Pip, a novel IRF family member, is a lymphoid-specific, PU.1-dependent transcriptional activator

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The immunoglobulin light-chain gene enhancers $E_{\kappa 3'}$, $E_{\lambda 2-4}$, and $E_{\lambda 3-1}$ contain a conserved cell type-specific composite element essential for their activities. This element binds a B cell-specific heterodimeric protein complex that consists of the Ets family member PU.1 and a second factor (NF-EM5), whose participation in the formation of the complex is dependent on the presence of DNA-bound PU.1. In this report we describe the cloning and characterization of Pip (PU.1 interaction partner), a lymphoid-specific protein that is most likely NF-EM5. As expected, the Pip protein binds the composite element only in the presence of PU.1; furthermore, the formation of this ternary complex is critically dependent on phosphorylation of PU.1 at serine-148. The *Pip* gene is expressed specifically in lymphoid tissues in both B- and T-cell lines. When coexpressed in NIH-3T3 cells, Pip and PU.1 function as mutually dependent transcription activators of the composite element. The amino-terminal DNA-binding domain of Pip exhibits a high degree of homology to the DNA-binding domains of members of the interferon regulatory factor (IRF) family, which includes IRF-1, IRF-2, ICSBP, and ISGF3 γ .

[**Key Words:** Pip protein; PU.1; lymphoid tissues; IRF family; transcriptional activator; immunoglobulin λ genes]

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The expression of immunoglobulin genes, which encode the heavy- and light-chain subunits of the antibody molecule, is tightly regulated in a cell type- and developmental stage-specific fashion. Expression of these genes in B cells is controlled both at the level of transcription and recombination. Multiple *cis*-regulatory elements, promoters, and enhancers appear to confer proper developmental regulation of transcription (Calame and Eaton 1988; Sen and Baltimore 1989; Staudt and Lenardo 1991). Immunoglobulin gene transcriptional enhancers also play a critical role in regulating *V(D)J* recombination (Ferrier et al. 1990; Engler et al. 1991; Takeda et al. 1993). Cell type-specific enhancers were first identified in the J-C introns of both the heavy (Banerji et al. 1983; Gillies et al. 1983; Neuberger 1983) and κ light chain (Queen and Baltimore 1983; Picard and Schaffner 1984) genes. More recently, additional enhancers have been identified 3' of the constant region genes in both the heavy (Matthias and Baltimore 1993; Pettersson et al. 1990) and κ light chain (Meyer and Neuberger 1989) loci. The λ light-chain gene locus, which is organizationally distinct (Storb et al. 1989), possesses two transcriptional enhancers, each located 3' of a J_{λ} - C_{λ} gene cluster (Hagman et al. 1990). These two enhancers, designated $E_{\lambda 2-4}$ and $E_{\lambda 3-1}$,

are >90% homologous, are thought to have arisen by an evolutionary gene duplication event, and presumably function in a similar manner.

We have demonstrated previously that the $E_{\lambda 2-4}$ enhancer contains two domains, λA and λB , which are essential for enhancer activity; mutation or deletion of either domain completely destroys enhancer function (Rudin and Storb 1992; Eisenbeis et al. 1993). Both λA and λB , which appear to be unrelated, have been shown to bind B cell-specific factors in nuclear extracts (Rudin and Storb 1992). The λB element consists of two juxtaposed but distinct transcription factor-binding sites (Eisenbeis et al. 1993). One site of the composite element is bound by PU.1, a member of the ETS family of transcription factors, that has been shown to be required for the development of lymphoid and myeloid lineages (Klemsz et al. 1990; Scott et al. 1994). The other site is likely bound by NF-EM5, a B cell-specific factor initially shown to form a ternary complex with PU.1 and a domain of the immunoglobulin $E_{\kappa 3'}$ enhancer (Pongubala et al. 1992). The λB and $\kappa 3'$ domains are homologous composite elements. Both sites of the composite element are required for enhancer function (Judde and Max 1992; Pongunbala et al. 1992; Eisenbeis et al. 1993). Intriguingly, whereas PU.1 can bind the composite element on its own, NF-EM5 is unable to do so (Pongubala et al. 1992; Eisenbeis

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et al. 1993). NF-EM5 is recruited onto the DNA by a specific interaction with PU.1. This protein-protein interaction is critically dependent on phosphorylation of PU.1 at serine residue 148 (Pongubala et al. 1993).

