

# The B Cell-specific Transcription Factor BSAP Regulates B Cell Proliferation

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## Summary

The B cell-specific activator protein (BSAP) is a DNA-binding transcription factor expressed in pro-B, pre-B, and mature B cells, but not in plasma cells. In this study, we explored the role of BSAP in B cell function by assessing how the content of this protein varies in cells driven by proliferative stimuli and, conversely, how artificial manipulation of BSAP activity affects cell proliferation. We found that BSAP activity of nuclear extracts increased when B cells were activated by mitogen (lipopolysaccharide [LPS]), antigen receptor-mediated signaling (surface immunoglobulin D [IgD] cross-linking) or T cell-dependent stimulation (CD40 cross-linking). We could suppress BSAP activity by exposure of B cells to phosphorothioate oligonucleotides antisense to the BSAP translation initiation start site, whereas control oligonucleotides were virtually inactive. Antisense-induced BSAP suppression was associated with a striking reduction in LPS-induced proliferation of splenic B cells and in the spontaneous proliferation of B lymphoma cells (CH12.LX), but the antisense oligonucleotide had virtually no effect on proliferation of two cell lines lacking BSAP: the T lymphoma line EL-4 and the plasma cell line MOPC-315. Overexpression of BSAP in splenic B cells or de novo expression in MOPC-315 plasma cells induced by transfection of a BSAP expression plasmid stimulated cell proliferation. Taken together, these results suggest that BSAP activity is a rate-limiting regulator of B cell proliferation. We also found that treatment with the antisense BSAP oligonucleotide downregulated Ig class switching induced by interleukin 4 plus LPS. This effect may be secondary to reduced proliferation or could be mediated through BSAP binding sites in the IgH locus.

**A** B cell lineage-specific transcription factor, B cell-specific activator protein (BSAP)<sup>1</sup> (1), was recently found to bind in regulatory regions of several B cell-specific genes, including the promoters of the CD19 gene (2), the CD20 gene (J. Kehrl, personal communication), the V<sub>preB1</sub> gene, and the λ5 gene (3). In addition, BSAP binding sites have been identified in several C<sub>H</sub> gene intronic regions (4–6), as well as at sites 5' to and within the Ig H chain 3'α enhancer (7, 8).

BSAP is encoded by the *Pax-5* gene (9) and is a member of the *Pax* gene family of homeodomain class transcription factors. All nine *Pax* genes described so far are important regulators of development and are highly conserved during evolution (10, 11). The *Pax-5* gene is classified in subclass III of these genes (along with *Pax-2* and *Pax-8*; 12), and contains a bipartite "paired domain" responsible for DNA binding

(9, 13), a sequence encoding a characteristic octapeptide, and a homeobox homology region (9). Individual *Pax* genes are temporally and spatially regulated during development: *Pax-2* and *Pax-8* are expressed in the developing excretory system and in kidney cells that lack BSAP (14, 15). In contrast, BSAP (*Pax-5*) is specifically expressed in the B cell lineage (1, 9). Within the B cell lineage, BSAP is found in pro-B, pre-B, and mature B cells, but not in plasma cells (1). In addition, it is not found in cells of hematopoietic lineage other than B cells, but is found in the developing central nervous system and adult testis (9, 16).

In this report we demonstrate that specific downregulation of BSAP expression is associated with greatly reduced B cell proliferation, whereas upregulation of BSAP expression is associated with increased B cell proliferation. This finding, combined with our recent observation that BSAP negatively regulates the Ig 3'α enhancer (8) suggests that BSAP regulates the differentiation of mature B cells on several levels, and thus is a key factor controlling B cell differentiation.

<sup>1</sup> Abbreviations used in this paper: BSAP, B cell-specific activator protein; EMSA, electrophoretic mobility shift assay.

## Materials and Methods

**Cell Preparation and Culture Conditions.** Purified splenic B cells were obtained from 4–8-wk-old female BALB/c mice (NIH stock) as follows: (a) splenocyte cell suspensions were depleted of RBCs by hypotonic lysis with ACK lysing buffer (B & B/Scott, Fiskeville, RI) and of T cells by complement-mediated cytotoxic lysis using various mAbs: anti-Thy 1.2 (New England Nuclear, Boston, MA), anti-CD3 (2C11; American Type Culture Collection [ATCC], Rockville, MD), anti-CD4 (RL1724), and anti-CD8 (3.155) kindly provided by Dr. E. Shevach, Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, Bethesda, MD) in conjunction with rabbit complement (Low-Tox M; Cedar Lane, Westbury, NY); (b) B cells thus obtained were further selected for high density (resting) cells by Percoll-gradient centrifugation (Pharmacia Fine Chemicals, Piscataway, NJ) using cells collected at 60/70% layer. B cell lymphoma cells CH12.LX (sIgM<sup>+</sup>) were obtained from Dr. G. Haughton (University of North Carolina, Chapel Hill, NC; 17) and maintained in this laboratory for several years. IgA-secreting myeloma cells (MOPC-315) and EL4 thymoma T cells were obtained from the ATCC. An L cell line expressing Fcγ II receptor (L<sub>CDw32</sub>) was kindly provided by Dr. Kevin Moore (DNAX, Palo Alto, CA; 18).

Cells were maintained in RPMI 1640 supplemented with 10% FCS, 5% NCTC 109 media (Whittaker M. A. Bioproducts, Walkersville, MD), 20 mM Hepes (Biofluids, Inc., Rockville, MD), 2 mM L-glutamine (GIBCO BRL, Gaithersburg, MD), and 50 μM β-ME (Sigma Chemical Co., St. Louis, MO). MOPC-315 myeloma cells were grown in DMEM containing the same ingredients, except for NCTC.

**Preparation of L Cells Expressing the CD40 Ligand.** L cells expressing the CD40 ligand were prepared as follows. First, DNA including the whole coding region of the CD40 ligand, was obtained by RT-PCR amplification of RNA extracted from PMA-stimulated EL4 T cells using specific primers (see Table 1) based on the published sequence of CD40 ligand (19). Next, the amplified product was first cloned into the pCR<sup>TM</sup> II vector (Invitrogen Corp., San Diego, CA) then recloned into the pcDNA I neo expression plasmid (Invitrogen Corp.), and the recombinant plasmids thus obtained were detected by restriction fragment analysis. Plasmid DNA from one such clone was then transfected by electroporation into L cells stably transfected with a plasmid expressing the FcγII receptor CDw32 (18). The cells were grown in G418 (400 μg/ml) to select for stably transfected L cells. Finally, stable transformants were screened for their ability to induce proliferation of spleen B cells, and the most potent cell line was recloned, designated L<sub>CD40L</sub>, and used in this study.

