Identification of BSAP (Pax-5) target genes in early B-cell development by loss- and gain-of-function experiments

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The *Pax-5* gene codes for the transcription factor BSAP which is essential for the progression of adult B lymphopoiesis beyond an early progenitor (pre-BI) cell stage. Although several genes have been proposed to be regulated by BSAP, *CD19* is to date the only target gene which has been genetically confirmed to depend on this transcription factor for its expression. We have now taken advantage of cultured pre-BI cells of wild-type and Pax-5 mutant bone marrow to screen a large panel of B lymphoid genes for additional BSAP target genes. Four differentially expressed genes were shown to be under the direct control of BSAP, as their expression was rapidly regulated in Pax-5-deficient pre-BI cells by a hormone-inducible BSAP–estrogen receptor fusion protein. The genes coding for the B-cell receptor component Ig- α (*mb-1*) and the transcription factors N-myc and LEF-1 are positively regulated by BSAP, while the gene coding for the cell surface protein PD-1 is efficiently repressed. Distinct regulatory mechanisms of BSAP were revealed by reconstituting Pax-5-deficient pre-BI cells with full-length BSAP or a truncated form containing only the paired domain. IL-7 signalling was able to efficiently induce the *N-myc* gene only in the presence of full-length BSAP, while complete restoration of *CD19* synthesis was critically dependent on the BSAP protein concentration. In contrast, the expression of the *mb-1* and *LEF-1* genes was already reconstituted by the paired domain polypeptide lacking any transactivation function, suggesting that the DNA-binding domain of BSAP is sufficient to recruit other transcription factors to the regulatory regions of these two genes. In conclusion, these loss- and gain-of-function experiments demonstrate that BSAP regulates four newly identified target genes as a transcriptional activator, repressor or docking protein depending on the specific regulatory sequence context.

Keywords: B lymphopoiesis/Pax-5 target genes/LEF-1/*mb-1*/N-myc

Introduction

The development of B lymphocytes from committed progenitors to terminally differentiated plasma cells is a multi-step process and involves the ordered expression of a large number of lineage-specific genes which are primarily controlled at the transcriptional level. The role of B-cell specific transcription factors in this developmental process has been analyzed by gene targeting in the mouse, which resulted in a hierarchical model for the transcriptional control of B lymphopoiesis (reviewed by Clevers and Grosschedl, 1996). However, it has proven difficult to proceed further down the genetic cascade and to identify the crucial genes regulated by these factors. The most commonly used approach for identifying target genes relies on the characterization of gene regulatory regions whereby a binding site for a given transcription factor is defined by protein–DNA binding assays and by functional analyses in transfected cells (Busslinger and Bergers, 1994). The identification of target genes by promoter analysis is, however, prone to artefacts as a factor-binding site identified in this manner may not be important for the regulation of the endogenous gene. Indeed, many potential target genes for B-cell specific transcription factors have been published, but only for a few examples has the role of these regulators been genetically confirmed by loss- and gain-of-function experiments. One such example is the germline *I μ* transcript of the immunoglobulin heavy-chain (*IgH*) locus which was shown to be under the control of the *E2A* gene. The expression of this transcript is lost in pro-B cells of *E2A* mutant mice (Bain *et al.*, 1994) and can furthermore be activated in heterologous cells by ectopic expression of the *E2A*-encoded transcription factor E47 (Schlüssel *et al.*, 1991; Choi *et al.*, 1996). Likewise, forced expression of the transcriptional regulators EBF and E47 induces the expression of the endogenous surrogate light chain genes $\lambda 5$ and *VpreB* in hematopoietic progenitor cells (Sigvardsson *et al.*, 1997). Moreover, *CD36* was identified as the first Oct-2-regulated gene, as its down-regulation in Oct-2-deficient B lymphocytes can be rapidly reversed by the use of an Oct-2 induction system (König *et al.*, 1995). Herein we describe the identification and characterization of endogenous target genes for BSAP by using such a combination of loss- and gain-of-function experiments.

BSAP is one of the transcription factors whose importance in B-lymphoid development has been demonstrated by gene targeting in the mouse (Urbánek *et al.*, 1994; reviewed by Morrison *et al.*, 1998). BSAP is encoded by the *Pax-5* gene which is expressed, in addition to all B lymphoid organs, also in the embryonic midbrain and adult testis of the mouse (Adams *et al.*, 1992). BSAP is known to recognize DNA via its N-terminal paired domain (Czerny *et al.*, 1993) and to control gene transcription

through a C-terminal regulatory module consisting of activating and inhibitory sequences (Dörfler and Busslinger, 1996). During B-cell development, the *Pax-5* gene is expressed from the earliest B lineage-committed precursor cell up to the mature B cell stage, but not in terminally differentiated plasma cells (Barberis *et al.*, 1990). Detailed analysis of the *Pax-5* mutant phenotype revealed a differential dependency of fetal and adult B lymphopoiesis on this transcription factor. *Pax-5* is essential for B-lineage commitment in the fetal liver, while in adult bone marrow this transcription factor is required for progression of B-cell development beyond the early pre-B (pre-BI) cell stage (Nutt *et al.*, 1997). *Pax-5*-deficient pre-BI cells exhibit a dramatic reduction in V-to-DJ recombination at the *IgH* locus and furthermore can be cultured *ex vivo* in the presence of stromal cells and IL-7 (Nutt *et al.*, 1997). The availability of *in vitro* cultured pre-BI cells which genetically differ only by the presence or absence of *Pax-5* therefore offers a unique tool for the identification of BSAP target genes (Nutt *et al.*, 1997).

Several B-cell specific genes have been proposed to be regulated by BSAP on the basis of paired domain-binding sites which were identified in their regulatory regions by the use of protein–DNA binding and transient transfection assays (reviewed by Busslinger and Nutt, 1998). Postulated BSAP target genes expressed in early B lymphopoiesis comprise the genes coding for the cell surface protein CD19 (Kozmik *et al.*, 1992), the tyrosine kinase Blk (Zwollo and Desiderio, 1994), the transcription factor XBP-1 (Reimold *et al.*, 1996) and the surrogate light chains $\lambda 5$ and VpreB (Okabe *et al.*, 1992). Of all these genes, only the expression of *CD19* was shown to critically depend on *Pax-5* function (Nutt *et al.*, 1997).

We have now screened a large panel of B lymphoid genes for differential expression in wild-type and *Pax-5*-deficient pre-BI cells which resulted in the identification of four new BSAP target genes. These genes code for the transcription factors *N-myc* and *LEF-1* and the cell surface proteins *Ig- α* (*mb-1*) and *PD-1*. The expression of *N-myc*, *LEF-1* and *mb-1* is rapidly activated by a hormone-inducible BSAP–estrogen receptor (BSAP–ER) fusion protein in *Pax-5*-deficient pre-BI cells, while the *PD-1* gene is efficiently repressed. The role of BSAP in the regulation of these genes was further studied by reconstituting *Pax-5*-deficient pre-BI cells with full-length and truncated BSAP proteins, indicating that BSAP functions as a transcriptional activator, repressor or docking protein depending on the regulatory sequence context of the target gene. Where known, the function of the identified BSAP target genes is discussed in the light of the early B-cell developmental block of *Pax-5* mutant mice.

Results

Experimental strategy for the identification of BSAP target genes

By genetic evidence we have recently identified *CD19* as a BSAP-regulated gene as its expression is lost in *Pax-5*-deficient pre-BI cells compared with wild-type cells (Nutt *et al.*, 1997). Moreover, the *CD19* gene was shown previously to contain a high-affinity BSAP-binding site in the –30 region which appears to be indispensable for

promoter activity (Kozmik *et al.*, 1992). Therefore, we used *CD19* as a genuine target gene to establish cell systems expressing a conditionally active BSAP protein. As the activity of many transcription factors has been successfully brought under hormonal control by fusing them to the ligand-binding domain of the human ER (reviewed by Picard, 1994), we used this post-translational induction system to generate an estrogen-inducible version of BSAP. To this end, BSAP was linked at its C-terminus to the hormone-binding domain of the ER (Figure 1C). Moreover, the control protein Δ BSAP–ER was rendered incapable of DNA binding by deletion of the paired domain (Figure 1C). The transactivation potential of BSAP–ER, Δ BSAP–ER and full-length BSAP was analyzed in SP2/0 plasmacytoma cells by transient transfection of the corresponding expression plasmids together with the BSAP-dependent luciferase reporter gene, *luc-CD19* (Czerny and Busslinger, 1995; Figure 1C). In the absence of estrogen (E2), the BSAP–ER fusion protein was inactive in this assay, resulting in the same low background activity observed with the empty expression vector pKW2T alone (Figure 1A). However, upon estrogen activation, BSAP–ER stimulated reporter gene transcription to the same degree as the constitutively active BSAP protein (Figure 1A). Moreover, estrogen-dependent transactivation of BSAP–ER was dependent on the DNA-binding function of the paired domain, as the Δ BSAP–ER protein was unable to induce luciferase expression. In conclusion, the BSAP–ER fusion protein can efficiently activate transcription from an artificial BSAP-responsive promoter in a hormone-regulable manner.