In this report we describe the cloning and characterization of a novel gene, *Pip* (PU.1 interaction partner) that encodes a PU.1-interacting protein with the properties expected of NF-EM5. PU.1 and *Pip* represent mutually dependent activators of the λ B and κ 3' enhancer elements.

Results

Isolation of the *Pip* gene

Of the many B-cell proteins that bind specifically to the λ A and λ B elements in vitro (Rudin and Storb 1992), only PU.1 represents a cloned transcription factor (Eisenbeis et al. 1993). In an effort to clone other factors that bind to the λ B elements of the $E_{\lambda 2-4}$ enhancer, we generated a λ gt11 cDNA expression library from poly(A)⁺ RNA extracted from J558L, a murine λ 1-producing myeloma cell line. We screened the library using an in situ filter-binding assay (Singh et al. 1988) and concatenated λ B probes. A screen of 1.2×10^6 plaques identified three short clones (all <800 bp). Sequence analysis confirmed that the three clones, designated λ 13, λ 15, and λ 37, contained extensively overlapping cDNA inserts that represented a portion of an incomplete open reading frame (ORF). The three clones were predicted to code for 134, 186, and 188 amino acids, respectively, with presumed DNA-binding activity.

To determine the DNA-binding characteristics of the cloned proteins, extracts were made from *Escherichia coli* lysogens harboring the λ 13 and λ 15 clones. These extracts were tested in electrophoretic mobility shift assays (EMSAs) for their ability to bind to the λ B site (Fig. 1A). Although there is no λ B-binding activity present in a λ gt11 control lysogen extract, the λ 13 extract contains a protein that binds the λ B element, forming a shifted complex in the gel. A similar result was obtained with the λ 15 extract (not shown), confirming that these two phage clones contain cDNA inserts that code for a protein that binds the λ B enhancer element in vitro.

Mapping the DNA binding site for *Pip*

To further define the binding site for the λ 13-encoded protein, we tested the ability of the λ 13 lysogen extract to bind a series of mutant λ B probes in an EMSA (Fig. 1B,C). Not only does the protein encoded by the λ 13 clone bind the wild-type λ B element, but it also binds, albeit less tightly, a λ B element containing a 4-base alteration in the core of the PU.1-binding site (Bm1, Fig. 1B,C). This protein is unable, however, to bind to probes containing mutations in the region 3' of the PU.1-binding site (Bm2 and Bm3, Fig. 1C) or to a shorter probe in which a portion of this 3' region has been deleted (Ets, Fig. 1C). These results suggest that unlike PU.1, whose binding requires the AGGA in the Ets consensus se-

Figure 1. The λ 13-encoded protein binds λ B specifically. (A) Gel mobility shift assay using the B probe and lysogen extracts from the parent vector (λ gt11) and the λ 13 clone. (B) Gel mobility shift assay using the λ 13 lysogen extract and a series of λ B probes. (C) Summary of the binding characteristics of the λ 13 clone. The sequences of the probes used in the gel shift analysis are shown. Dashes indicate residues that are identical to those at the analogous position in the B probe. The ability of the λ 13-encoded protein *Pip* to bind to each probe is indicated.

quence core but not the 3' sequence that is mutated in the Bm2 and Bm3 probes (Eisenbeis et al. 1993), the λ 13 protein binds the sequence 3' of the PU.1 site.

To identify guanine residues involved in contacting the protein, a methylation interference assay was performed using the λ 13 lysogen extract and a methylated λ B site probe. We analyzed the top (Fig. 2A) and bottom (Fig. 2B) strands of the λ B probe; the results are summarized in Fig. 2C. On the top strand, methylation of the first guanine 3' of the AGGA Ets consensus sequence core partially interferes with complex formation (shown as an open circle in Fig. 2C). Methylation of the next guanine completely interferes with the ability of the λ 13 protein to bind the λ B DNA (shown as a solid circle, Fig. 2C), indicating that the λ 13 protein comes into close contact with the major groove of the DNA at this position. On the bottom strand only two adjacent guanines exist for analysis, and these lie 3' of the PU.1-binding site. Methylation of either of these G's abolished λ 13