**Electrophoretic Mobility Shift Assay.** Nuclear proteins were prepared from cultured cells as described elsewhere (20). Protein concentrations were measured with the Bradford assay (21). Oligonucleotides for electrophoretic mobility shift assays (EMSAs) (Table 1) were endlabeled with γ-[<sup>32</sup>P]ATP (>5,000 Ci/mmol; Amersham Corp., Arlington Heights, IL) using bacteriophage T4 polynucleotide kinase (New England Biolabs Inc., Beverly, MA) and gel purified. EMSA reactions contained 1–3 fmol double-stranded DNA, 2 μg synthetic DNA duplex of poly (dI-dC) (Pharmacia Fine Chemicals), 10 mM Hepes, pH 7.9, 100 mM NaCl, 10% glycerol, 0.5 mM MgCl<sub>2</sub>, and 1 mM dithiothreitol. After preincubation without protein for 15 min at room temperature, crude nuclear proteins in indicated concentrations were added to the reaction for an additional 15 min. The mixture was then subjected to electrophoresis on native 4% polyacrylamide gels in 1× Trisborate/EDTA (TBE) buffer to separate protein-DNA complexes. Finally, the gels were dried and exposed to Kodak films on inten-

sifying screens overnight at –80°C. Autoradiographs were analyzed by densitometry using Image Quant (Molecular Dynamics, Inc., Sunnyvale, CA).

**Coincubation with Oligonucleotides.** Phosphorothioate oligonucleotides were prepared by Genosys Biotechnologies, Inc. (Woodland, TX). These consisted of 18 mer that were antisense and sense for the RNA sequence that spans the translation initiation site of murine BSAP (see Table 1; these sequence data are available from EMBL/GenBank/DDBJ under accession number M97013). In addition, a control (non-sense) oligonucleotide mixture was prepared consisting of scrambled 18 mer containing the same G + C and A + T content of the antisense oligonucleotide (see Table 1). Coincubation of cells with phosphorothioate oligonucleotides was performed as previously described (22). In brief, B cells were washed twice with HBSS and Opti-MEM, then cultured in serum-free Opti-MEM containing indicated concentrations of oligonucleotides for various lengths of time.

**Proliferation and Viability Assays.** Proliferation of cells was assessed by measuring [<sup>3</sup>H]TdR incorporation during the final 8 h of cell culture. In brief, 5 × 10<sup>4</sup> B cells/ml were cocultured with irradiated (10 krad) L cells (5 × 10<sup>3</sup>/ml) in flat-bottomed 96-well plates (Costar Corp., Cambridge, MA) for 72 h. During the last 8 h of culture, 1 μCi of [<sup>3</sup>H]TdR (sp act 6.7 Ci/mmol; New England Nuclear) was added to each well; incorporated <sup>3</sup>H radioactivity was measured in a scintillation counter (LS2800; Beckman Instruments, Inc., Fullerton, CA). Each incubation experiment was done in triplicate. The cell viability at the end of culture was assessed by trypan blue exclusion.

In experiments examining the effects of CD40L stimulation, resting B cells were cocultured with an indicated (see Results) number of L<sub>CD40L</sub> cells. For anti-IgD stimulation, resting B cells were cocultured with L<sub>CDw32</sub> (5 × 10<sup>3</sup>/ml) in media containing 10 μg/ml of anti-IgD (AMS 9.1.1.1.; obtained from the ATCC). For LPS stimulation, resting B cells were cultured in media containing 20 μg/ml of *Escherichia coli* LPS (B4:0111; Sigma Chemical Co.); to control for the costimulatory activity of the L cells, L<sub>CDw32</sub> cells (5 × 10<sup>3</sup>/ml) were included in the LPS-stimulated cell cultures. L cells were omitted in the proliferation assays using cell lines. In coincubation studies of resting B cells and S-oligonucleotides, an indicated concentration of oligonucleotides was added to each culture at the start of the culture period.

**Construction of BSAP Expression Plasmid.** cDNA for the coding region of BSAP was obtained by subjecting total RNA extracted from CH12.LX B cells to RT-PCR using primers (Table 1) derived from previously published sequence data (9). The resulting cDNA was first cloned into the pCR<sup>TM</sup> II vector (Invitrogen Corp.), then recloned in sense orientation between the NotI and XhoI sites of the BCMGSNeo cDNA expression vector (kindly donated by Dr. H. Karasuyama, Basel Institute of Immunology, Basel, Switzerland; 23). The transcription unit of BCMGSNeo consisted of the CMV promoter region, a leader sequence interrupted by an intron, a multiple cloning site, and a polyadenylation signal. The BCMGSNeo plasmid containing the BSAP cDNA was designated BCMGSNeo-BSAP. For control studies, a derivative of the BCMGSNeo plasmid containing a 350-bp stuffer fragment (BCMGSNeo-control) was also prepared.

**Transfection Conditions.** 2 × 10<sup>7</sup> spleen B cells or MOPC-315 plasma cells in the logarithmic growth phase were transfected with 10 μg of the BCMGSNeo-BSAP or BCMGSNeo control plasmid. Transfection of plasmids was performed by electroporation in 800 μl phosphate-buffered sucrose media (272 mM sucrose, 7 mM sodium phosphate, and 1 mM MgCl<sub>2</sub>, pH 7.4) using a Gene Pulser

(Bio-Rad, Richmond, CA) at 300 V and 25  $\mu$ F capacitance. After electroporation, cells were kept for 10 min on ice and then cultured in conventional media.