The paired domain is known to lack any transactivation function (Dörfler and Busslinger, 1996), and hence we investigated whether the paired domain polypeptide PRD (Figure 1C) is able to act as a passive repressor by competing away full-length BSAP from a BSAP-responsive promoter. For this purpose, increasing amounts of the PRD expression plasmid were transiently transfected together with a constant amount of the BSAP expression vector into RAC65 embryonal carcinoma cells where transcription of the reporter gene *luc-CD19* could be stimulated 18-fold by BSAP (Figure 1B). This BSAP-dependent transactivation was efficiently blocked, even at low concentrations, by the PRD polypeptide (Figure 1B). Hence, the PRD protein can antagonize functional BSAP in transient transfection experiments.

To study the potential of BSAP and its derivatives BSAP–ER, Δ BSAP–ER and PRD to regulate the transcription of endogenous genes, we infected cultured pre-BI cells from *Pax-5*-deficient bone marrow (Nutt *et al.*, 1997) with retroviral vectors coding for all four proteins. The resulting cell pools were analyzed for the expression of the different BSAP proteins by electrophoretic mobility shift assay (EMSA). To simplify the nomenclature of the different cell lines, we refer to the *Pax-5*-deficient pre-BI cells expressing BSAP, BSAP–ER and PRD as KO–BSAP, KO–BSAP–ER and KO–PRD cell lines. As shown by the DNA-binding assay in Figure 2A, the KO–BSAP cell line #2 and the KO–PRD cells expressed the BSAP proteins at levels comparable with that of wild-type pre-BI cells, while BSAP expression was 10-fold lower in a second KO–BSAP cell pool (#1). As the absence of a functional DNA-binding domain prevents detection of the Δ BSAP–

Fig. 1. Estrogen-inducible and dominant negative versions of BSAP. (A) Transactivation potential of the BSAP-ER, ΔBSAP-ER and full-length BSAP proteins in SP2/0 cells. The expression plasmids pKW2T (empty vector), pBSAP, pBSAP-ER and pΔBSAP-ER (2 μg each) were electroporated into the plasmacytoma cell line SP2/0 together with the reporter gene *luc-CD19* (5 μg) and the reference *CMV-CAT* gene (0.1 μg; transfection efficiency control). The transfected cells were divided in two halves, and one pool was treated with 1 μM β-estradiol (E2) for 24 h prior to harvesting. Luciferase activities of three independent transfection experiments were normalized to measured CAT activities and are shown as average values relative to the basal activity observed with the pKW2T vector alone. (B) Repression of BSAP-dependent transactivation by the paired domain polypeptide PRD. The plasmids *luc-CD19* (5 μg), *CMV-CAT* (0.1 μg) and pBSAP (0.5 μg) were transfected into RAC65 cells together with increasing amounts of the expression vector pPRD (0.2–2 μg). Normalized luciferase activities of three independent experiments are shown as values relative to the basal expression level of the *luc-CD19* gene (in the absence of BSAP). (C) Schematic representation of the different BSAP constructs. The full-length BSAP protein (amino acids 1–391; Adams *et al.*, 1992) is shown together with its domain structure. The abbreviations for the different domains are: PRD, paired domain (DNA-binding motif); OP, conserved octapeptide; HDH, homeodomain homology region; TA, transactivation domain; ID, inhibitory domain. The polypeptide PRD consists of amino acids 1–145 of BSAP linked to the SV40 nuclear localization signal (NLS; Kalderon *et al.*, 1984). The BSAP-ER protein was generated by fusing BSAP at its C-terminus to the hormone-binding domain (HBD) of the human estrogen receptor (hER) (Superti-Furga *et al.*, 1991). The amino acids joining the two proteins are shown together with the respective amino acid positions of the two proteins. The DNA-binding function of the paired domain was inactivated by deletion of 105 amino acids in construct ΔBSAP-ER. The reporter gene *luc-CD19* contains three copies of the high-affinity BSAP-binding site CD19-2 (A-ins) upstream of a TATA box and the luciferase gene (Czerny and Busslinger, 1995).

ER protein by EMSA analysis, we measured expression of the fusion proteins by intracellular staining with a polyclonal anti-BSAP antibody (Adams *et al.*, 1992) followed by flow-cytometric analysis. As shown in Figure 2B, the expression level of the fusion proteins in KO-BSAP-ER and KO-ΔBSAP-ER cell lines was similar to that of BSAP in wild-type cells. Moreover, the expression

of the BSAP-ER protein was rather homogeneous within each cell population, which was also confirmed by immunohistochemical analysis with the same anti-BSAP antibody (Figure 2B). In contrast, the BSAP expression level displayed a high degree of heterogeneity among individual cells of the KO-BSAP pool #2, as shown by flow-cytometric and immunohistochemical methods. This heterogeneous expression pattern was maintained in KO-BSAP cells despite strong antibiotic selection. As expected, the endogenous and retrovirally expressed BSAP proteins were localized in the nucleus (Figure 2B). In contrast, the estrogen receptor fusion protein was predominantly retained in the cytoplasm in the absence of ligand (Figure 2C) and was only efficiently transported to the nucleus upon estrogen treatment (Figure 2B). These data indicate therefore that the transcriptional inactivity of BSAP-ER is partially determined by its cytoplasmic sequestration in the absence of hormone.

Induction of CD19 expression on the cell surface of reconstituted Pax-5-deficient pre-BI cells

Restoration of CD19 expression provided a convenient assay for measuring the activity of the different BSAP proteins in reconstituted Pax-5 (–/–) pre-BI cells, as CD19 can be readily detected on the cell surface by flow-cytometric analysis with an anti-CD19 monoclonal antibody (Krop *et al.*, 1996). In agreement with previous data (Nutt *et al.*, 1997), the CD19 protein was completely absent on Pax-5 (–/–) pre-BI cells in contrast to wild-type (+/+) cells (Figure 3). Low but significant CD19 expression was observed on the two reconstituted KO-BSAP cell lines (#1 and #2), indicating that retroviral BSAP transcription could only partially restore CD19 expression in Pax-5 (–/–) pre-BI cells (addressed in detail at the end of the Results section). Importantly, CD19 expression was efficiently induced on KO-BSAP-ER cells within 18 h of estrogen addition, while it was undetectable on these cells in the absence of ligand (Figure 3). CD19 induction was also observed with the estrogen antagonist 4-hydroxy-tamoxifen (data not shown), which is unable to induce the hormone-dependent transactivation function of the estrogen receptor (Webster *et al.*, 1988). Furthermore, the KO-ΔBSAP-ER cells failed to activate CD19 expression upon estrogen treatment, thus indicating that the DNA-binding domain is required together with the transactivation function of BSAP for direct regulation of the *CD19* gene. In summary, these data fully validate the combined use of Pax-5-deficient pre-BI cells and the BSAP-ER induction system for the identification of target genes.