Figure 2. Methylation interference analysis of the $\lambda 13$ -encoded DNA-binding protein. Analysis of the upper (A) and lower (B) strands of the B probe is shown. The $\lambda 13$ lysogen complex was isolated and analyzed. (B) The complexed probe; (F) the free probe recovered from the native gel. Corresponding sequences are shown for reference. (C) Summary of the methylation interference analysis of the $\lambda 13$ complex in comparison to methylation interference results for the JB2 and PU.1 complexes (Eisenbeis et al. 1993). (Pip) The $\lambda 13$ complex. Sites of close contact between protein and DNA upon complex formation are indicated. (●) G residues whose methylation results in a complete loss of complex formation; (●) Residues whose methylation results in a substantial decrease in complex formation; (○) residues whose methylation results in a modest decrease in complex formation. Protein binding sites are boxed.

protein binding, again suggesting that the $\lambda 13$ protein makes intimate contacts in the major groove at these two positions. The interference patterns delimit a minimal binding site for the $\lambda 13$ protein, which is adjacent to that determined previously for PU.1 and is identical to that determined previously for the 3'-binding factor in the JB2 complex (Fig. 2C; Eisenbeis et al. 1993).

These binding site experiments demonstrate that the protein encoded by the $\lambda 13$ cDNA insert is capable of binding the λB element at a site adjacent to the PU.1-binding site and may therefore be a component of the JB2 complex described previously (Eisenbeis et al. 1993), which contains a factor (NF-EM5) that binds this se-

quence. Interestingly, in EMSAs with J558L nuclear extract, we have not observed a binding activity that interacts with this site in the absence of simultaneously bound PU.1. These observations raised the possibility that whereas the truncated cloned protein could bind the λB element on its own, the full-length native molecule requires PU.1 for interaction with DNA.

Isolating a complete Pip ORF

To investigate the possibility of isolating a complete ORF, we screened the same J558L cDNA library with the $\lambda 13$ cDNA insert as a probe to obtain the complete cDNA sequence. A screen of 1×10^6 plaques identified ~200 clones containing inserts with homology to $\lambda 13$. Twenty of these were purified, and the four clones with the largest cDNA inserts were subcloned for sequencing. The longest of the clones, $\lambda 13.9$, had an insert of 1783 bp (sequence submitted to GenBank), which contained a 64-bp 5'-untranslated region (UTR), a long ORF of 1353 bp (65–1417), and a 365-bp 3' UTR. Because no poly(A) addition signal was found, this is unlikely to be the complete 3' UTR. The deduced ORF codes for a protein of 450 amino acids with a predicted molecular mass of 51.5 kD. We have named this protein Pip (see below). All of the sequenced clones (including the short $\lambda 13$, $\lambda 15$, and $\lambda 37$ clones) share nearly identical 5' ends. Thus, we believe that the 5' end of the sequence is at or near the cap site. The sequence surrounding the putative initiator methionine is consistent with the loosely defined consensus sequence for eukaryotic translational initiation (Kozak 1987). In addition, the 64 bp of 5' UTR contains two translational stops in-frame with the coding sequence, suggesting that this is the true start of the ORF. It should be noted, however, that a second methionine occurs as the fourteenth amino acid in this sequence (Fig. 3A), and we have not ruled out the possibility that this may be an alternative translational start.

The shortest of the original clones, $\lambda 13$, codes for the first 134 amino acids of the Pip protein. Because this clone was identified by its ability to bind specifically to the λB element in vitro, it localizes the DNA-binding domain to the first 134 amino acids of the Pip protein. Interestingly, this portion of the Pip protein sequence exhibits a considerable degree of sequence homology to the DNA-binding domains of the interferon consensus sequence-binding protein (ICSBP; Driggers et al. 1990) and other, related members of the interferon regulatory factor family (IRF) (Fig. 3A). In addition, Pip shares a series of small blocks of homology with ICSBP at similar positions in the carboxy-terminal half of each protein (Fig. 3B).