**Enrichment of Plasmid-transfected Cells.** Since only a minority of cells are successively transfected under the above transfection conditions, an attempt was made to study the proliferation of a cell population enriched for transfection-competent cells by marking these cells with dextran-FITC (24, 25). In these experiments, splenic B cells were electroporated with the test plasmids in the presence of 0.2 mg/ml of dextran-FITC (10,000  $M_r$ ; Sigma Chemical Co.). The cells were then incubated for 2 h in culture medium, treated for 1 min with 0.01% trypsin to remove nonspecifically bound dextran-FITC, and washed three times in HBSS containing 0.1% BSA. Finally, the cells were sorted using a FACStar Plus<sup>®</sup> (Becton Dickinson & Co., San Jose, CA). Gates were set by side and forward scattergram, as well as with propidium iodide staining to exclude dead cells. For sorting, one gate was set to collect the FITC-positive cells which appeared in the top 15th percentile of the fluorescence intensity histogram. These were labeled "FITC-High" cells. Another sorting gate was set to collect the cells in the lowest 15th percentile of the histogram and labeled, "FITC-Low" cells. The FITC-High and FITC-Low populations were then cultured separately and assayed for proliferation as described above. The ability of FITC staining to mark transfection-competent cells was assessed by a control experiment in which cells were transfected with 10  $\mu$ g/ml of pSV- $\beta$ -galactosidase Control Vector (Promega Corp., Madison, WI) in the presence of dextran-FITC and sorted as described above. After 24 h of culture, both cell populations were fixed and incubated with X-Gal, according to the manufacturer's protocol, to measure  $\beta$ -galactosidase expression.

**Flow Cytometric Analysis of B Cell Isotype Switching.** To examine oligonucleotide effects on switching of B cells to IgG1 expression, resting B cells were cultured in media containing LPS (20  $\mu$ g/ml), IL-4 (400 U/ml of mL-4; Genzyme Corp., Cambridge, MA) and 30  $\mu$ M of phosphorothioate oligonucleotides. After 4 d of incubation, the B cells were washed twice in HBSS (Biofluids Inc.),

resuspended in HBSS containing 0.2% BSA without phenol red (NIH Media Unit) and stained with FITC-labeled goat anti-IgG1 and PE-labeled goat anti-IgM (both obtained from Southern Biotechnology Associates, Birmingham, AL). In most cases, 30  $\mu$ g/ml of anti-Fc $\gamma$ R (2.4G2; Pharmingen, San Diego, CA) was added to each tube before addition of fluorochrome-labeled antibodies to prevent cytophilic binding of fluorochrome-labeled antibody to the cell surface. Propidium iodide (1  $\mu$ g/ml) was added to the final cell suspension so that gates could be set to exclude dead cells. Surface immunofluorescence was assessed using a flow cytometer (EPICS 753; Coulter Electronics Inc., Hiialeah, FL).

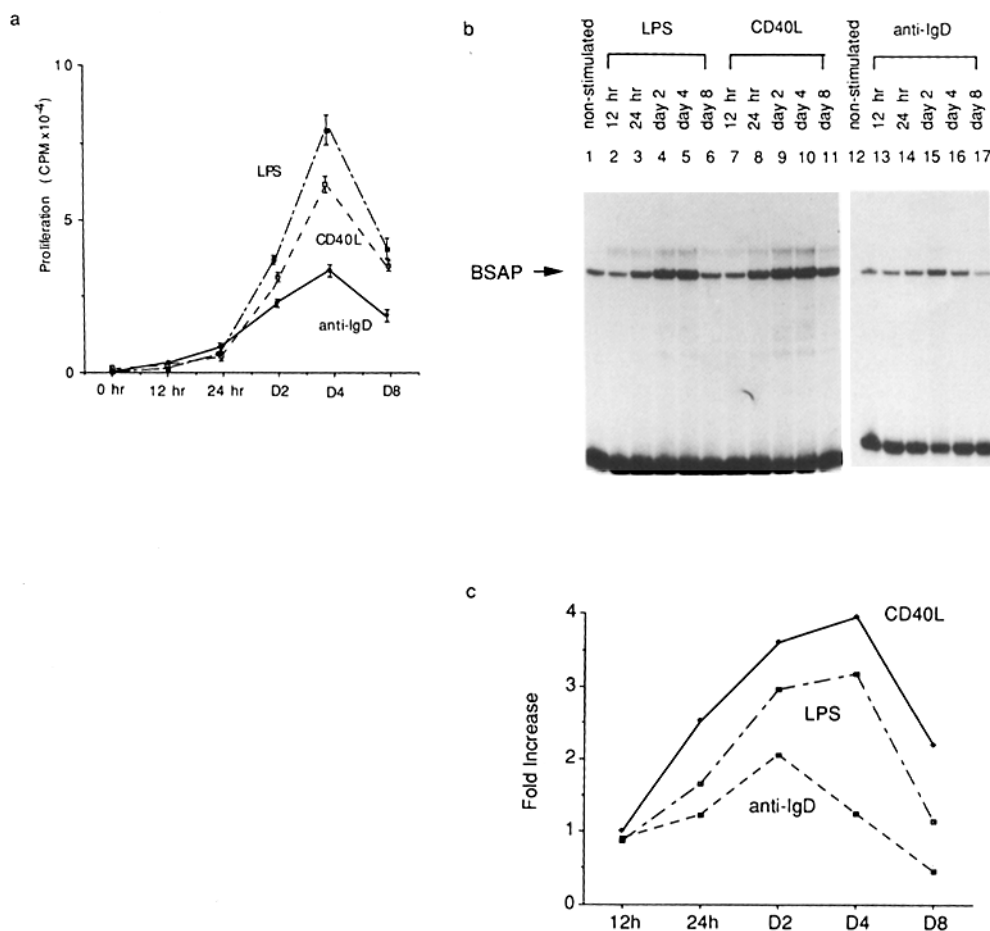
**Data Analysis.** Statistical analysis was done by the paired Student's *t* test.