Identification of potential BSAP targets by a candidate gene approach

The availability of cultured pre-BI cell lines of wild-type (+/+) and Pax-5 (–/–) genotype made it possible to search for additional BSAP-regulated genes using a candidate gene approach. To this end, a large panel of genes which are known to be important for various aspects of B lymphopoiesis was chosen for comparative expression analysis in wild-type and Pax-5-deficient pre-BI cells. The results of these RNase protection analyses are summarized in Table I where the expression of the different genes is indicated as relative mRNA levels. The expression of the

Fig. 2. Expression of constitutively active and estrogen-inducible BSAP proteins in Pax-5-deficient pre-BI cells. **(A)** Quantitation of the BSAP proteins by DNA-binding assay. Pax-5-deficient pre-BI cell lines were infected with retroviruses expressing the PRD, BSAP, BSAP-ER or Δ BSAP-ER proteins, and stable cell pools were subsequently established by puromycin selection. Nuclear extracts were prepared from the parental Pax-5 ($-/-$) pre-BI cells, the KO-PRD cells and two KO-BSAP cell pools. Equivalent amounts of each extract were analyzed for the DNA-binding activity of BSAP by EMSA analysis with a labelled oligonucleotide containing the paired domain-binding site 1 of the *CD19* gene (Kozmik *et al.*, 1992). **(B)** Nuclear localization and quantitation of BSAP proteins by immunofluorescence analysis. The KO-BSAP-ER and KO- Δ BSAP-ER cells were treated with 1 μ M estrogen for 24 h prior to fixation. Cells were stained with affinity-purified polyclonal rabbit antibodies directed against the C-terminal part of BSAP (amino acids 189–391; Adams *et al.*, 1992) followed by detection with FITC-conjugated goat anti-rabbit antibodies. The stained cells were examined by fluorescence microscopy and flow cytometry. For microscopy, the nuclei were visualized by DAPI staining of chromosomal DNA. Flow-cytometric analysis was used to quantitate the BSAP protein levels in the reconstituted pre-BI cells relative to the background staining of the parental Pax-5 ($-/-$) cells. **(C)** Cytoplasmic localization of the unliganded BSAP-ER protein. KO-BSAP-ER cells were cultured in the absence of estrogen prior to detection of the BSAP-ER protein by fluorescence microscopy as described in (B).

majority of the 49 genes analyzed was unaffected by the absence of Pax-5 in pre-BI cells. These precursor cells are characterized by the expression of the early markers VpreB, λ 5, RAG-1, RAG-2, TdT, CD37 and CD22 and by the absence of transcripts for the more mature B-cell markers CD20, CD21, CD23, CD40 and CD72. Moreover, most genes coding for intracellular kinases and hematopoietic transcription factors were equally expressed in the pre-BI cells of both genotypes. In conclusion, these data provide a fairly detailed description of the gene expression pattern in pre-BI cells and furthermore demonstrate that the Pax-5-deficient pre-BI cells closely resemble their wild-type counterparts with regard to the expression of most genes.

Apart from *CD19*, the expression of six additional genes was affected in pre-BI cells by the absence of Pax-5 (Figure 4; bold lettering in Table I). The expression of

the genes coding for the transcription factors LEF-1 (Travis *et al.*, 1991) and N-myc (DePhino *et al.*, 1986), the signalling component mb-1 (Ig- α) of the pre-B- and B-cell receptors (Sakaguchi *et al.*, 1988) and the anti-apoptotic regulator Bcl- x_L (Boise *et al.*, 1993) was reduced ~10-fold in Pax-5-deficient pre-BI cell (Figure 4A). In contrast, two genes, coding for the transcription factor c-Myc (Stanton *et al.*, 1984) and the cell surface protein PD-1 (Ishida *et al.*, 1992), were consistently up-regulated in Pax-5-deficient pre-BI cells (Figure 4A). Of all these genes, only *CD19* lost even its basal level of expression in the absence of BSAP, while the expression of *PD-1* was below detection in the presence of BSAP. Recently, Stuart *et al.* (1995) suggested on the basis of transient transfection data that Pax-5 (BSAP) is a potent repressor of *p53* gene transcription. However, *p53* expression is totally unaffected by the loss of Pax-5 function, thus

Fig. 3. Restoration of CD19 expression on Pax-5-deficient pre-BI cells by BSAP reconstitution. Wild-type (+/+) and Pax-5 mutant (-/-) pre-BI cells as well as the indicated KO-BSAP(ER) cell lines were analyzed for CD19 expression by flow cytometry with a biotinylated anti-CD19 antibody (1D3) which was detected by incubation with PE-coupled streptavidin. The KO-BSAP-ER and KO-ΔBSAP-ER cells were treated with 1 μ M β -estradiol for 18 h prior to analysis. Unstained control cells are shown by a stippled line.

providing genetic evidence against an involvement of BSAP in *p53* gene regulation (Figure 4B).

Rapid and direct regulation of *CD19*, *mb-1*, *LEF-1*, *N-myc* and *PD-1* by BSAP-ER

The induction kinetics of the differentially expressed genes was next studied in KO-BSAP-ER cells to identify those genes which are rapidly and thus directly regulated by BSAP. To this end, the KO-BSAP-ER cells were treated with 1 μ M estrogen for different time periods up to 24 h, and the response of the putative target genes to estrogen stimulation of BSAP-ER was analyzed by RNase protection assay (Figure 5). Two of the differentially expressed genes, *bcl-x_L* and *c-myc*, could neither be activated nor repressed by BSAP-ER during the time interval studied, indicating that both genes are unlikely to be under the direct control of the transcription factor BSAP (S.Nutt, data not shown). In contrast, transcription of the *CD19*, *mb-1*, *LEF-1* and *N-myc* genes was rapidly induced by estrogen addition, although the kinetics of mRNA accumulation varied slightly for the different genes (Figure 5). *CD19* expression increased throughout the 24 h period analyzed, while the *mb-1* and *LEF-1* mRNAs reached their maximal levels in 12 h and the *N-myc* transcript in only 8 h. Importantly, none of these genes was activated by estrogen treatment in KO-ΔBSAP-ER cells, indicating that the DNA-binding function of BSAP is essential for their transcriptional activation (Figure 5). Next we have

Table I. Gene expression pattern in wild-type and Pax-5-deficient pre-BI cells

Gene	pre-BI cells		Reference
	<i>Pax-5</i> (-/-)	<i>Pax-5</i> (+/+)	
Target genes			
<i>CD19</i>	—	+++++	a
<i>mb-1</i>	+	+++	
<i>PD-1</i>	++	—	
<i>LEF-1</i>	+	+++	
<i>N-myc</i>	+	+++	
Miscellaneous			
<i>bcl-x_L</i>	+	+++	a
<i>bcl-2</i>	++	++	
<i>Iμ</i>	+++++	+++++	
<i>Iε</i>	—	—	
<i>Iγ1</i>	++	++	
<i>RAG-1</i>	++	++	a
<i>RAG-2</i>	+	+	a
<i>TdT</i>	+++	+++	b
<i>PLRLC</i>	+++	+++	
<i>Mil</i>	++	++	
Cell surface proteins			
<i>λ5</i>	+++	+++	a
<i>VpreB</i>	+++++	+++++	a
<i>B29</i>	+++	+++	
<i>CD20</i>	—	—	
<i>CD21</i>	—	—	
<i>CD22</i>	+	+	
<i>CD23</i>	—	—	
<i>CD37</i>	+++++	+++++	
<i>CD40</i>	—	—	
<i>CD72</i>	—	—	
Kinases			
<i>lyn</i>	++	++	a
<i>fyn</i>	++	++	
<i>syk</i>	++	++	
<i>btk</i>	++	++	
<i>blk</i>	++	++	
Transcription factors			
<i>c-myc</i>	+++	+	a
<i>XBP-1</i>	++	++	
<i>p53</i>	+++	+++	
<i>Sox-4</i>	++	++	
<i>c-myb</i>	++	++	
<i>Oct-1</i>	++	++	a
<i>Oct-2</i>	++	++	a
<i>Ikaros</i>	+++	+++	a
<i>E2A</i>	+++	+++	a
<i>OBF-1</i>	++	++	a
<i>WT-1</i>	—	—	
<i>PU.1</i>	++	++	a
<i>Blimp-1</i>	—	—	
<i>EBF</i>	++	++	a
<i>Id-1</i>	+	+	
<i>Id-2</i>	++	++	b
<i>Id-3</i>	++	++	b
<i>Id-4</i>	+	+	
<i>ER</i>	+	+	

The mRNA levels of the indicated genes were quantitated by RNase protection assay in cultured pre-BI cells of wild-type (+/+) and Pax-5 mutant (-/-) genotypes (see Materials and methods). The *S16* mRNA was co-mapped as internal reference transcript. The results are summarized as relative mRNA levels using a scale ranging from rare (+) to abundant (++++). Some genes were not expressed (-) in the pre-BI cells of both genotypes. a; previously published expression data (Nutt *et al.*, 1997). b; significant fluctuation of gene expression between cell lines of the same genotype.

