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17. For comparison, k_{cat}/k_{uncat} for EcCM under these conditions is 3×10^6 [calculated from the activation parameters reported in C. C. Galopin, S. Zheng, D. B. Wilson, B. Ganem, *Tetrahedron Lett.* **37**, 8675 (1996); *ibid.* **38**, 1467 (1997)]. The efficiency of MjCM at 85°C, the optimal growth temperature for *M. jannaschii*, actually decreases somewhat ($k_{cat}/k_{uncat} = 9 \times 10^4$) because of the steeper temperature dependence of the uncatalyzed reaction.
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20. Libraries were constructed in plasmid pKMCMT-W, which features the chorismate mutase gene (*aroQ*$_f$) from *M. jannaschii* under control of the *trc* promoter. Duplication of Leu^{20}-Lys^{21}, introduction of a Hind III site, and mutation of Leu^{20a} to Glu and Ile^{77} to Arg was accomplished by use of the polymerase chain reaction (PCR) with template pKMCMT-W and primers MNEG-S (5′-ATAAGATACTTAAGGAAAAGCTTATTGCTGAAAGAAATAGTTTAGCT-3′) and MNEG-N (5′-CTTTTGGAGGGCCTTATTATGCTCTATAAGACGTTGAAATATTTTAATGCCAAT-3′). Cloning of this product into pKMCMT-W with restriction enzymes Afl II and Eco 0109I gave pKMCMT-NEG. Six random codons were then introduced into the modified *aroQ*$_f$ using the PCR with template pKMCMT-W and primers SEQT-S (5′-GTGAGCGGATAACAATTTCACA-3′) and MJMA-N (5′-TCAGCAATAAGCTTTTCSNNSNNSNNSNNSNNSNNCTTAAGGATCTTATTGTCAATCTCATCAATCT-3′) (N = A, C, G, or T; S = C or G). Cloning of this library of products into pKMCMT-NEG with restriction enzymes Nde I and Hind III gave library pKMCMT-LibA.
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24. Sedimentation equilibrium runs were performed from 3000 to 17,000 revolutions per minute with 2, 10, and 30 μM protein concentrations in phosphate-buffered saline (PBS) at 20°C. Partial specific volumes and solvent densities were calculated with the program Sednterp [T. M. Laue, B. D. Shah, T. M. Ridgeway, S. L. Pelletier, in *Analytical Ultracentrifugation in Biochemistry and Polymer Science*, S. E. Harding, A. J. Rowe, J. C. Horton, Eds. (Royal Society of Chemistry, Cambridge, England, 1992), pp. 90–125] and data were analyzed by nonlinear least-squares fitting with the Origin software provided by Beckman.
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29. Reaction kinetics were measured with 50 nM enzyme at 20°C in PBS, supplemented with bovine serum albumin (0.1 mg/ml). Inclusion of bovine serum albumin did not alter the aggregation state of the protein as verified by analytical size-exclusion column chromatography. Initial rates were determined by monitoring the disappearance of chorismate spectrophotometrically at 274 nm ($\varepsilon_{274} = 2630\ M^{-1}\ cm^{-1}$). Steady-state kinetic parameters k_{cat} and K_m were calculated from the initial rates.
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31. Removing six amino acids from the carboxyl terminus of MjCM increased its resistance to proteolytic cleavage without affecting the properties of the protein. Consequently, all detailed biochemical and biophysical characterizations of mMjCM were also carried out on a truncated derivative, which behaved identically to the full-length mMjCM in all aspects tested.

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Dependence of BSAP Repressor and Activator Functions on BSAP Concentration

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During a B cell immune response, the transcription factor BSAP maintains its activator functions but is relieved of its repressor functions. This selective targeting of BSAP activities was shown to be regulated by a concentration-dependent mechanism whereby activator motifs for BSAP had a 20-fold higher binding affinity than repressor motifs. An exchange of activator and repressor motifs, however, showed that the context of the motif, rather than the affinity, determined whether BSAP operated as an activator or repressor.