## Results

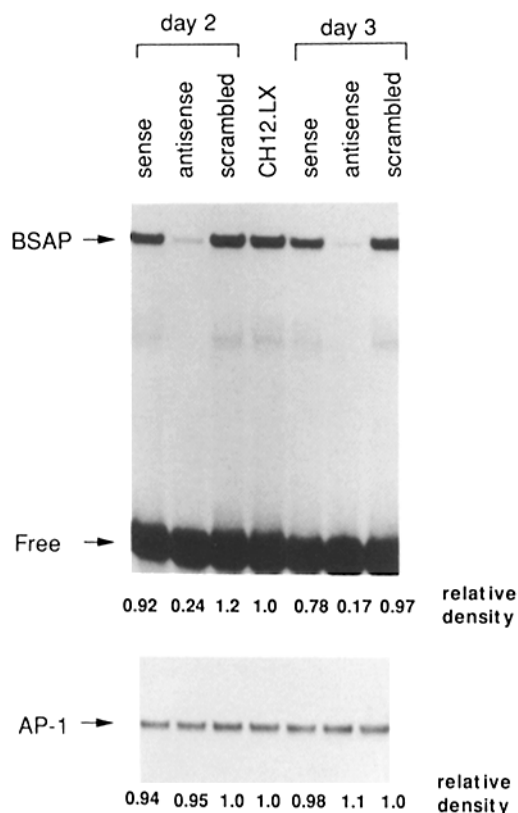
**BSAP Expression in Resting and Stimulated B Cells.** In initial studies, we explored changes in BSAP activity during the exposure of murine spleen B cells to three activation stimuli: (a) CD40 ligand, expressed on the surface of murine L cells stably transfected with a CD40 ligand cDNA construct ( $L_{CD40L}$ ); (b) monoclonal anti-IgD (AMS9.1.1.1) bound to mouse L cells stably transfected with a gene encoding the CDw32 molecule (Fc $\gamma$  II receptor) ( $L_{CDw32}$ ); and (c) LPS (in the presence of  $L_{CDw32}$ ). As shown in Fig. 1 a, all three methods of stimulation induced B cell proliferation under the culture conditions used. Nuclear proteins extracted from B cells stimulated under these conditions were analyzed for BSAP content by EMSAs at various time points. As shown in Fig. 1 b, BSAP is constitutively expressed in spleen B cells, and all three methods of stimulation induced increases in BSAP expression compared with nonstimulated B cells. Quantification of EMSA bands, as shown in Fig. 1 c, revealed that the

**Table 1.** Synthetic Oligonucleotides Used in this Paper

	Reference
S-oligo DNAs for coinubation study	
Sense oligo DNA: 5'-CGAAATGGATTTAGAGAA-3'	
Antisense oligo DNA: 5'-TTCTCTAAATCCATTTTCG-3	
Non-sense (scrambled) oligo DNA: 5'-WRWWRWRRSWRWRWSRWR-3' (W = A + T; R = A + G; S = G + C)	
Double-stranded DNAs for EMSA probes	
H2A-2.2 site: CAGGGTCTGACGCAGCGGTGGGTGACGACTGTCG CTCCCAACACTGCGTCGCCACCCACTGCTGACAGC	1
AP-1 site: CTAGTGATGAGTCAGCCGGATC GATCACTACTCAGTCGGCCTAG	38
PCR primers	
BSAP cDNA 5' primer: 5'-ATCGTCTCGAGTCATCAAGTCCTGAAATATCG-3'	9
BSAP cDNA 3' primer: 5'-TATGCGGCCGCGTCAGCGTCGGTGCAGAGTA-3'	9
CD40 ligand 5' primer: 5'-CCCTTAAGCTTGCATGATAGAAACATA-3'	19
CD40 ligand 3' primer: 5'-TAGAGCTCGAGTTTCAGAGTTTGAGTAAGCC-3'	19



**Figure 1.** Time course of BSAP induction by three different methods of B cell stimulation. Percoll gradient-separated, small, high density (resting) spleen B cells (at  $10^5$ /ml) were stimulated by LPS ( $20 \mu\text{g}/\text{ml}$ ) in the presence of L cells expressing Fc $\gamma$ II receptors ( $L_{CDw32}$ ) ( $10^4$ /ml);  $L_{CDw32}$  stably transfected with CD40 ligand ( $L_{CD40L}$ ) ( $10^4$ /ml); and anti-IgD ( $10 \mu\text{g}/\text{ml}$ ) in the presence of  $L_{CDw32}$  cells ( $10^4$ /ml). (a) Effect of the three stimuli on B cell proliferation. (b) EMSA analysis of nuclear proteins extracted from stimulated B cells for BSAP expression. Nuclear proteins were extracted at indicated time points and EMSA was performed using  $5 \mu\text{g}$  of crude nuclear extract and a labeled H2A-2.2 BSAP probe. (c) BSAP EMSA signals from b were quantified by densitometry and the values obtained were plotted as fold increases relative to BSAP EMSA signals of nonstimulated B cells.



time course of BSAP expression in stimulated cells roughly correlated with the proliferation data.

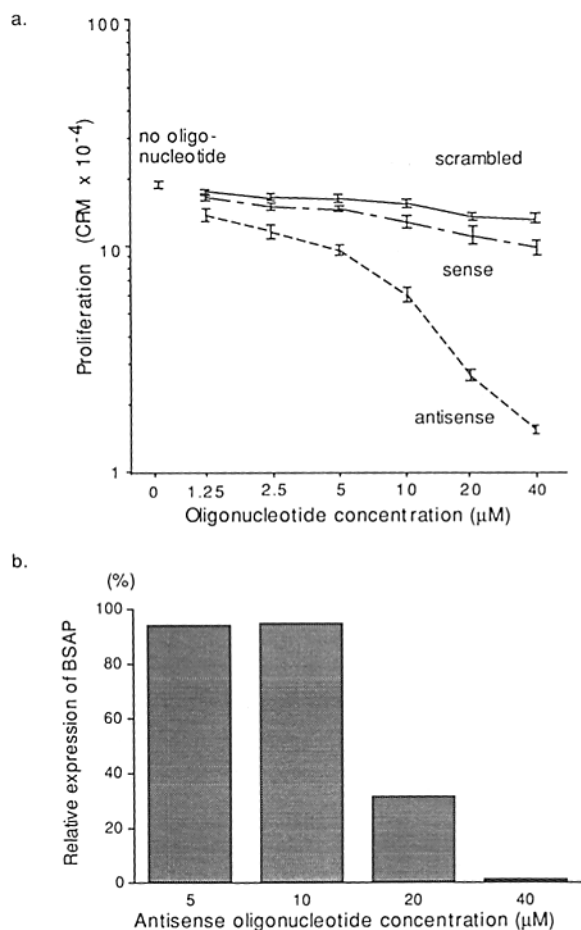
**BSAP Antisense Oligonucleotide Specifically Reduces BSAP Expression and Inhibits LPS-induced B cell Proliferation.** The parallel changes in BSAP activity and proliferation rate observed after B cell activation with all three stimuli suggested the possibility that BSAP might have a role in the regulation of B cell proliferation. We therefore investigated whether proliferation could be inhibited by exposing cells to an antisense oligonucleotide designed to downregulate BSAP expression. In these studies, we incubated cells with a phosphorothioate-substituted oligonucleotide that is complementary to BSAP mRNA overlapping the translation initiation site (antisense oligonucleotide, Table 1), as well as oligonucleotides corresponding to the sense strand sequence (sense oligonucleotide) and non-sense (scrambled) oligonucleotides having the same G + C and A + T composition as the antisense oligonucleotide. As shown in Fig. 2, incubation of LPS-treated