Fig. 4. Regulation of gene expression by BSAP in pre-BI cells. (A) 10-fold reduction of *mb-1*, *LEF-1*, *N-myc* and *bcl-x_L* transcription and derepression of the *PD-1* and *c-myc* genes in Pax-5-deficient pre-BI cells. Bone marrow pre-BI cells from wild-type (+/+), heterozygous (+/-) and two homozygous Pax-5 mutant (-/-) mice were propagated on stromal ST2 cells in the presence of IL-7. Total RNA prepared from these pre-BI cells was analysed by RNase protection assay for the presence of the indicated gene transcripts (see Materials and methods). Transcripts coding for the small ribosomal protein S16 were co-mapped and used as an internal reference for quantitation of the RNA signals by PhosphorImager analysis. (B) *p53* expression is not effected by the absence of BSAP. The same pre-BI cell lines described above were used for RNase protection analysis with a mouse *p53* and reference *S16* probe.

taken advantage of the fact that the BSAP-ER protein is already present in an inactive form before estrogen stimulation of the KO-BSAP-ER cells. Hence, the BSAP-ER protein can be activated by estrogen addition even in the presence of the translational inhibitor cycloheximide (CHX) blocking all protein synthesis. Under these conditions, BSAP-ER is still able to induce transcription of *CD19*, *mb-1*, *N-myc* and *LEF-1*, thus demonstrating that all four genes are directly controlled by BSAP (Figure 6A).

The BSAP-ER induction system was useful even for studying the repression of the *PD-1* gene. The mRNA level of this gene was rapidly reduced in KO-BSAP-ER cells, resulting in efficient repression within 24 h of estrogen addition (Figure 5). This transcriptional repression also requires the DNA-binding function of BSAP, as it was not observed in estrogen-treated KO-ΔBSAP-ER cells. The rapid kinetics of *PD-1* down-regulation strongly predict that its mRNA must decay with a short half-life in order to provide a read-out for transcriptional repression in our mRNA analysis. Indeed, direct measurement of the mRNA stability by actinomycin D treatment of Pax-5-deficient pre-BI cells revealed a half-life of <1 h for the *PD-1* transcript (S.Nutt, data not shown). Moreover, the level of *PD-1* mRNA was substantially increased by cycloheximide treatment of KO-BSAP-ER cells, thus

suggesting that the *PD-1* transcript is stabilized in the absence of on-going protein synthesis (Figure 6B). As a short half-life coupled with mRNA stabilization in the presence of translational inhibitors is a classical hallmark of immediate early genes, it is likely that *PD-1* also belongs to this gene family, which is further supported by the specific induction of *PD-1* expression in stimulated B and T cells (Agata *et al.*, 1996). Hence, BSAP appears to be involved in the repression of the immediate early gene *PD-1*.

Differential regulation of target gene expression by BSAP

As the cell surface expression of CD19 could be restored only partially by reintroduction of BSAP in the Pax-5-deficient pre-BI cells (Figure 3), we next investigated the responsiveness of the newly identified target genes to regulation by full-length BSAP. To this end, the expression of the *mb-1*, *LEF-1*, *N-myc* and *PD-1* genes was compared by RNase protection assay with that of *CD19* in KO-BSAP, KO-PRD and wild-type pre-BI cells (Figure 7). The transcript levels in the different cell lines were quantitated and normalized to the expression of the small ribosomal protein S16 gene. This analysis revealed that the expression of the *mb-1*, *LEF-1* and *N-myc* genes could be almost fully reconstituted to wild-type levels in both KO-BSAP cell lines. Although some fluctuation in the expression of these genes was observed, it did not correlate with the 10-fold difference in BSAP expression of the two cell lines (Figure 2A). In contrast, the repression of the *PD-1* gene consistently correlated with BSAP protein levels, as it was minimal in the KO-BSAP cell pool #1 and 5-fold in the KO-BSAP cells #2 relative to the Pax-5-deficient pre-BI cells. The inability of some cells to fully repress or activate gene transcription may be caused by the heterogeneity of the KO-BSAP cells, as the expression of BSAP differed considerably among individual cells of these cell pools (Figure 2B). Despite this cellular heterogeneity, the newly identified target genes proved to be efficiently regulated by full-length BSAP in contrast to *CD19* which was expressed at a 50-fold lower level in KO-BSAP cells compared with wild-type cells (Figure 7).

Surprisingly, the PRD protein was almost as efficient as full-length BSAP in activating the endogenous *mb-1* and *LEF-1* genes and in repressing the *PD-1* gene (Figure 7). In contrast, expression of the *CD19* and *N-myc* genes could be activated only by full-length BSAP. These observations are particularly interesting as the PRD polypeptide lacks any transactivation function (Dörfler and Busslinger, 1996; Figure 1B). Hence, the paired domain constituting only one third of the entire BSAP protein is already sufficient for transcriptional regulation of the *mb-1*, *LEF-1* and *PD-1* genes. Detailed analysis of the *mb-1* promoter revealed a functional BSAP-binding site at position -80 adjacent to a suboptimal recognition sequence for Ets transcription factors at position -70 (Fitzsimmons *et al.*, 1996). On the basis of biochemical and cell transfection data, these authors postulated that BSAP regulates the *mb-1* gene by recruiting Ets proteins to the promoter and that this recruitment activity depends solely on the DNA-binding function of BSAP. In agreement with this hypothesis we have now observed that the PRD polypeptide is indeed able to activate the endogenous

Fig. 5. Rapid transcriptional induction of endogenous target genes by BSAP-ER. The pre-BI cell lines KO-BSAP-ER and KO-ΔBSAP-ER were treated with 1 μ M β -estradiol (E2), and RNA was isolated at the indicated time intervals after estrogen addition. Total RNA (10 μ g) was analysed by RNase protection assay with *CD19*, *mb-1*, *N-myc*, *LEF-1* and *PD-1* riboprobes in conjunction with a *S16* reference probe. The RNA signals were quantified on a PhosphorImager, normalized to the *S16* transcript and displayed as a bar graph to the right. Black and grey bars indicate the effect of BSAP-ER and ΔBSAP-ER on gene expression, respectively.

mb-1 gene in pre-BI cells (Figure 7). In summary, the reconstitution of BSAP expression in Pax-5-deficient pre-BI cells demonstrated a differential requirement of functional BSAP domains for the regulation of endogenous target genes which is most likely determined by the distinct regulatory sequence context of each individual gene.

BSAP is essential for efficient IL-7 induction of *N-myc* expression

The expression of both the *N-myc* and *c-myc* genes is known to be rapidly induced in pre-BI cells by IL-7 stimulation (Morrow *et al.*, 1992). Hence, *N-myc*, like *c-myc*, is an early response gene in these cells. The down-regulation of *N-myc* expression in Pax-5-deficient pre-BI cells (Figure 4) therefore raises the question as to whether

BSAP controls *N-myc* expression as part of an IL-7-dependent or -independent regulatory pathway. To address this issue, pre-BI cells of wild-type and Pax-5 mutant genotype were deprived of IL-7 for 6 h, before they were re-stimulated by the addition of IL-7 for various time intervals (Figure 8A). The *N-myc* mRNA levels were subsequently measured and normalized to *S16* expression by RNase protection assay. In wild-type pre-BI cells, the *N-myc* gene behaved as previously described (Morrow *et al.*, 1992), as its mRNA level was reduced ~8-fold upon IL-7 withdrawal and was rapidly restored to maximal expression levels within 30 min of IL-7 re-addition (Figure 8A). Interestingly, Pax-5-deficient pre-BI cells showed, under normal IL-7 culture conditions, only basal *N-myc* expression which was not further reduced in the absence