BSAP, the transcription factor encoded by the *Pax5* gene, is a key regulator of B lymphocyte development (*1*). Its critical role in early B cell lymphopoiesis has been established by targeted disruption of the mouse *Pax5* gene (*2*). B cells from the mutant mice are blocked at the pro-B stage and do not develop further. BSAP also participates in the later antigen-driven stages of B cell differentiation. Analysis of the changes in gene expression induced by antigen and cytokine signals has shown that BSAP positively regulates the expression of the CD19 component of the B cell antigen coreceptor and germ-line transcription of the ε gene (Iε), which is a prerequisite for a switch to immunoglobulin E (IgE) synthesis (*3*, *4*). BSAP can also serve as a negative regulator during development. During an immune response, BSAP blocks are removed from the heavy chain 3′ enhancer to allow isotype switching and from the J chain gene promoter to allow synthesis and secretion of pentamer IgM antibody (*5*–*7*).

Because the positive functions of BSAP are maintained at the same time that its negative functions are relieved, we investigated how these different regulatory activities are accomplished. BSAP concentrations are high in pre-B, immature B, and mature B cells; once an antigen signal is delivered, however, the concentrations progressively decrease from the presecretor to the mature plasma cell stage, in which BSAP is almost undetectable (*7*, *8*). The decrease in BSAP expression is induced by signals from interleukin-2 (IL-2) or IL-5, which can down-regulate BSAP RNA expression to 20 to 25% of untreated cells (*7*, *9*). Overexpression of BSAP in plasma cell lines increases BSAP repressor activity: J-chain RNA and secreted IgA decrease (*7*, *10*). This expression pattern suggested that BSAP activities might be selectively targeted during antigen-driven differentiation by competition among the BSAP sites for the diminishing amounts of factor (*7*, *11*).

We obtained experimental evidence for a concentration-dependent regulation by determining the relative affinities of BSAP activator and repressor elements from gel-shift assays (Fig. 1). BSAP binding to a consensus recognition sequence (*12*) was competed by adding increasing amounts of the BSAP recognition motifs with estab-

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lished functions such as the CD19, Iε, and J chain gene promoters and the 3′ α enhancer (*13*). The BSAP sites with positive regulatory activity had 20 times the affinity for the factor as those sites with repressor activity (Fig. 1, A and B). A fivefold excess of the CD19 or Iε oligonucleotide blocked BSAP binding to the probe by 80 to 90%, whereas a 100-fold molar excess of the J chain or 3′ α-enhancer oligonucleotides was required to achieve the same degree of binding inhibition (Fig. 1C). This difference in affinity indicated that the activator sites could out-compete the repressor sites when the supply of BSAP was limiting.

We obtained further evidence for a concentration-dependent mechanism by examining the effects of BSAP overexpression on both its repressor and activator functions. For the repressor experiments, representative plasma cell lines were stably transfected with BSAP expression vector or vector alone, and G418-resistant clones were selected for analysis (*14*). The amounts of BSAP were determined in protein immunoblots of nuclear extracts, and the amount of heavy chain and J chain in protein immunoblots of cytoplasmic extracts. Because light chain synthesis was unaffected by changes in BSAP expression, the light chain content of the cytoplasmic samples was used to normalize the densitometry data for differences in sample loading.

A concentration-dependent regulation predicts that BSAP repressor activities can be restored by increasing BSAP concentrations in plasma cells. This prediction was borne out by protein immunoblots of heavy chain isotypes regulated by the 3′ heavy chain enhancer. Of four transfected clones of a MPC11 line producing a γ2b heavy chain, the two that expressed moderate BSAP concentrations reduced γ2b content by one-half to two-thirds (Fig. 2A). In three clones of an α-producing MOPC315 cell line that expressed moderate amounts of BSAP, the α–heavy chain content was reduced by one-half to one-quarter (Fig. 2B). Similarly, J chain expression was depressed in all clones expressing transfected BSAP, and the extent of suppression roughly correlated with BSAP concentration. In S194 clones, for example, relatively low BSAP concentrations decreased J chain content by 15 to 45%, whereas high amounts decreased J chain content by 85 to 90% (Fig. 2C).

