimeric partner of ABF-1, the bHLH protein E47. Complex III was supershifted with antibodies to AP-4, a member of the bHLH-Zip subgroup of E-box binding proteins²¹. These data indicate that at least two E-box proteins show allele-specific recruitment to *LTA*+80A *in vitro* using crude nuclear extracts.

Figure 4 Differential binding of *in vitro* expressed E-box binding proteins. We analyzed total protein extracts from COS-7 cells transfected with either an empty vector (V) or with plasmids expressing ABF-1, E47 or AP-4 by EMSA. Control lanes show, in order, radioactively labeled probe alone and radioactively labeled probe incubated with protein extracts from nontransfected cells. (a) Protein extracts from COS-7 cells transfected with E47 and ABF-1 expression plasmids singly or together and incubated with a radioactively labeled probe for consensus E-box binding element, E5. (b) Protein extracts from COS-7 cells transfected with E47 and ABF-1 expression plasmids singly or together and incubated with radioactively labeled probe spanning *LTA*+80 with either C or A at the site of the SNP. (c) Protein extracts from COS-7 cells transfected with AP-4 expression plasmid analyzed for binding to radioactively labeled probes for consensus E-box binding element, E5, or probes spanning *LTA*+80 with either C or A at the site of the SNP.

To examine further allele-specific binding by candidate E-box binding proteins, we expressed ABF-1, E47 and AP-4 in COS-7 cells. We incubated protein extracts with radioactively labeled oligoduplexes corresponding to sites *LTA*+80C, *LTA*+80A and E5, and analyzed them using EMSAs (Fig. 4a,b). For ABF-1 expressed alone, we observed no binding to any of the probes, consistent with previous observations that ABF-1 normally binds as a heterodimer²⁰. In contrast, E47, which is capable of binding as a homodimer²², bound the consensus E5 probe and, more weakly, the *LTA*+80A probe; no bind-

Figure 5 Allele-specific modulation of gene expression by ABF-1 and E47. We transiently transfected COS-7 cells with pGL3.prom reporter gene alone or with pGL3.prom reporters containing a 20-bp DNA fragment matching the *LTA* 5' UTR (nucleotides +72 to +91) bearing C or A at the site of the *LTA*+80 SNP placed upstream of the SV40 promoter (designated pGL3.prom*LTA*+80C and pGL3.prom*LTA*+80A, respectively). We transfected increasing amounts of empty expression vector or expression vectors for ABF-1 and E47 with the reporter constructs, including pBSK as required to keep the total amount of plasmid transfected constant (0.5 μ g). The mean $\pm$ s.e.m. of five independent transient transfection experiments are shown together with paired *t*-tests comparing constructs pGL3.prom*LTA*+80A and pGL3.prom*LTA*+80C (NS, $P > 0.05$; * $P = 0.02$; ** $P = 0.005$; *** $P = 0.003$). Results are shown for pGL3.prom*LTA* constructs relative to pGL3.prom using values derived from luciferase activity normalized to *Renilla* and expressed relative to empty expression plasmid. When we transfected E47 expression construct in the absence of ABF-1, we found no difference between levels of activity of the two *LTA* constructs using 0.0625, 0.125 or 0.25 μ g of E47 expression plasmid. There was, however, a small but significant difference between *LTA* constructs using 0.5 μ g of E47 expression plasmid ($P = 0.043$ in a paired *t*-test of five independent transfection experiments): mean luciferase activity of pGL3.prom*LTA*+80A relative to pGL3.prom was 1.27 (s.d. 0.16) compared with 1.00 (s.d. 0.28) for pGL3.prom*LTA*+80C relative to pGL3.prom. For pGL3.prom transfected alone into 4×10^5 COS-7 cells, mean luminometer values of 2,398 light units were obtained.

ing was seen for *LTA*+80C. When we coexpressed ABF-1 with E47, we observed binding to E5 and *LTA*+80A but no binding to the *LTA*+80C allele probe. The complex was supershifted in the presence of antibody to ABF-1 but not E47. The specificity of the antibody to ABF-1 was resolved by western blotting (see **Supplementary Fig. 1** online). AP-4 bound strongly to both the E5 and *LTA*+80A allele probes but only very weakly to the *LTA*+80C allele probes (**Fig. 4c**). These data indicate that the sequences spanning *LTA*+80 show allele-specific differences in their ability to bind E-box proteins *in vitro*.