Fig. 6. Direct regulation of target gene expression by BSAP-ER. (A) Transcriptional induction in the absence of protein synthesis. KO-BSAP-ER cells were treated for 8 h with the translational inhibitor cycloheximide (CHX; 50 μ g/ml). During the last 4 h of this treatment, β -estradiol (E2; 1 μ M) was added to one aliquot of the cells prior to RNA preparation and RNase protection analysis. (B) Increase of the *PD-1* mRNA level in the absence of protein synthesis. KO-BSAP-ER cells were either treated with CHX alone or simultaneously with CHX and E2 for 8 h. Untreated KO-BSAP-ER cells were used as control for RNase protection analysis of the *PD-1* transcript. The same amount of *S16* mRNA was detected in all cases. *PD-1* gene repression by estrogen-induced BSAP-ER could not be observed under these conditions, as CHX treatment resulted in considerable stabilization of the *PD-1* mRNA.

of IL-7. Pax-5-deficient cells were, however, still able to accumulate *N-myc* transcripts upon IL-7 stimulation, although with considerably delayed kinetics and to lower levels than wild-type pre-BI cells (Figure 7A and B). Importantly, reconstitution of BSAP expression in the KO-BSAP cells could fully restore IL-7 responsiveness to the *N-myc* gene (Figure 8A). Together these data demonstrate therefore that BSAP is indispensable for efficient IL-7 induction of *N-myc* expression. In this context it is interesting to note that IL-7 treatment of both wild-type as well as Pax-5-deficient pre-BI cells induced expression of the *c-myc* gene with similar kinetics (Figure 8A). Hence, the absence of BSAP did not result in a general impairment of IL-7 signalling. Moreover, the steady-state levels of the *c-myc* and *N-myc* transcripts were inversely correlated between wild-type and mutant pre-BI cells (Figures 4A and 8A), implying that increased *c-myc* expression may functionally compensate for the loss of *N-myc* mRNA in Pax-5-deficient pre-BI cells.

***CD19* expression is sensitive to the dosage of BSAP**

Of all the BSAP target genes analyzed, *CD19* proved to be a special case for the following two reasons. First, the expression of this gene was strictly dependent on BSAP, as even basal-level transcription was lost in Pax-5-deficient pre-BI cells (Nutt *et al.*, 1997). Secondly, the restoration

Fig. 7. Different target genes require distinct functional domains of BSAP for their regulation. The expression of the *CD19*, *mb-1*, *LEF-1*, *N-myc* and *PD-1* genes was analyzed and quantified by RNase protection assay in wild-type (+/+), Pax-5 deficient (-/-), KO-BSAP and KO-PRD pre-BI cells as described in the legend to Figure 4. The expression level of each gene was determined by at least two different RNase protection experiments and is shown below each lane as average value relative to that of the Pax-5 (-/-) pre-BI cells. A representative experiment is shown for each transcript. The arbitrary background of the autoradiographic exposure (indicated by an asterisk) had to be used as reference for the *CD19* and *N-myc* signals as the level of these two transcripts was below detection in Pax-5 (-/-) pre-BI cells.

of *CD19* expression was inefficient in KO-BSAP cells, even though the overall level of retroviral BSAP expression in cell pool #2 was comparable with that of wild-type cells (Figures 2A and 3). The variability of *CD19* expression in these reconstituted cells suggests therefore that the transcription of the *CD19* gene may be highly sensitive to fluctuations of BSAP expression at the single cell level. To investigate this phenomenon, we used fluorescence-activated cell sorting to separate the KO-BSAP cell pool #2 into *CD19*⁺ and *CD19*⁻ cells. The fate of *CD19* expression in the two separated cell fractions was then followed at different time intervals by flow-cytometric analysis. As shown in Figure 9A, all the sorted *CD19*⁺ cells initially expressed *CD19*, but then rapidly down-regulated the expression of this gene within 14 days in culture, until <20% of the cells remained *CD19*⁺. Conversely, a low percentage (9.3%) of the *CD19*⁻ cells started within the same time period to re-express *CD19* on the cell surface (S.Nutt, data not shown). We conclude therefore that the majority of the KO-BSAP cells are indeed unable to stably maintain their *CD19* expression pattern.

To investigate a possible correlation between the *CD19* and BSAP expression levels, we derived clonal cell populations from individual *CD19*⁺ cells by single cell

Fig. 8. BSAP is required for efficient IL-7 induction of *N-myc* expression in pre-BI cells. **(A)** IL-7 stimulation of *N-myc* and *c-myc* expression. Wild-type (+/+), *Pax-5* mutant (-/-) and KO-BSAP pre-BI cells were cultured on stromal ST2 cells in the presence of IL-7, then divided into four plates and subjected to the treatment depicted in the bottom part of (A). Total RNA was isolated from cells which were grown either continuously in the presence of IL-7 (lane 1), without IL-7 for 6 h (lane 2), or following re-addition of IL-7 for 30 min (lane 3) and 4 h (lane 4). The *N-myc* and *c-myc* mRNA levels were analysed by RNase protection assay together with the reference *S16* mRNA. All autoradiographs were exposed for the same time period. **(B)** Time course of IL-7 stimulated *N-myc* expression. Wild-type (+/+) and *Pax-5*-deficient (-/-) pre-BI cells were subjected to the above treatment with the exception that the time course of IL-7 stimulation was extended to 24 h. The *N-myc* mRNA levels were quantitated and normalized to those of the *S16* transcript by PhosphorImager analysis. The *N-myc* mRNA concentration is plotted relative to the basal mRNA level observed after IL-7 withdrawal.

sorting of KO-BSAP cells (#2). As shown by the representative examples in Figure 9B, a large degree of variability in CD19 expression was observed among the 24 clones which were analyzed three weeks after single cell cloning. These cell clones could be divided roughly into three categories. The first category consisted of six clones (25%) which were either CD19-negative or expressed CD19 only at a low level in <20% of the cells (Figure 9B, clone 4). With the exception of clone 4, however, these clonal populations expressed, relatively high levels of the BSAP protein which was detected by intracellular staining and flow-cytometric quantitation with an anti-BSAP antibody (Figure 9B; data not shown). We conclude therefore that BSAP was unable to activate *CD19* transcription in these clones which lost their BSAP responsiveness for unknown reasons within the 3-week culture period. The second and largest category comprised 12 clones (50%) which were characterized by heterogeneous CD19 expression on 20–80% of all cells (Figure 9B, clones 2 and 3). Where analyzed, these clones showed good correlation between

the expression levels of BSAP and CD19. The third and most interesting group contained six clones (25%) which exhibited homogeneous CD19 expression on 80–100% of all cells (Figure 9B, clone 1). The level of CD19 expression on these cells was comparable with that of wild-type pre-BI cells and furthermore could be stably maintained for at least 5 weeks in culture. Most interestingly, even the BSAP expression level of these clones was homogeneous and similar, if not identical, to that of wild-type pre-BI cells (Figure 9B, clone 1). In conclusion, the transcription of the *CD19* gene appears to be highly sensitive to the dosage of BSAP, as stable and efficient *CD19* expression was observed in KO-BSAP cells only at the optimal BSAP concentration present in wild-type B lymphocytes.

Discussion

The transcription factor BSAP (*Pax-5*) is known to be essential for B lineage commitment in the fetal liver and for B-lymphoid development beyond the early pro-B (pre-BI) cell stage in adult bone marrow (Nutt *et al.*, 1997). However, the molecular mechanisms which depend on BSAP activity at these early developmental stages have not as yet been defined. Although several genes have been proposed to be regulated by BSAP, only the expression of *CD19* was affected in *Pax-5*-deficient pre-BI cells (Nutt *et al.*, 1997). Here we describe the identification of additional BSAP target genes by a candidate gene approach which relies on the comparative analysis of gene expression between wild-type and *Pax-5*-deficient pre-BI cells. Moreover, we have generated a hormone-inducible BSAP-ER fusion protein which was used in *Pax-5*-deficient pre-BI cells to demonstrate rapid and direct regulation of endogenous target genes by BSAP. Out of 49 genes, which were mainly selected on the basis of their known function in B-cell development, we have identified four additional target genes which can be rapidly induced (*mb-1*, *N-myc*, *LEF-1*) or repressed (*PD-1*) by BSAP-ER in *Pax-5*-deficient pre-BI cells. In addition, the *c-myc* gene was up-regulated and the *bcl-x_L* gene down-regulated in the absence of BSAP. However, these two genes were refractory to regulation by both inducible BSAP-ER activity and constitutive BSAP expression, implying that their deregulation in *Pax-5*-deficient pre-BI cells is an indirect consequence of the loss of BSAP function. Interestingly, the expression of the *p53* gene was not affected by the absence of BSAP in pre-BI cells (Figure 4B), thus providing strong genetic evidence against the hypothesis of Stuart *et al.* (1995) that *Pax-5* (BSAP) is a potent repressor of *p53* gene transcription.