**Figure 2.** Specificity of downregulation of BSAP by antisense oligonucleotides. Resting spleen cells were stimulated with LPS in the presence of BSAP oligonucleotides ( $30 \mu\text{M}/\text{ml}$ ) (antisense, sense, and non-sense). Nuclear proteins were extracted at days 2 and 3, and analyzed by EMSA using labeled oligonucleotides specific for BSAP (H2A-2.2 site) and AP-1 (see Table 1) as probes. The values in the figure indicate the relative density of each retarded band to that of the control ( $CH12.LX$ ) obtained by densitometry of EMSA signals.

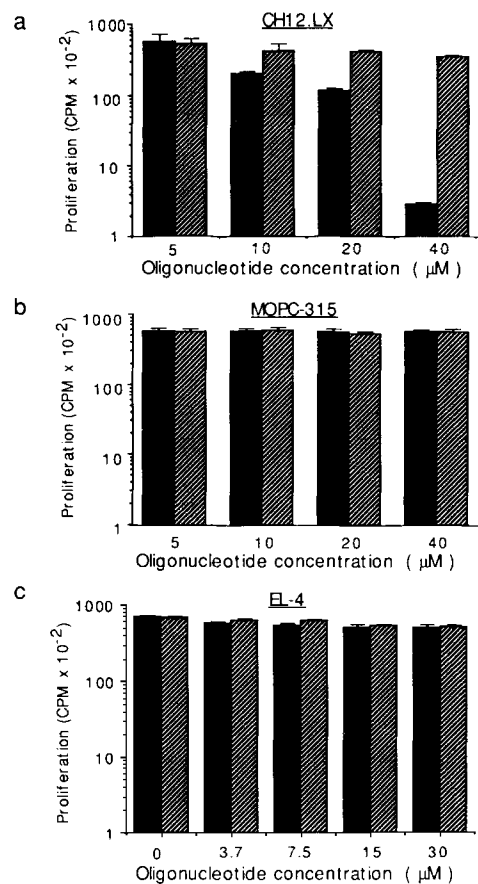
spleen B cells with 30  $\mu\text{M}$  antisense oligonucleotide for 12 h significantly reduced the BSAP activity detected in nuclear extracts isolated from the cells after 2 or 3 d of culture, in comparison to the activity from cells incubated with the sense oligonucleotide or the non-sense (scrambled) oligonucleotides. This effect was specific for BSAP, as the antisense oligonucleotide had no effect on the AP-1 activity in the same extracts (Fig. 2, *bottom*).

Having demonstrated the ability of antisense oligonucleotides to downregulate BSAP, we then determined the effect of such downregulation on B cell proliferation. In these studies, we incubated LPS-stimulated B cells with oligonucleotides over a range of concentrations, and assayed proliferation by incorporation of [ $^3\text{H}$ ]thymidine after 4 d of culture. As

shown in Fig. 3 *a*, the antisense oligonucleotide caused a marked dose-dependent decrease in cell proliferation compared with the control non-sense (scrambled) oligonucleotides, and, as shown in Fig. 3 *b*, the dose dependence of this decrease in proliferation correlated with that of the decrease in BSAP expression detected by EMSA. The sense oligonucleotide caused a smaller but reproducible reduction in proliferation, consistent with a small reduction in BSAP levels detected by densitometry of corresponding EMSA signals (Fig. 2). As shown in Fig. 4 *a*, the antiproliferative effect of the antisense oligonucleotide was also seen in the culture of BSAP-expressing B lymphoma cells (CH12.LX) growing spontaneously. This effect was not due to nonspecific toxicity of the antisense oligonucleotide, since cell viability was never <85% even in cell cultures exposed to high (40  $\mu\text{M}$ ) concentrations of antisense oligonucleotide, in which proliferation was inhibited more than 100-fold (data not shown). Furthermore, proliferation of two cell lines lacking measurable BSAP, the murine T cell line EL-4 and the myeloma MOPC-315, was virtually



**Figure 3.** (a) Proliferation of spleen B cells stimulated by LPS in the presence of oligonucleotides sense and antisense to the BSAP translation initiation site and non-sense (*scrambled*) oligonucleotides. Resting spleen B cells ( $5 \times 10^4$ /well) were stimulated with LPS (20  $\mu\text{g}/\text{ml}$ ) and coincubated with oligonucleotides at different concentrations as indicated. Proliferation was assayed at day 4 by pulsing cells with [ $^3\text{H}$ ]thymidine for 8 h (see Materials and Methods). (b) Dose-dependent reduction of BSAP activity in spleen B cells after coincubation with antisense oligonucleotide. Nuclear proteins were extracted 4 d after coincubation and BSAP activity was measured by EMSA using a labeled H2A-2.2 probe. Retarded EMSA bands were quantified by densitometry, normalized to the total radioactivity loaded per lane, and compared with noncoincubated cells.