We then investigated whether the allele-specific recruitment of E-box binding proteins observed *in vitro* modulated levels of gene expression. We carried out transient transfection assays in which these proteins were overexpressed in the presence of constructs bearing the *LTA*+80 SNP. We engineered reporter gene constructs in which 20-bp DNA fragments of the *LTA* 5' untranslated region (UTR) corresponding to each of the two allelic variants of the *LTA*+80 SNP were placed upstream of an SV40 promoter in a pGL3 luciferase reporter. We then transiently transfected these constructs into COS-7 cells. When

cotransfected with ABF-1 and E47, the constructs bearing *LTA*+80A relative to the SV40 promoter alone had significantly lower reporter gene activity than did the *LTA*+80C constructs (**Fig. 5**). We observed a dose-dependent effect, with increasing repression when we cotransfected increasing amounts of ABF-1/E47 expression plasmids (**Fig. 5**). We found no significant difference between *LTA* reporter gene plasmids ($P > 0.05$) at any amount of AP-4 expression plasmid cotransfected (0.0625–0.5 μ g). These data provided evidence that ABF-1 has the capacity to modulate reporter gene expression in an allele-specific manner, in contrast to AP-4, which showed higher DNA binding affinity in the presence of the *LTA*+80A allele but no difference in expression after cotransfection.

The functional data from assays of protein-DNA binding demonstrated the capacity *in vitro* for E-box binding proteins ABF-1 and AP-4 to bind in an allele-specific manner, modulated by the *LTA*+80 polymorphism. To seek evidence that these E-box binding proteins were recruited *in vivo*, we investigated binding of ABF-1 and AP-4 at the *LTA* promoter or 5' UTR by chromatin immunoprecipitation (ChIP) using a

Figure 6 Allele-specific occupancy by ABF-1 *in vivo*. (a) We analyzed recruitment of ABF-1 and AP-4 at the *LTA* promoter and 5' UTR (nucleotides +171 to +46) by ChIP using a LCL stimulated with ionomycin and PMA (lanes 1–6) for the times shown. Input shows the products of PCR reactions containing 0.5% of total amount of chromatin used in immunoprecipitation reactions. Mock immunoprecipitation reactions using irrelevant control antibodies (against T antigen pAb101) together with immunoprecipitations done using antibodies specific for ABF-1 or AP-4 are shown. (b) Allele-specific loading of ABF-1 analyzed for *LTA*+80 SNP by PE-MS. We stimulated four cell lines heterozygous with respect to *LTA*+80 SNP with ionomycin and PMA. The overall geometric mean ratios ($\pm$ 95% c.i.) of ABF-1 loading between the C and A alleles are shown.

LCL heterozygous with respect to *LTA*+80 (Fig. 6a). ABF-1 and AP-4 were recruited to the region, with AP-4 present constitutively but not after mitogen stimulation. We then carried out haplotype-specific ChIP (haploChIP) using the *LTA*+80 polymorphism as a marker to analyze allele-specific occupancy by ABF-1 and AP-4 in each of four B cell lines heterozygous with respect to *LTA*+80. We previously showed that accurate quantification of relative allelic abundance can be assayed by this method using primer extension–mass spectrometry (PE-MS)¹⁷. We generated standard curves for the *LTA*+80 polymorphism by analyzing differing known input ratios of plasmid DNA for the two allelic forms from 1:4 to 4:1. This analysis showed a strong linear relationship ($r^2 = 0.96$) with the ratios observed by PE-MS. We then analyzed allele-specific occupancy by ABF-1 using haploChIP. This analysis showed progressively more binding by ABF-1 to the *LTA*+80A allele over time after mitogen stimulation (one-way analysis of variance test for linear trend over time $P = 0.004$, $r^2 = 0.349$; Fig. 6b). In contrast, the constitutive binding that we observed for AP-4 showed no significant allelic difference, with a mean ratio *LTA*+80C:*LTA*+80A of 1.08 (95% confidence interval (c.i.) = 0.56–1.25).