We examined the effect of BSAP overexpression on its activator functions by stably transfecting the BSAP expression vector and vector control into a CH12LX subline that expresses IgM (*15*). Unlike plasma cells, CH12LX-IgM cells synthesize CD19, express low amounts of J chain but do not secrete significant amounts of pentamer IgM, and have not undergone isotype switching. The phenotype of such CH12 cells is consistent with their BSAP content, which has been reduced by the partial activation to 30% of that present in lines representative of mature, unactivated B cells (*7*). RNA blots were used to assay the results of the transfections (*16*) because they allowed the amounts of endogenous and transfected BSAP RNA to be readily distinguished: a 9- to 10-kb endogenous RNA versus a 1.2-kb transcript of the transfected BSAP cDNA (Fig. 3).

A concentration-dependent control mechanism would predict that expression of

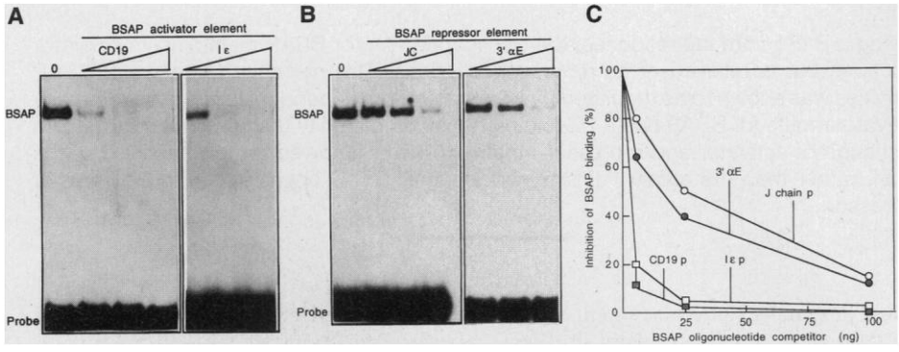

Fig. 1. Affinity of BSAP binding to its recognition sites. BSAP recognition motifs were examined in gel-shift assays for their abilities to compete with the consensus recognition sequence from the sea urchin promoter, H2A2.2, for the endogenous BSAP in extracts from the mature B cell line, K46R. The competitors were added in a 5, 25, and 100 molar excess over probe. (**A**) Representative competition by activator elements. (**B**) Representative competition by repressor elements. (**C**) Quantitation of the competition assays by PhosphorImager measurements of the BSAP-probe complexes formed. Each point represents an average of three independent gel-shift determinations.

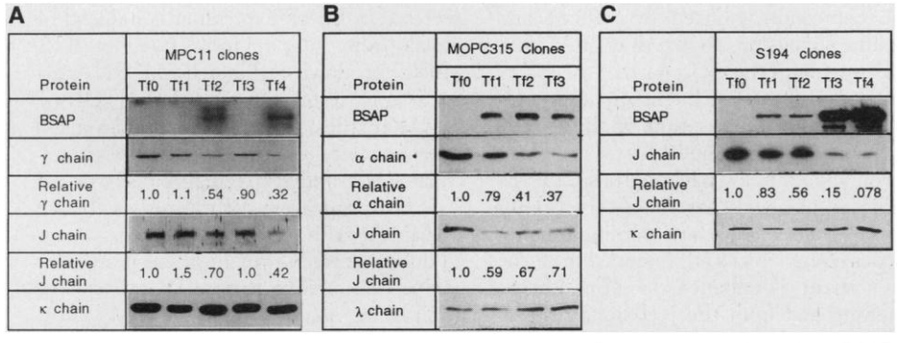

Fig. 2. Effect of BSAP overexpression on its repressor functions. Protein immunoblots of the BSAP, heavy, J, and light chain contents in (**A**) MPC11, (**B**) MOPC315, and (**C**) S194 plasmacytomas stably transfected with a BSAP expression vector (Tf1 to Tf4) or with vector alone (Tf0). The amounts of each protein were measured by densitometry, normalized to light chain content, and expressed relative to the value obtained with the vector-transfected control, which was set to 1.0. Endogenous BSAP in the three plasma cell lines was too low to be detected by the conditions used to evaluate the concentrations of transfected BSAP. No heavy chain data are included in the S194 assays because the cells synthesize too little α chain protein for quantitative measurements.