Function of BSAP target genes in B-cell development

Three of the BSAP-regulated genes (*mb-1*, *CD19* and *PD-1*) code for cell surface molecules involved in signal transduction, while the products of two target genes (*N-myc* and *LEF-1*) are nuclear transcription factors. The question therefore arises whether the function of these genes is related to the early B-cell developmental block of *Pax-5* mutant mice.

The *mb-1* gene encodes the transmembrane molecule Ig- α which, together with the Ig- β (B29) protein, forms a heterodimer mediating signal transduction through the

Fig. 9. The expression of *CD19* is dependent on the concentration of BSAP. (A) Instability of *CD19* expression in sorted *CD19*⁺ cells of the KO-BSAP pool #2. At day 0, the *CD19*⁺ cells, which were located within the indicated gate, were isolated by fluorescence-activated cell sorting and proven to be all *CD19*⁺ by re-analysis of an aliquot of the sorted cells (shown in the insert). The sorted *CD19*⁺ cells were cultured on stromal ST2 cells in the presence of IL-7 for the indicated time period and then analyzed for *CD19* expression by flow cytometry (see legend to Figure 3). (B) Correlation between *CD19* and BSAP expression in individual KO-BSAP cell clones. Single *CD19*⁺ cells were sorted from the KO-BSAP cell pool #2 and grown into clonal cell populations in the presence of ST2 cells and IL-7. After 21 days, each cell clone was analyzed for cell surface *CD19* and intracellular BSAP expression by flow-cytometric analysis as described in the legends to Figures 3 and 2B, respectively. The analysis of four representative cell clones is shown.

pre-B- and B-cell receptors (reviewed by Borst *et al.*, 1996). The role of the *mb-1* gene in B-cell development has so far been analyzed in a mouse mutant containing a deletion of the cytoplasmic tail of Ig- α . This modification does not correspond to a null mutation, and indeed early B-cell development was only mildly impaired in this mouse strain (Torres *et al.*, 1996). However, the synthesis of a functional pre-B cell receptor is known to correspond to an important checkpoint in B-cell development, as it initiates the transition from the pro-B- (pre-BI) to the pre-B- (pre-BII-) cell stage (Borst *et al.*, 1996). Indeed, B lymphopoiesis is arrested at the pro-B cell stage in mice lacking the Ig- β (*B29*) gene (Gong and Nussenzweig, 1996), which superficially resembles the developmental block of Pax-5-deficient mice. The 10-fold reduction observed in Ig- α (*mb-1*) expression is, however, unlikely to be the principle cause for the differentiation arrest in Pax-5-deficient bone marrow for the following reason. The fusion protein μ -Ig β , which consists of the external and transmembrane domains of the immunoglobulin μ heavy chain linked to the intracellular domain of the Ig- β protein, is known to signal the transition from the pro-B- to the pre-B-cell stage even in the absence of the Ig- α or Ig- β protein (Sanchez *et al.*, 1993; Papavasiliou *et al.*,

1995). The expression of a functional μ -Ig β transgene was, however, unable to advance B-cell development to the pre-B-cell stage in Pax-5 mutant mice, indicating that the developmental block cannot be caused by the impaired Ig- α (*mb-1*) expression in these mice (C.Thévenin and M.Busslinger, unpublished data).

The *CD19* protein forms a complex with *CD21*, *CD81* and *Leu-13* on the surface of mature B cells. This complex is known to associate with the B-cell receptor whereby *CD19* acts as a co-stimulatory molecule to lower the threshold for antigen-dependent signalling (reviewed by Tedder *et al.*, 1997). In agreement with this function, the processes of B-cell activation, selection and maturation are severely impaired in mice lacking *CD19* (Engel *et al.*, 1995; Rickert *et al.*, 1995). However, B-lymphoid development up to the mature B-cell stage was unperturbed in the bone marrow of these mice. Hence, the lack of *CD19* expression cannot explain the early developmental block in the bone marrow of Pax-5-deficient mice.

The transmembrane protein *PD-1* is a member of the immunoglobulin superfamily which has been implicated in signal transduction due to the presence of tyrosine kinase association motifs in its cytoplasmic domain (Ishida *et al.*, 1992). *PD-1* was originally cloned as a gene that

is rapidly up-regulated in a T-cell line upon induction of apoptosis (Ishida *et al.*, 1992). Subsequently, the PD-1 protein was shown to be expressed on stimulated B and T cells in different lymphoid organs (Agata *et al.*, 1996). *PD-1* therefore appears to be an lymphocyte-specific early response gene. This notion is supported by the characteristic short half-life of the *PD-1* mRNA (S. Nutt, data not shown), which made it possible to study the rapid transcriptional repression of this gene by BSAP-ER in our steady state mRNA analysis. Given the observation that *PD-1* is an early response gene, it is unlikely that the initial cloning as an apoptosis-induced transcript is indicative of the function of this gene. In fact, mature B cells are generated in mice lacking *PD-1*, although they show an augmented response to antigenic stimulation (T.Honjo, personal communication).

The *N-myc* gene is tightly regulated during B lymphopoiesis, as it is expressed only in pro-B and pre-B cells, but not at more mature stages of B-cell differentiation (Zimmerman *et al.*, 1986; Smith *et al.*, 1992). The role of *N-myc* in B-cell development, however, could not be investigated by targeted gene inactivation in the mouse germline, as *N-myc*-deficient embryos die at ~11.5 days of gestation due to multiple developmental defects (Charron *et al.*, 1992; Stanton *et al.*, 1992). Hence, the RAG-2 blastocyst complementation assay was used to demonstrate that *N-myc* (−/−) ES cells could give rise to normal B lymphopoiesis in RAG-2-deficient chimeric mice. Therefore, *N-myc* does not appear to be essential for B-cell development (Malynn *et al.*, 1995). One possible reason for the apparent redundancy of *N-myc* in B lymphocytes is the expression of the closely related *c-myc* gene, which may compensate for the loss of *N-myc* function. Indeed, negative cross-regulation between the two genes has been reported in mice overexpressing an *N-myc* transgene in the B lymphoid lineage which resulted in transcriptional silencing of the endogenous *c-myc* gene (Dildrop *et al.*, 1989; Rosenbaum *et al.*, 1989). However, this cross-regulation was not observed in pre-B cells which were derived from the same *N-myc* transgenic mice by transformation with the Abelson murine leukaemia virus (A-MuLV) (Ma *et al.*, 1991). Likewise, no increase in *c-myc* expression was detected in *N-myc* (−/−) pre-B cell lines which were established from RAG-2-deficient chimeras by A-MuLV infection (Malynn *et al.*, 1995). Our own data would, however, suggest that the Abelson virus itself may be responsible for the lack of cross-regulation in immortalized B lymphocytes. Intriguingly, we observed that the 10-fold reduction of *N-myc* expression in Pax-5-deficient pre-BI cells was matched by an equivalent increase in *c-myc* transcription (Figure 4A). This therefore implies that a functional cross-talk between the two *myc* genes does indeed exist in non-transformed pre-BI cells.