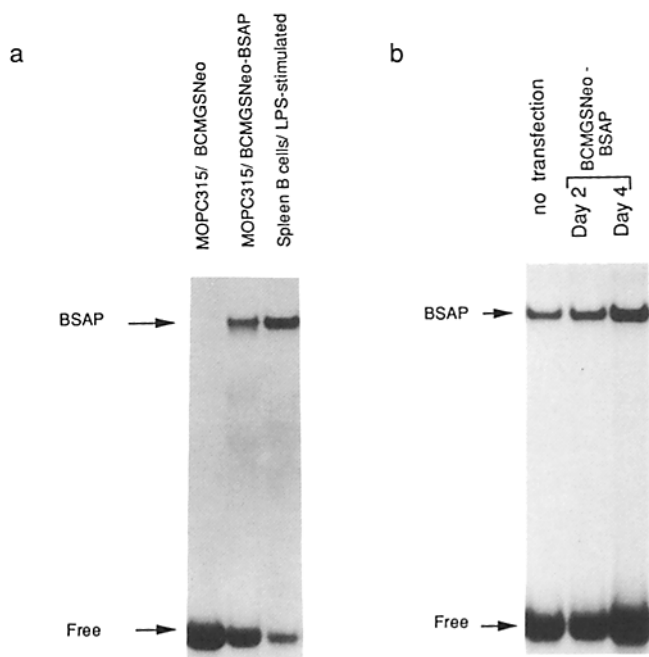


**Figure 4.** Effects of coincubation of BSAP phosphorothioate oligonucleotides on cell proliferation. (a) B cell lymphoma cells (CH12.LX), (b) MOPC-315 plasma cells, and (c) T cell lymphoma cells (EL-4) were cultured in microwells ( $5 \times 10^4$  cells/well) in the presence of various concentrations of BSAP oligonucleotides. On day 3, cell proliferation assays ([ $^3\text{H}$ ]thymidine incorporation) were performed as described in Materials and Methods. (Filled columns) Antisense oligonucleotide; (shaded columns) sense oligonucleotide.

unaffected by incubation with the antisense oligonucleotide (Fig. 4, *b* and *c*). Apparently, proliferation in EL-4 and MOPC-315 cell lines is regulated by mechanisms independent of BSAP.

**Effect of BSAP Upregulation on B Cell Proliferation.** In the light of the above findings, we sought to determine the effect of increased BSAP expression on B cell proliferation. For these studies we first constructed a BSAP expression plasmid, BCMGSNeo-BSAP, as described in Materials and Methods. We then verified that this cDNA expression plasmid encoded functional BSAP by transfecting it into MOPC-315 plasma cells, which lack endogenous BSAP, and testing those cells for BSAP expression. As shown in Fig. 5 *a*, nuclear protein extracted from transfected MOPC-315 plasma cells generated an EMSA band that comigrated with the endogenous BSAP band from LPS-stimulated B cells, whereas nuclear proteins extracted from MOPC-315 cells transfected with an empty plasmid had no detectable BSAP. In addition, as shown in Fig. 5 *b*, nuclear protein extracted from splenic B cells transiently transfected with the BSAP expression plasmid generated a BSAP EMSA band of increased intensity.

The demonstration that the BCMGSNeo-BSAP plasmid encoded assayable BSAP protein allowed us to test the effect

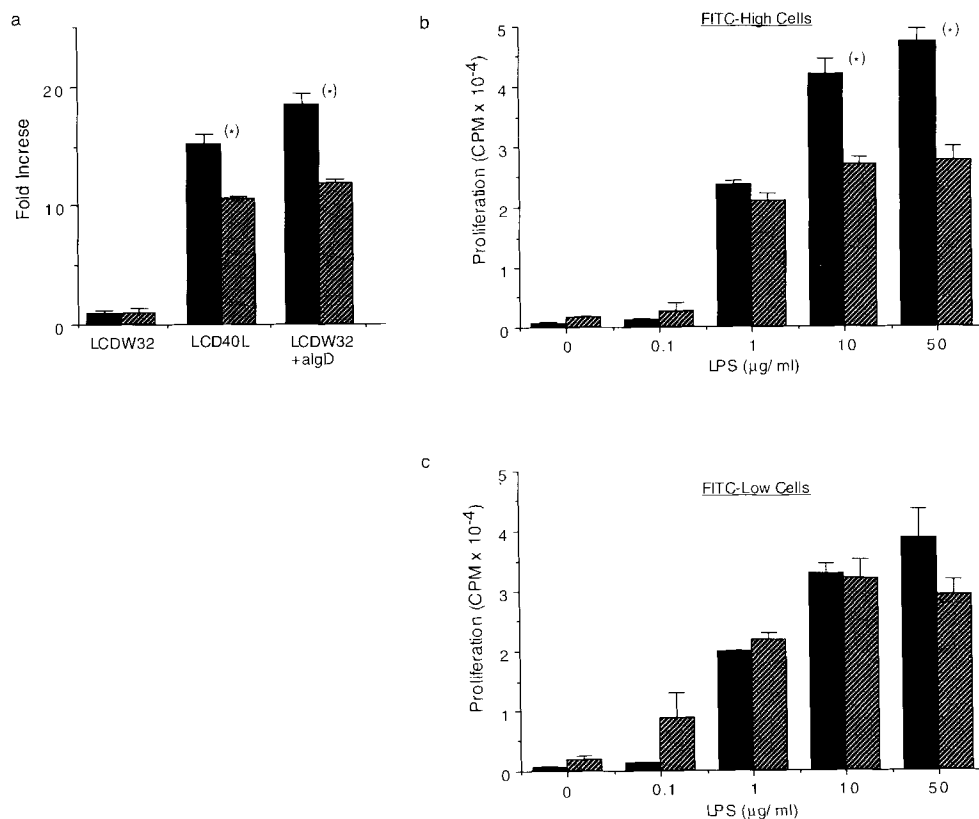


**Figure 5.** Expression of BSAP in MOPC-315 plasma cells and purified spleen B cells transiently transfected with BCMGSNeo-BSAP or BCMGSNeo-control plasmid. (*a*) The cDNA expression plasmid BCMGSNeo-BSAP or BCMGSNeo-control plasmid (10  $\mu$ g) were transfected into the BSAP-nonproducing plasma cell line MOPC-315. Nuclear proteins were extracted 2 d after transfection and EMSA was performed using a labeled H2A-2.2 probe. In BCMGSNeo-BSAP-transfected MOPC-315 cells, a retarded band was observed that comigrated with the BSAP signal obtained with nuclear extracts from LPS-stimulated spleen B cells. (*b*) Nuclear proteins from purified spleen B cells transiently transfected with 10  $\mu$ g of the BCMGSNeo-BSAP plasmid were extracted at the indicated time points after transfection and subjected to EMSA analysis using the H2A-2.2 probe. Nontransfected purified B cells served as controls.