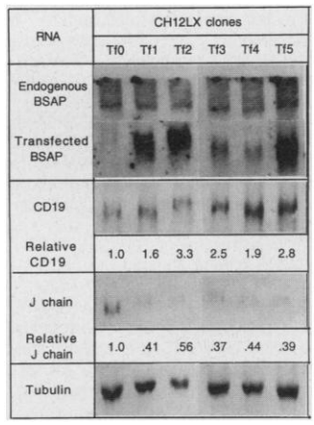

Fig. 3. Effect of BSAP overexpression on its activator functions. RNA blot analyses of endogenous and transfected BSAP, CD19, and J chain transcripts in CH12LX-IgM cells stably transfected with a BSAP expression vector (Tf1 to Tf5) or with vector alone (Tf0). The relative CD19 and J chain RNA values were determined from PhosphorImager measurements normalized to the tubulin RNA content. The density of the BSAP and CD19 blots was measured after a 24-hour exposure, whereas the density of the J chain blots was determined after a 4-day exposure.

the target genes under positive BSAP control would increase, or at least remain the same, with increasing BSAP concentrations. Again, the prediction was matched by the experimental data (Fig. 3). CD19 RNA was 1.6 to 3.3 times higher in the BSAP-transfected clones than in the vector control. Thus, maximum expression of CD19 is not required to maintain its coreceptor functions during the presecretor–germinal center stage of a B cell primary immune response (*17*). J chain RNA was also regulated in a concentration-dependent manner. Increasing BSAP in these cells increased its repressor activities so that even the low expression of the target J chain gene was reduced by 45 to 60% (Fig. 3). The μ heavy chain, however, was not affected, consistent with studies showing that the μ isotype expression is not significantly regulated by the 3′ α enhancer and thus is independent of any BSAP-induced changes (*18*).

We next asked whether the affinity of a BSAP site determined its activator or repressor action. The CD19 BSAP recognition sequence was substituted for the BSAP sequence (JC) in the J chain gene promoter. Nine of the 18 base pairs (bp) that make up the minimum consensus BSAP site were replaced (Fig. 4A). We evaluated the effect of the replacement by inserting the J2 (nucleotide −168 to −84) test and control fragments from the J chain promoter upstream of a minimum γ-fibrinogen promoter and the chloramphenicol acetyltransferase (CAT) reporter gene (*19*). These constructs were then assayed for CAT activity after transfection into J chain–expressing S194 cells (*20*). The BSAP element, which functioned as an activator binding site in the CD19 promoter, was converted to a repressor binding site in the J chain gene promoter (Fig. 4A). The CD19 site–containing J2 fragment induced a 90% decrease in CAT expression compared with an 84% decrease obtained with the counterpart endogenous J chain–containing J2 fragment.

To determine whether the change in function of the CD19 BSAP site was accompanied by a change in affinity, we used the J chain promoter fragments containing the CD19 and JC binding elements as competitors in the gel-shift assay described in Fig. 1. The patterns of competition obtained with the 85-bp fragments (Fig. 4B) duplicated those observed with the 34-bp CD19 and JC oligonucleotides (Fig. 1), indicating that the affinity of the BSAP site did not change when its context was altered. Despite this switch in affinity, the BSAP site still functioned as a repressor in the J chain promoter, specifying that affinity alone was not sufficient to dictate the repressor or activator function of a BSAP site within an individual promoter. Thus, although BSAP sites with high affinity correlate with positive regulatory function, and low-affinity sites with negative regulatory functions, the context of a site is crucial, even though the occupancy of a site is determined by the gradient of BSAP concentration.

Here, we have defined a concentration-dependent mechanism that allows a single transcription factor to alter its coordinate regulation of gene expression at different stages during B cell differentiation. A similar mechanism operates in the developing embryo of *Drosophila* where concentration gradients of transcription factors, such as the dorsal activator, are established along the dorsoventral axis (*21*). In this mammalian example, the gradient of BSAP concentration is instituted by signals from IL-2 or IL-5 (*7*), so that the mechanism allows a single external cytokine to regulate the various BSAP-controlled events in the B cell immune response. In view of these features, concentration-dependent regulation of gene expression may be a generally used mechanism in the developmental systems of vertebrates to control the coordinated activation and repression of multiple genes during development.