The amino-terminal half of the Pip protein is highly hydrophilic and is separated from the carboxy-terminal half by a potential PEST domain (a region rich in proline, glutamate, serine, and threonine residues whose presence in intracellular proteins correlates with a short half-life; Rogers et al. 1986). This 31-amino-acid domain (208–238) contains 45% P, E, S, and T and is flanked by basic amino acids. The region of the Pip protein that

contains this putative PEST domain is in general proline-rich (151–237, 20% proline), representing a potential transcriptional activation domain (Mermod et al. 1989). In addition, there is a region in the carboxyl terminus of Pip that is rich in glutamine residues (354–419, 15% glutamine). Glutamine-rich domains have also been shown to function as transcriptional activation domains (Courey and Tjian 1988).

Full-length Pip does not bind λ B independently of PU.1

To test the binding activity of the full-length Pip protein, we subcloned the complete ORF (from amino acid 2) and 218 bp of 3'-untranslated sequence into pBSATG, which provides a strong initiator codon for in vitro translation (Baldwin et al. 1990). Recombinant Pip protein was produced and analyzed by EMSA for its ability to bind to the λ B element (Fig. 4A,B). Although Pip was initially cloned by virtue of its ability to bind λ B, the full-length protein produced in vitro (Fig. 4B) does not display detectable binding activity in the EMSA (Fig. 4A). However, if recombinant PU.1 is supplied along with Pip, a complex migrating slower than the PU.1/ λ B complex appears in the gel. This complex, which has an electrophoretic mobility identical to that of JB2, appears only when PU.1 and Pip are present in the reaction together and is de-

pendent on the presence of both the PU.1-binding site and the Pip-binding site, as mutation of either (Bm1 and Bm2, see Fig. 1C) abolishes its formation. In addition, the formation of the Pip/PU.1 complex is dependent on the presence of a serine residue at amino acid position 148 of the PU.1 protein, as mutation of this serine to alanine leads to a protein that can no longer recruit Pip to the λ B site, even though its own DNA-binding ability is unaltered (Fig. 4A, S>A). This serine has been shown to be phosphorylated both in reticulocyte lysate-produced PU.1 and in PU.1 produced in vivo and, in its phosphorylated form, is required for the essential interaction between PU.1 and NF-EM5, which recruits NF-EM5 to its binding site on the κ 3' enhancer DNA (Pongubala et al. 1993). Therefore, phosphorylation of Ser-148 in PU.1 is also required for interaction with Pip.

The Pip protein binds specifically to the λ B element, but only after interacting directly with a PU.1 protein whose intact binding site is nearby. This interaction requires serine at position 148 of the PU.1 protein and results in the formation of a ternary complex with a mobility identical to that of the previously described complex JB2. It is likely that Pip is the second component of the JB2 complex. It is the correct size and displays all of the unique DNA-binding characteristics that have been described for that factor. In addition, because the NF-EM5 protein displays the same set of characteristics and

Figure 4. Binding characteristics of the Pip and ICSBP proteins. (A) In a gel mobility shift assay, the full-length Pip protein binds to the B probe in a PU.1-dependent fashion. (B, Bm1, and Bm2) Probes used in the assay. (J) J558L nuclear extract; (PU.1, S>A, and Pip) recombinant proteins. (B) SDS-PAGE analysis of [³⁵S]methionine-labeled recombinant proteins. Protein size markers are indicated. (C) ICSBP interacts with PU.1 to form a ternary complex on the B probe. A gel mobility shift assay using the B probe and recombinant ICSBP and PU.1 proteins is shown. (IBP) Recombinant ICSBP protein. The mobility of the PU.1 and JB2 complexes is indicated.

participates with PU.1 to form a λ B complex with a mobility identical to that of JB2, it is likely that Pip and NF-EM5 are the same protein.

ICSBP binds λ B in conjunction with PU.1

Because ICSBP is the protein related most closely to Pip with a nearly identical DNA-binding domain, we tested its ability to bind the λ B element in the gel shift assay. ICSBP protein produced in the reticulocyte lysate system was unable to bind λ B independently but was able to interact with PU.1 to form a complex that has a mobility identical to that of the Pip/PU.1 and JB2 complexes. (The size of the ICSBP protein produced in vitro is indistinguishable by SDS-PAGE from that of the Pip protein; C.F. Eisenbeis, unpubl.). The formation of the ICSBP/PU.1 complex is also dependent on the presence of serine at position 148 of the PU.1 protein (Fig. 4C). Thus, ICSBP and Pip are identical in terms of their size and DNA-binding characteristics. Experiments with anti-ICSBP antibodies, however, indicate that ICSBP is not a major component of the JB2 complex (C.F. Eisenbeis, unpubl.). This suggests that ICSBP is not NF-EM5.