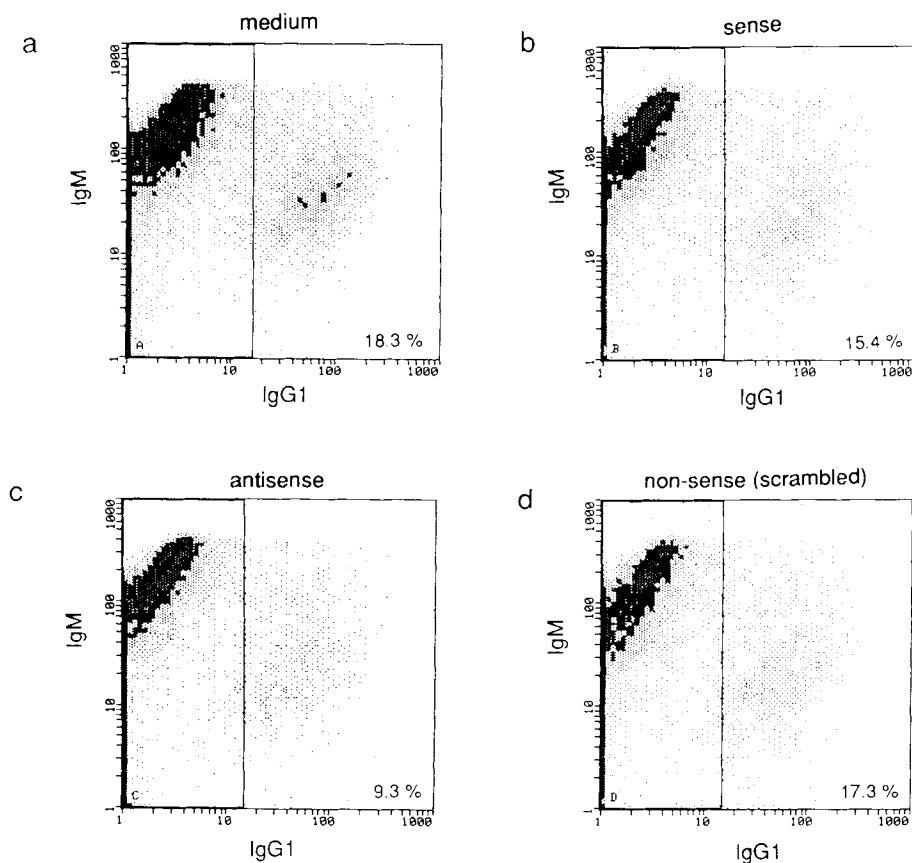
of upregulating BSAP levels in B cells. Accordingly, we transiently transfected spleen B cells with the BCMGSNeo-BSAP plasmid and, after 24 h, subjected the cells to various proliferation stimuli. We then measured proliferation after 72 h of additional incubation. As shown in Fig. 6 *a*, BCMGSNeo-BSAP-transfected B cells exhibited significantly greater ( $p < 0.01$ ) proliferation than B cells transfected with control plasmids. To confirm and amplify these results, we also determined LPS-induced proliferation in BCMGSNeo-BSAP-transfected versus control plasmid-transfected cells in cell populations enriched for transfected and nontransfected cells. Enrichment of transfected and nontransfected cells was accomplished by electroporation in the presence of dextran-FITC followed by cell sorting and isolation of FITC-High and FITC-Low cells (see Materials and Methods). The efficiency of this method was verified in a control experiment in which a pSV- $\beta$ -galactosidase control vector was transfected in the presence of dextran-FITC and  $\beta$ -galactosidase activity was measured in FITC-High and FITC-Low cell populations.  $\beta$ -galactosidase activity was detected in 40% of FITC-High and 5% of the FITC-Low cell populations, indicating an eightfold enrichment of transfected cells in the FITC-High cell population compared with the FITC-Low cell population. As shown in Fig. 6, *b* and *c*, we found that in the FITC-High cell population, transfection with BCMGSNeo-BSAP caused significantly higher proliferation than transfection with control plasmid. In contrast, in FITC-Low cell population, no significant differences between BCMGSNeo-BSAP and control transfected cells were observed. Taken together, these results show that upregulation of BSAP expression in spleen B cells results in substantially increased B cell proliferation responses, regardless of the method of cell stimulation.

In further studies we determined the effect of de novo BSAP expression on MOPC-315 plasma cells, which as mentioned above, do not normally express BSAP. In these studies, equal numbers of BCMGSNeo-BSAP-transfected and control plasmid-transfected MOPC-315 plasma cells were placed into microwells, and after 72 h, proliferation of cells was assessed by [ $^3$ H]thymidine uptake. Significantly more [ $^3$ H]thymidine uptake occurred in plasma cells transfected with the BSAP-expressing plasmid ( $52,118 \pm 459$ ) than in plasma cells transfected with the non-BSAP-expressing plasmid ( $29,078 \pm 2763$ ) ( $p < 0.005$ ).

**Effect of Downregulation of BSAP by Antisense Oligonucleotide on B Cell Isotype Switch.** To explore further the role of BSAP expression on B cell function, we determined the effect of BSAP downregulation on B cell isotype switch. This was of considerable interest because binding sites for BSAP have recently been found 5' to and within the switch region of Ig H chain genes (4–6) and in the 3' $\alpha$  enhancer (7, 8), suggesting that BSAP plays a role in H chain isotype switching. In these studies, we first stimulated purified resting spleen B cells with LPS and cytokines in the presence or absence of the phosphorothioate oligonucleotides for 4 d. We then stained the cells with isotype-specific antibodies to determine the percentage of various surface Ig-positive B cells present at the end of the culture period. As shown in the representative experiment depicted in Fig. 7, the incubation of cells



**Figure 6.** Effects of BSAP up-regulation on proliferation of spleen B cells. (a) Fold increase in proliferation of B cells transfected with BCMGSNeo-BSAP (solid bar) or BCMGSNeo-control plasmid (shaded bar) when cells are cocultured with L cells (*L<sub>CDW32</sub>*), L cells transfected with a plasmid expressing CD40 ligand (*L<sub>CD40L</sub>*), and L cells (*L<sub>CDW32</sub>*) plus anti-IgD (means of three experiments). The baseline counts of BCMGSNeo-BSAP and control plasmid-transfected B cells were  $5,458 \pm 69.0$  and  $7,246 \pm 89.1$ , respectively. (b and c) LPS-stimulation of spleen B cells transfected with BCMGSNeo-BSAP (solid bar) or control plasmid (shaded bar) and enriched by cotransfection of dextran-FITC followed by cell sorting (see Materials and Methods). Fluorescence-high (b) and -low (c) cells were seeded at  $5 \times 10^4$  cells/well and stimulated with various concentrations of LPS. (Asterisks)  $p < 0.01$ .