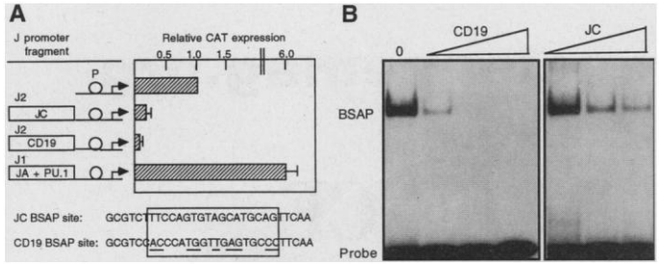

Fig. 4. Exchange of an activator motif for a repressor motif. (**A**) Effect of exchange on the function of the activator motif. Constructs of the J2 promoter fragment (nucleotide −168 to −84) containing either the endogenous JC BSAP repressor element or the CD19 BSAP activator element, and constructs of the J1 fragment (nucleotide −83 to −9) containing the positive-acting JA and PU.1 elements were assayed for CAT activity after transfection into S194 plasmacytoma cells. CAT activity is expressed relative to the value obtained with the γ-fibrinogen promoter alone, which was set to 1.0. All values are the mean of at least three independent determinations. Bars indicate the SEM. (**B**) Effect of exchange on the affinity of the activator motif. J2 fragments containing the CD19 and JC elements were used as competitors in gel-shift assays as described in Fig. 2.

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13. For gel mobility–shift assays, the H2A oligonucleotide (*11*) was end-labeled with [α-^{32}P]dCTP (deoxycytidine 5′-triphosphate) and Klenow enzyme (*22*). The binding reactions with crude nuclear extracts were performed as described (*9*), with 8 μg of nuclear extract and 4 μg of poly(dI-dC) nonspecific competitor. The protein complexes formed were resolved from free probe by electrophoresis through 5% polyacrylamide gels (29:1) containing 0.25× tris-borate EDTA buffer. Top strands of the oligonucleotides used for competition reactions are as follows: CD19, 5′-CTAGACAGACACCCATGGTTGAGTGCCCTCCAGT-3′; Iε, 5′-GTTAGCTGAGGGCACTGAGGCAGAGCGGCCCCTAGG-3′; JC, 5′-GGTGTGCGTCTTTCCAGTGTAGCATGCAGTTCAA-3′; and 3′ α E, 5′-CTAGATCACTTCCCTGGGGTGTTGAGCCACCCAT-3′.
14. For the generation of stable transfectants, 8 μg of the BSAP-BCMGSNeo construct (*7*) or the BCMGSNeo vector was introduced into S194, MPC11, MOPC315, or CH12 cells by electroporation; 1.5 × 10^7 cells in 270 μl of RPMI were transfected at 250 V and 960 μF with a Gene Pulser (Bio-Rad). Transfected cells (1:10 dilution) were aliquoted into 96-well plates and 24 hours later were mixed with an equal volume of 2× G418-sulfate selection medium. G418-resistant clones were visible by microscopy after 2 to 3 weeks of culture. At that time, cells from positive wells were replated at a concentration of less than one cell per well, and positive samples were expanded and maintained in the selection media. For protein immunoblot analyses, nuclear extracts (25 μg) from stably transfected clones were boiled for 5 min, size-fractionated by SDS–polyacrylamide gel electrophoresis (12.5%), and transferred to a nitrocellulose filter. After pretreatment with 5% dry milk in 1× phosphate-buffered saline, the filters were incubated for 3 hours with a 1:10,000 dilution of antibody specific for the BSAP paired domain (gift of M. Busslinger) or antibody specific for γ or α immunoglobulin heavy chain, or κ or λ light chain (Zymed).
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20. Transient transfections of S194 myeloma cells were performed with DEAE-dextran (*25*). In each case, 10^7 cells in log-phase were transfected with 9 μg of supercoiled test plasmid. Cell extracts were prepared 44 to 48 hours after transfection and assayed for CAT activity (*26*).
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