The transcriptional regulator LEF-1 belongs to the small family of TCF proteins which contain the high-mobility group (HMG) domain as their characteristic DNA-binding motif (Travis *et al.*, 1991). LEF-1, which lacks a 'classical' transactivation function, is known to regulate gene expression via two different mechanisms. LEF-1 plays an architectural role in regulating the T cell antigen receptor (TCR) α enhancer as it facilitates, through DNA bending, the assembly of multiple transcription factors into a

functional higher-order complex (Giese *et al.*, 1992). In addition, LEF-1 directly interacts with β -catenin which acts as a transcriptional co-activator to regulate gene expression in response to Wnt signalling (Behrens *et al.*, 1996). In the B lymphoid lineage, *LEF-1* is only expressed in pro-B and pre-B cells, but not during the late phases of B-cell differentiation (Travis *et al.*, 1991). Mice lacking *LEF-1* exhibit severe abnormalities in organs that depend on inductive epithelial–mesenchymal interactions for their development (van Genderen *et al.*, 1994). However, an initial analysis of the lymphoid system did not reveal any obvious phenotype in the absence of LEF-1 (van Genderen *et al.*, 1994), indicating that the down-regulation of *LEF-1* is unlikely to play an important role in the bone marrow phenotype of Pax-5-deficient mice.

In summary, none of the identified BSAP target genes seems to be a candidate gene that may on its own cause the early B-cell developmental block as a consequence of its deregulation in Pax-5 mutant mice. However, it is conceivable that the cumulative deregulation of several of these target genes may create the Pax-5 mutant phenotype. Moreover, it is important to note that three of the identified target genes (*CD19*, *mb-1*, *PD-1*) are known to play a critical role in late B-cell differentiation, thus pointing to a late function of BSAP (Pax-5) in B lymphopoiesis.

Regulation of target gene expression by BSAP

Previous structure–function analyses of BSAP have revealed a potent C-terminal transactivation function which is essential for transcriptional stimulation of artificial, BSAP-dependent reporter genes in transient cell transfection assays (Dörfler and Busslinger, 1996). The identification of BSAP target genes has now enabled us to directly investigate the role of BSAP and its transactivation domain *in vivo*. For this purpose, the restoration of endogenous target gene expression was analyzed in Pax-5-deficient pre-BI cells which were complemented with the full-length BSAP protein or the truncated paired domain polypeptide PRD. Intriguingly, the expression of only two of the BSAP target genes (*CD19* and *N-myc*) was strictly dependent on full-length BSAP, while the PRD polypeptide alone was already able to partially reconstitute the expression of *mb-1*, *LEF-1* and *PD-1* in Pax-5-deficient pre-BI cells.

The mouse *CD19* gene lacks a TATA-box, but instead contains a high-affinity BSAP-binding site in the −30 promoter region. This proximal binding site was shown to be fully occupied *in vivo* by BSAP (Kozmik *et al.*, 1992) and hence is likely to play a central role in mediating the effect of BSAP on *CD19* transcription. The complete loss of *CD19* expression in Pax-5-deficient pre-BI cells suggests therefore that the interaction of BSAP with the −30 region is essential for recruiting the basal transcription machinery to the *CD19* promoter. This recruitment process appears, however, to be highly sensitive to the dosage of BSAP protein, as the expression of the endogenous *CD19* gene was efficiently reactivated and stably maintained in Pax-5-deficient pre-BI cells only at concentrations of full-length BSAP which were comparable with those in wild-type B lymphocytes. The function of mammalian Pax genes is well known to be exquisitely sensitive to gene dosage which is responsible for the frequent association of these genes with mouse developmental mutants and

human disease syndromes (reviewed by Strachan and Read, 1994). To our knowledge, *CD19* is the first Pax target gene whose regulation is subject to and thus reflects this dosage sensitivity at the molecular level.

In contrast to *CD19*, *N-myc* expression is completely reconstituted to wild-type levels by complementation of Pax-5-deficient pre-BI cells with full-length BSAP protein. To date, little is known about the regulation of *N-myc* expression except that 2.5 kb of 5' flanking sequences are sufficient for correct transcription initiation of this gene (Hiller *et al.*, 1991) and that regulatory elements in the first intron are involved in the attenuation of *N-myc* transcription (Morrow *et al.*, 1992). Precursor B cells express relatively high levels of *N-myc* mRNA in response to IL-7 signalling which results in relief of the transcriptional attenuation block (Morrow *et al.*, 1992). Here we have demonstrated that BSAP is essential for efficient IL-7 induction of *N-myc* transcription. In the absence of BSAP, the *N-myc* gene is induced by IL-7 with considerably delayed kinetics and to a lower expression level, indicating that BSAP may either directly mediate the effect of IL-7 signalling or more likely cooperate with an IL-7-inducible transcription factor. The lack of any detailed characterization of the *N-myc* regulatory sequences has precluded the identification of a functional BSAP-binding site which may be involved in transcriptional initiation in the promoter region or in the relief of transcriptional attenuation in the first intron. Interestingly, in an analogous situation, the IL-4 plus LPS stimulation of the immunoglobulin germline ϵ transcript was shown to depend on the cooperation of a BSAP-binding site with adjacent IL-4-responsive elements which are located in the proximal promoter region (reviewed by Busslinger and Nutt, 1998).

Curiously, the expression of the endogenous *mb-1* and *LEF-1* genes is reactivated in Pax-5-deficient pre-BI cells already by the paired domain polypeptide PRD which lacks any transactivation function. The recent identification of a BSAP-binding site in the -80 region of the *mb-1* promoter offers a molecular explanation for this phenomenon (Fitzsimmons *et al.*, 1996). Mutation of this binding site reduces the activity of the *mb-1* promoter ~5-fold in transfected pre-B cells (Fitzsimmons *et al.*, 1996) which compares favourably with the 10-fold lower expression level of the endogenous *mb-1* gene in Pax-5-deficient pre-BI cells. In the context of the *mb-1* promoter, BSAP was shown to function as a docking protein which efficiently recruits Ets transcription factors to an adjacent, suboptimal Ets-binding site. In *in vitro* binding studies, the paired domain of BSAP was already sufficient for the formation of ternary complexes with Ets proteins and DNA (Fitzsimmons *et al.*, 1996). Our finding that the PRD polypeptide is able to transcriptionally activate even the endogenous *mb-1* gene is therefore in good agreement with this recruitment model. No characterization of the *LEF-1* regulatory sequences has yet been reported. However, the observation that the PRD protein also stimulates the expression of *LEF-1* strongly predicts that BSAP regulates this gene by a similar recruitment mechanism.

BSAP has been proposed to function as a negative regulator of gene expression in late B-cell differentiation by repressing the J-chain gene (Rinkenberger *et al.*, 1996) and the activity of the 3' enhancers of the immunoglobulin heavy-chain (Singh and Birshtein, 1993; Neurath *et al.*,

1994) and κ light-chain genes (Roque *et al.*, 1996). While the postulated role of BSAP in the regulation of these genes could not be investigated in Pax-5-deficient mice due to the early developmental block, our analysis of the *PD-1* gene has now provided the first genetic evidence for a repression function of BSAP. In the absence of this regulator, *PD-1* expression was not only activated, but it could also be rapidly repressed again upon estrogen induction of BSAP-ER.

In summary, the analysis of endogenous BSAP target genes by loss- and gain-of-function experiments has revealed a complex and pleiotropic role of BSAP in the transcriptional regulation of early B-cell development. BSAP was shown to function as a transcriptional activator, repressor or docking protein depending on the regulatory sequence context of the target gene. Moreover, each identified target gene on its own is unlikely to explain the early block of B lymphopoiesis in Pax-5-deficient mice. However, we have now established a powerful induction system allowing the identification of novel BSAP target genes, which should result in the dissection of the genetic pathway controlling early B-cell development.

Materials and methods

Cell lines and culture

Pre-BI cells were derived from bone marrow of 2-week-old wild-type and Pax-5 mutant mice and propagated on a semi-confluent layer of stromal ST2 cells in the presence of IL-7 exactly as described (Nutt *et al.*, 1997). The IL-7-containing medium consisted of Iscove's modified Dulbecco's medium (IMDM; Gibco-BRL) supplemented with 50 μ M 2-mercaptoethanol, 1 mM glutamine, 2% heat-inactivated fetal calf serum, 0.03% (w/v) primatone RL (Quest International, Naarden, The Netherlands) and 1% conditioned supernatant of rIL-7-secreting J558L cells (Rolink *et al.*, 1993). The capacity of BSAP-ER to potentiate target gene transcription was investigated by the addition of 1 μ M 17 β -estradiol (E2, Sigma) to KO-BSAP-ER cells for various time intervals (0–24 h) prior to harvesting. To investigate the effect of IL-7 on *N-myc* transcription, cells were washed twice with PBS and kept for 6 h in normal growth medium lacking IL-7 before the re-addition of IL-7. The murine plasmacytoma cell line SP2/0 and the embryonal carcinoma cell line RAC65 were cultured as described (Dörfler and Busslinger, 1996).