Phenotypic variation in *LTA* production has previously been correlated with the *LTA*+252 polymorphism^{4,13,14,16}. Here we identified nine different polymorphisms that correlate with *LTA* protein production. When *LTA* sequence variation is analyzed in detail using multivariate statistics, this complex pattern of association can be reduced to the effects of a specific low-producing haplotype clade. This commonly occurring haplotype is uniquely defined by two markers, one of which, *LTA*+80, was found *in vitro* to modulate allele-specific binding of two E-box binding proteins, ABF-1 and AP-4, in the presence of the *LTA*+80A allele. ABF-1 is a recently described transcription factor that has been particularly associated with lymphoid tissue^{20,23}, raising the possibility that the functional effects of the *LTA*+80 polymorphism are tissue-specific. ABF-1 repressed transcription in our reporter gene model when the *LTA*+80A allele was present, consistent with previous reports that it acts as a transcriptional repressor^{20,23}, whereas AP-4 had no effect on allele-specific expression.

The haploChIP method provides a powerful new tool for investigating whether regulatory polymorphisms alter specific DNA–protein interactions *in vivo* (i.e., to investigate polymorphisms in their natural chromatin structure in a living cell). The method was initially developed to assess the effects of allelic variation on polymerase II binding, as a surrogate marker of gene expression¹⁷. Here we show how it may be used to acquire specific information about the mode of action of a putative regulatory polymorphism. When we tested the hypothesis generated from our *in vitro* data that the *LTA*+80 polymorphism modulates binding of ABF-1 and AP-4, we found allele-specific differences in binding *in vivo* by haploChIP for ABF-1 whereas AP-4 showed constitutive binding that was unaffected by the polymorphism. The AP-4 signal on chromatin immunoprecipitation may emanate from a non-polymorphic binding site in the same vicinity, such as the E-box motif that is located 317–312 nucleotides upstream of the transcriptional start site, which would be present on the same amplified DNA fragments after sonication as these are typically 1 kb in length.

Can the functional effects of the *LTA*+80A allele explain the reported epidemiological associations between polymorphisms in the *LTA* region and susceptibility to common diseases^{4–12}? Our analysis of haplotype structure in 52 chromosomes of European origin confirmed high linkage disequilibrium across the 10-kb region. The *LTA*+80A allele was present on 70% of chromosomes analyzed. The *LTA*+252G allele was not present on any of the chromosomes that carried the *LTA*+80A allele but was present on 57% of chromosomes that did not. Thus, the significant association that we observed between the

LTA+252G allele and high *LTA* protein production could entirely be explained, when examined by multivariate analysis, by the ability of the *LTA*+80A allele to suppress *LTA* production. Analysis of larger data sets or different experimental models may identify other functional variants at the *LTA* locus⁴, but based on these data, we can at least state that *LTA*+80A is a good functional candidate to explain the reported disease associations of the *LTA*+252G allele, and this needs to be addressed in future epidemiological studies.