**Figure 7.** Effect of BSAP oligonucleotides on B cell isotype switching. High density (resting) spleen cells were purified and cultured (at  $10^5/\text{ml}$ ) in media containing LPS ( $20 \mu\text{g/ml}$ ) and rIL-4 ( $400 \text{ U/ml}$ ) in the presence of indicated oligonucleotides ( $30 \mu\text{M}$ ) for 4 d. On day 4, the cells were stained with PE-labeled anti-IgM and FITC-labeled anti-IgG1, after which flow cytometry was performed in an EPICS 753 flow cytometer. Percent sIgG1-positive B cells are indicated.

with anti-sense oligonucleotide led to a reduction in the percentage of sIgG1<sup>+</sup> cells in B cells cultured with LPS plus IL-4. Similarly, reduced numbers of sIgG2a B cells were found in cells cultured with LPS and IFN- $\gamma$ , and reduced numbers of sIgG3<sup>+</sup> B cells were noted in cells cultured with LPS alone. Thus, downregulation of BSAP led to reduced B cell isotype switching of B cells treated by LPS plus cytokines.

## Discussion

The B cell-specific activator protein BSAP is a mammalian homolog of the sea urchin DNA-binding protein TSAP (tissue-specific transcription activator protein), a regulator of a nonallelic pair of late histone genes (H2A-2 and H2B-2) (26). Several findings suggest that BSAP is a transcriptional regulator of B cell-specific genes. For instance, BSAP has been shown to regulate transcription of the CD19 gene, a gene encoding a B cell transmembrane glycoprotein involved in cell activation (27). In addition, BSAP is probably identical to EBB-1, a protein which has been shown to regulate transcription of the early B cell signal transduction molecules, V<sub>preB1</sub> and  $\lambda$ 5 (3). Finally, BSAP has been shown to repress the Ig H chain 3' $\alpha$  enhancer at early stages of B cell differentiation (7, 8), with probable but as yet unknown effects on Ig gene transcription. The fact that BSAP binds to various intronic regions of the Ig constant region gene segments leads to the expectation that it also plays a role in regulating isotype switching, but this remains to be proven.

In this study, we provide several kinds of evidence for a new role for BSAP as a regulator of B cell proliferation. Initially, we showed that stimulation of spleen B cells by three representative mechanisms resulted in an increase in BSAP expression that correlated with an increase in B cell proliferation. We then demonstrated that down- or upregulation of BSAP expression during B cell stimulation led to decreased or increased cell proliferation, respectively. Downregulation of BSAP levels, induced by exposure of stimulated B cells to an antisense oligonucleotide, resulted in a more than 100-fold reduction in the proliferation of LPS-activated normal B cells, as well as greatly reduced proliferation of spontaneously growing lymphoma B cells (CH12.LX B cells). This effect of the antisense oligonucleotide was shown by previously established criteria (28) to be both effective and specific: (a) the EMSA levels of functional BSAP protein were greatly reduced in cells exposed to the antisense oligonucleotide; (b) exposure of B cells to the antisense oligonucleotide had no effect on a nontargeted protein (AP-1); (c) treatment of B cells with non-sense (scrambled) oligonucleotides only minimally affected BSAP levels; and (d) antisense oligonucleotide treatment had no effect on proliferation of two cell lines that lack endogenous BSAP. Finally, we found that upregulation of BSAP in spleen B cells or de novo expression of BSAP in MOPC-315 plasma cells, achieved by transfection of a BSAP expression plasmid, leads to a significant increase in cell prolifer-

ation. In control experiments using a transfected  $\beta$ -galactosidase expression plasmid, we found that our protocol of electroporation in the presence of dextran-FITC followed by cell sorting to obtain the FITC-High cell population enriched transfected cells to only 40% of the FITC-High cell population. Thus the increased proliferation we observed probably underestimates the ability of increased BSAP levels to upregulate proliferation. Taken together, these findings demonstrate that either down- or upregulation of BSAP induced a decrease or increase in B cell proliferation, respectively. As such, they provide strong evidence that BSAP is an important and rate-limiting factor in B cell proliferation.

Our findings on the relation of BSAP to B cell proliferation place BSAP in the company of several protooncogene products including *c-fos*, *c-myc*, and *c-myb* (29-34), which have also been shown by techniques similar to the ones used here to regulate cell proliferation. In addition, the effect of BSAP on cell proliferation can be related to recent findings which indicate that deregulation of several PAX genes promotes malignant transformation (35). Of interest, this oncogenesis depended on a functional paired domain that is unique to PAX genes and that mediates DNA binding of PAX gene products. At the moment it is not known which gene or genes mediate BSAP's effect on B cell proliferation. It is clear, however, that this function is very different from that of the above noted oncogenes or other PAX genes since the proliferative effect of BSAP is B cell specific.

Because BSAP has known binding sites near various switch regions, BSAP may play a role in the regulation of isotype switching. In fact, we found that downregulation of BSAP by antisense oligonucleotides does inhibit isotype switching induced by various stimuli, most notably, LPS plus IL-4. However, it is not clear whether this effect is mediated by BSAP binding sites in the Ig H chain locus or by a more general effect on cell proliferation, since proliferation has been shown to be a prerequisite of switch rearrangement (36, 37).

Recently, we (8) and Singh and Birshtein (7) have shown that BSAP binding sites in the Ig H chain 3' $\alpha$  enhancer have a negative effect on enhancer activity. For instance, in the experiments we performed, expression of a reporter gene driven by the 3' $\alpha$  enhancer plus  $\kappa$  L chain promoter was inhibited by cotransfection of a BSAP expression plasmid in MOPC-315 plasma cells. The fact that BSAP may repress the 3' $\alpha$  enhancer and thereby downregulate Ig gene transcription would explain why BSAP is not expressed in plasma cells, cells whose main function is high-rate Ig gene transcription (and subsequent Ig secretion). In summary, it appears that BSAP effects are quite complex and are dependent on the stage of B cell development. Early on, BSAP is necessary for B cell proliferation and differentiation. Later, after the B cell has terminally differentiated, BSAP is downregulated in order to enable high level Ig gene expression. In either case, the function of BSAP is closely linked to the two most important functions of B cells: proliferation and synthesis of Igs.



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