Plasmid constructs

The BSAP-dependent reporter construct *luc-CD19* and the *CMV-CAT* reference gene were previously described (Dörfler and Busslinger, 1996). The expression plasmid pBSAP-ER was constructed by linking a *SaII-EcoRI* fragment of human BSAP cDNA (encoding amino acids 1–383) in-frame to a *BamHI-SacI* fragment of human estrogen receptor cDNA (amino acids 282–595; Superti-Furga *et al.*, 1991) via a double-stranded linker oligonucleotide (5'-AATTATGCTGCCGCAAGCTGCCGCTGCG3' annealed with 5'-GATCCGACGCGCAGCTGCGGCAGCAT3'). The plasmid pABSAP-ER contains a 105 amino acid deletion of the paired domain (amino acids 49–153) which was generated by fusing C- and N-terminally resected BSAP cDNA fragments (Adams *et al.*, 1992) in-frame via a linker oligonucleotide (5'-AATTG-AACCAACAGTA3' annealed with 5'-AATTTACTGGTTGGTTCA3'). The *SaII-EcoRI* cDNA inserts of pBSAP-ER, pABSAP-ER and pBSAP (containing the entire coding sequences of human BSAP) were inserted into the polylinker downstream of the CMV enhancer/promoter region of the eukaryotic expression vector pKW2T (Dörfler and Busslinger, 1996). The BSAP cDNA sequences encoding amino acids 1–145 were linked in-frame to the SV40 nuclear localization signal (Kalderon *et al.*, 1984) in the vector pKW2T to generate the expression construct pPRD. The cDNA inserts of pBSAP, pBSAP-ER, pABSAP-ER and pPRD were cloned into the *EcoRI* and *HindIII* sites of the retroviral vector pBabe-Puro (Morgenstern and Land, 1990).

Transient cell transfection assays

Transient cell transfection experiments were performed and analyzed exactly as described (Dörfler and Busslinger, 1996) with the exception

that 1 μ M 17 β -estradiol (E2) was added to the cell cultures 24 h prior to harvesting (where indicated). RAC65 and SP2/0 cells were transfected by the calcium phosphate co-precipitation and electroporation method, respectively.

Generation of stable pre-BI cell lines

pBabe-puro vectors expressing the different BSAP constructs were transfected into GP+E-86 packaging cells (Markowitz *et al.*, 1988) which were subsequently selected with puromycin (5 μ g/ml; Sigma). The retroviral supernatants were used to infect murine pre-BI cells (grown on puromycin-resistant ST2 feeder cells) followed by puromycin selection (2.5 μ g/ μ l). Single cell clones were generated by fluorescence-activated cell sorting of KO-BSAP cells.

EMSA analysis

Nuclear extracts were prepared according to the method of Andrews and Faller (1991). Equal amounts of protein were analysed by EMSA according to Barberis *et al.* (1990) except for the further addition of 20 μ g BSA to the binding reaction. An end-labelled oligonucleotide containing the Pax-binding site of the *CD19* gene (Kozmik *et al.*, 1992) was used as a probe to detect the DNA-binding activity of BSAP proteins.

Antibodies and flow cytometry

The anti-CD19 mAb (1D3; Krop *et al.*, 1996) was purified from hybridoma cell supernatants on protein G-Sepharose columns (Pharmacia, Uppsala, Sweden) and conjugated with sulfo-NHS-biotin (Pierce Chemical Co). Streptavidin-PE was obtained from Southern Biotechnologies Inc. (Birmingham, AL, USA). Antibody staining and flow-cytometric analyses were performed on a FACScan flow cytometer (Becton Dickinson) as previously described (Urbánek *et al.*, 1994). Single cell and bulk sorting was carried out on a FACS Vantage TSO flow cytometer (Becton Dickinson). For intracellular antibody staining, pre-BI cells were fixed and permeabilized in 2% paraformaldehyde (Fluka), 0.1% Triton X-100 for 30 min at 4°C except for the untreated KO-BSAP-ER cells (Figure 2C) which were fixed in 4% paraformaldehyde. Fixed cell were incubated with a 100-fold dilution of a rabbit polyclonal, affinity-purified anti-BSAP antibody (raised against amino acids 189–391; Adams *et al.*, 1992) for 1 h on ice followed by incubation with a FITC-conjugated goat anti-rabbit polyclonal antibody (1:200; Vector Laboratories) for 1 h on ice.

Immunohistochemical analysis of BSAP

Pre-BI cells were fixed, permeabilized and stained with the affinity-purified anti-BSAP antibody exactly as described above for the intracellular antibody staining. The fixed cells were air-dried onto Tespa-coated slides (Sigma) and mounted in Vector Shield (Vector Laboratories) and DABCO (10 μ g/ μ l, Sigma)-DAPI (0.15 μ g/ μ l, Sigma) solutions at a ratio of 1:1. Images were obtained on a fluorescence microscope and processed on a CCD camera.

Riboprobes and RNase protection assay

The following oligonucleotide pairs were used for PCR amplification of the indicated murine riboprobes:

<i>N-myc</i>	5'GCGGAATTCAGATCAAGAGCGAGGCTTCTC3' 5'GCGAAGCTTTCTTACCAGCTCAGGCACAT3'
<i>mb-1</i>	5'GCGGAATTCACGAAGACACAGGGGCTTGTAC3' 5'GCGAAGCTTAAGGTTTCAGGCCCTCATAGAG3'
<i>p53</i>	5'GCGGAATTCAGACTCCAGTGGGAACCTTCT3' 5'GCGAAGCTTCATTAGCTCCCGAACATCT3'
<i>LEF-1</i>	5'GCGGAATTCACAGAGAAAGGAGCAGGAGC3' 5'GCGAAGCTTTGACCAGCCTGGATAAAGCTG3'
<i>Bcl-x_L</i>	5'GCGGAATTCGATTCCTCCATGGCAGCAGTGA3' 5'GCGAAGCTTAACACACACAGCCACAGTCAT3'
<i>PD-1</i>	5'GCGGAATTCCTAGCTGTCTTCTGCTCAAC3' 5'GCGAAGCTTCCAGCCCTTCAGTGAAGACAA3'

cDNA synthesized from total RNA of the murine pre-B cell line 70Z/3 was used as a template for the amplification of *N-myc* (DePhino *et al.*, 1986), *mb-1* (Sakaguchi *et al.*, 1988), *p53* (Arai *et al.*, 1986), *LEF-1* (Travis *et al.*, 1991), *bcl-x_L* (Boise *et al.*, 1993) and *PD-1* (Ishida *et al.*, 1992) sequences. The amplified cDNA was inserted in antisense orientation into the *Hind*III and *Eco*RI sites of pSP64. A 353 bp *Aval*-*Pst*I fragment of *c-myc* cDNA (Stanton *et al.*, 1984) was cloned into pSP64 to obtain the *c-myc* riboprobe. The mouse *S16* (Urbánek *et al.*, 1994) and *WT-1* (Kozmik *et al.*, 1993) riboprobes were previously described. *Mil* and *Id-1,2,3,4* probes were provided by P.Ferrier and R.Benezra, respectively. The cloning of the *I μ* , *λ 5*, *VpreB*, *B29*, *CD19*,

blk, *RAG-1*, *RAG-2*, *XBP-1*, *Sox-4*, *Oct-1*, *Oct-2*, *Ikaros*, *E2A*, *PU.1* and *EBF* riboprobes has previously been reported (Nutt *et al.*, 1997). Information on the cloning of the following riboprobes is available on request: *CD20*, *CD21*, *CD22*, *CD37*, *CD40*, *CD72*, *lyn*, *fyn*, *btk*, *TdT*, *PLPRC*, *c-myb*, *Blimp-1*, and *mER*.

Total RNA was prepared from pre-BI cell lines using the TRIzol Reagent (Gibco-BRL) and 10 μ g of each RNA preparation were used for RNase protection assay according to the method of Vitelli *et al.* (1988) except that a hybridization temperature of 60°C was used.

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