METHODS

Plasmids and haplotype construction. To generate the pGL3.prom*LTA*+80C and pGL3.prom*LTA*+80A constructs, we designed oligonucleotides spanning the *LTA*+80 site (sequences are available on request), which we annealed and cloned into a *KpnI*–*BglII*-digested dephosphorylated pGL3-promoter vector (Promega) upstream of the SV40 promoter. We verified all constructs by DNA sequencing. Expression plasmids used were pHBA.FLAG-ABF-1 (ref. 20), pHBA-E47 (ref. 20), pHBApR-1-neo²⁴ and pcDNA3-AP-4MD²⁵. To generate the haplotypic data for the panel of LCLs, we carried out long-range PCR from genomic DNA, cloning and sequencing as previously described¹⁷ and used polyphred alignment to create haplotypes from each hemizygous clone. Contig locations (GenBank accession number Y14768) for the SNPs described were as follows: *LTA*–294, 47622; *LTA*–293, 47621; *LTA*+10, 47318; *LTA*+80, 47248; *LTA*+252, 47076; *LTA*+368, 46960; *LTA*+438, 46890; *LTA*+495, 46833; *LTA*+723, 46605; *TNF*–1031, 45081; *TNF*–863, 44913; *TNF*–308, 44358.

Nuclear extracts, EMSAs, western blotting and antibodies. We annealed oligonucleotide probes (sequences are available on request) and used Klenow to radioactively label the resulting oligoduplexes *LTA*+80C, *LTA*+80A, *LTA*+368G, *LTA*+368C, *LTA*–293G, *LTA*–293A, Oct-1 and E5 with [α -³²P]dCTP (Amersham Pharmacia Biotech). We prepared nuclear extracts from 20–10⁶ LCLs as previously described^{26,27}. We mixed 2 μ g of nuclear extract with 0.2–0.5 ng of labeled probe (1–5 $\times 10^4$ c.p.m.) in a 10- μ l reaction containing 12 mM HEPES buffer (pH 7.8), 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 12% glycerol and 0.5 μ g poly(dA-dT) (Amersham Pharmacia Biotech) and incubated it at room temperature for 20 min. We analyzed the reaction by electrophoresis in a nondenaturing polyacrylamide gel at 4 °C in 0.5 TBE buffer. For supershift analysis, we preincubated nuclear extract with appropriate antiserum at room temperature for 20 min before adding the radioactively labeled probe. We used the following antibodies for supershift studies: ABF-1 (sc9555) and Oct-1 (sc232; Santa Cruz); human monoclonal antibody to E47/E12 (554199; BD Biosciences); AP4 (ref. 28). We transfected COS-7 cells with constructs expressing pHBA.FLAG-ABF-1, pHBA-E47, pHBApR-1-neo, pcDNA3-AP-4MD and pcDNA3 and then prepared total protein extracts by lysing cells in lysis buffer (20 mM Tris-HCl (pH 8), 300 mM NaCl, 0.1% Nonidet P-40, 10% glycerol) supplemented with Complete protease inhibitors (Boehringer Mannheim). We carried out western blotting for total protein in COS-7 cell extracts by fractionation on a 4–12% NuPage Bis-Tris polyacrylamide gel (Invitrogen) in 1 MOPS buffer, followed by transfer to a PVDF membrane (Invitrogen) and incubation with primary antibody in 4% dried milk powder with 0.1% Tween in phosphate-buffered saline. We used primary antibodies to ABF-1 (sc9555) at 1:500 dilution and to β -tubulin 1 (T-7816; Sigma) at 1:1,000,000 dilution. We visualized bands by chemiluminescence with horseradish peroxidase-conjugated rabbit antibody to goat and goat antibody to mouse, respectively, using ECL Plus (Amersham).

Cell culture, transfections and luciferase assay. We obtained EBV-transformed B cell lines and cultured them as described previously¹⁷. We stimulated cells with 200 nM PMA (Sigma) and 125 nM ionomycin (Sigma) unless otherwise stated. We assayed *LTA* concentration in cell culture supernatants in duplicate using an *LTA* ELISA kit (AMS Biotechnology) according to the manufacturer's instructions. We analyzed data by linear regression using SPSS software. We cultured COS-7 cells in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 100 U ml^{–1} penicillin, 100 mg ml^{–1} streptomycin, 0.2 mM L-glutamine at 37 °C in 5% humidified CO₂. We used Polyfect (Qiagen) for transient transfection of expression constructs, with and without reporter gene constructs, into COS-7 cells following the manufacturer's protocol. We transfected 4 $\times 10^5$

COS-7 cells with 1.5 μ g of total DNA (0.5 μ g of expression plasmid or pBSK, 0.167 μ g of pGL3 reporter and 0.833 μ g of pRL.TK) and collected them after 48 h. We cotransfected pRL.TK to normalize for transfection efficiency. We kept the total amount of DNA used in each transfection experiment constant by addition of pBSK. We lysed cells in 500 μ l of 1 lysis buffer (Promega) and analyzed 10 μ l using the Dual-Luciferase reporter assay kit (Promega) and a Turner luminometer model 20 (Promega) following the manufacturer's protocol.

Chromatin immunoprecipitation. We prepared chromatin from 5 $\times 10^8$ cells, sonicated it and purified it on a cesium-chloride gradient as previously described¹⁷. We incubated Dynabeads M-280 precoated with appropriate sheep antibodies to mouse or to rabbit IgG overnight with primary antibodies at 4 °C in phosphate-buffered saline containing 5 mg ml⁻¹ bovine serum albumin (Sigma). To generate magnetic beads coated with antibody to goat, we incubated Dynabeads M-280 with biotinylated antibodies to goat IgG (227-AB-2G2; AMS Biotechnology) for 30 min at room temperature and then incubated washed beads with primary antibody as described. We used specific antibodies against ABF-1 and AP-4 as described for supershift analysis and also did mock antibody controls using antibody to actin (A2066; Sigma). After washing to remove unbound antibody, we incubated antibody beads overnight at 4 °C with 50 μ g of chromatin in 1 RIPA buffer²⁹ containing 1 complete protease inhibitors (Roche) and 5 μ g ml⁻¹ pepstatin (Roche) on a nutator. We washed bead-chromatin immunoprecipitations in 1 RIPA buffer containing 500 mM LiCl (Sigma) eight times with a final wash in Tris-EDTA buffer. We resuspended beads in 120 μ l of Tris-EDTA digestion buffer containing 1% SDS, incubated them at 65 °C for 10 min, pelleted beads by centrifugation and incubated the supernatant at 65 °C overnight to reverse cross-links. We added proteinase K to 200 μ g ml⁻¹ final concentration, incubated at 37 °C for 2 h, extracted the DNA using phenol and chloroform and precipitated it with ethanol in the presence of 20 μ g of glycogen. We resuspended pellets in 30 μ l of Tris-EDTA containing 10 μ g of RNase A, incubated them at 37 °C for 1 h, purified the reactions by spin column and assayed them by semiquantitative PCR³⁰. We amplified the *LTA* region between -46 and +171 relative to the transcriptional start site (primer sequences available on request) with PCR cycling parameters of 96 °C for 1 min then 5 cycles of 96 °C for 35 s, 68 °C for 45 s, 72 °C for 35 s; then 20 cycles of 96 °C for 35 s, 64 °C for 45 s, 72 °C for 40 s; and then a final 6 cycles of 96 °C for 35 s, 56 °C for 60 s, 72 °C for 90 s. We carried out haploChIP analysis using PE-MS as previously described¹⁷. We carried out first-round amplification with primers and cycling parameters as described for semiquantitative PCR except we used 27 cycles rather than 20 cycles annealing at 64 °C. We carried out primer extension for *LTA*+80 (extension primer sequence available on request) using MassEXTEND (Sequenom) as described previously¹⁷. We immunoprecipitated each cell line twice and analyzed the products of immunoprecipitation by four independent PCR reactions, which were in turn each spotted in quadruplicate on the chip before PE-MS. As we have observed previously for certain SNPs¹⁷, we found a systematic allelic difference in PE-MS signal intensity for *LTA*+80 when we compared observed ratios with a range of known input ratios (from 1:4 to 4:1). ChIP sample ratios given were normalized by the input ratios.

Note: Supplementary information is available on the Nature Genetics website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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