The expression of Pip mRNA is tissue restricted

To determine the pattern of Pip gene expression, we performed Northern blot analysis and ribonuclease protection assays (RPAs) on a variety of RNAs from murine tissues and cultured cell lines. Northern blot analysis of polyA⁺ RNA from cultured murine cell lines (Fig. 5A)

reveals that a 5-kb message is present in all of the B-cell lines examined (J558L, WEHI 231, and lipopolysaccharide (LPS)-stimulated 70Z/3), but not in an erythroid line [myeloid erythroleukemia (MEL)]. An additional, abundant message of 2.5 kb and a less abundant message of 1.5 kb are present only in J558L, a myeloma line that expresses λ 1 light chains. The significance of these additional transcripts is unclear at the present time. Clearly, the complete Pip message is much longer (5 kb) than the largest cDNA clone that we have obtained (1.8 kb). Because numerous cDNA clones have a common 5' end, the Pip message appears to contain a long 3' UTR.

Analysis of total RNA from cultured murine cell lines reveals a similar pattern of expression (Fig. 5B): A 5-kb message is present in all B-cell lines analyzed, whereas additional 2.5- and 1.5-kb messages are present in the myeloma lines only (J558L, S194, and MOPC315). No Pip message was detectable by Northern blotting in EL4 (a T-cell lymphoma) or NIH-3T3 (a fibroblast) cell lines.

To achieve greater sensitivity, further analysis of Pip gene expression was performed using an RPA. The Pip riboprobe used in this assay is 400 nucleotides in length, 350 of which will be protected by specific hybridization to Pip mRNA. A mouse β -actin probe was employed to control for RNA loading. Analysis of tissue RNAs shows that the Pip gene is expressed in murine spleen, thymus, and bone marrow but not liver, kidney, brain, or testes (Fig. 5C). Analysis of cultured cell line RNAs confirms that while expression of Pip RNA is greatest in the three myelomas, there is expression of the Pip gene in all B-cell types tested. The Pip message is also present, al-

Figure 5. Analysis of the expression of *Pip* mRNA. A Northern blot analysis of 5 µg of polyA⁺ RNA (A) and 10 µg of total RNA (B) from cultured cell lines is shown. Blots were probed with a 679-bp *Nco*I fragment corresponding to the carboxy-terminal half of the *Pip*-coding sequence. Blots were rehybridized with a GAPDH probe to control for differences in sample loading. RNA size markers are indicated. An RPA of 5 µg of total RNA from tissues (C) and cultured cell lines (D) is shown. A β-actin riboprobe was included in the RPA to control for RNA quantity. The time of exposure of each set of samples is indicated.

beit at a much lower level than in any of the B-cell lines, in two T-cell lines, EL4 and PGL2 (Fig. 5D). There is no detectable expression of *Pip* RNA in MEL or NIH-3T3 (Fig. 5D). Preliminary evidence also suggests that the *Pip* gene is not expressed in macrophage lines (A. Brass, unpubl.).

The *Pip* gene appears to be expressed exclusively in lymphoid cells. In addition, it appears to be expressed at a higher level in B cells than in T cells. The comparable levels of expression in the spleen and thymus could either be attributable to high levels of *Pip* RNA in thymocytes or, more likely, contaminating B cells in the tissue preparation (the thymus RNA sample had large amounts of κ RNA). Because PU.1 is restricted in its expression to the B-cell and macrophage lineages, the PU.1/*Pip* complex will only form in B cells, the only cell type in which the expression of both genes appears to overlap. Thus, this complex may play a critical role in determining the B-cell specificity of the λ and κ3' enhancers.

In conjunction with PU.1, Pip functions as a transcriptional activator

We have reported here that *Pip* and PU.1 interact to form a complex on the λB element of the E_{λ2-4} enhancer. We had shown previously that when multimerized, this element was sufficient to drive transcription of a reporter gene transiently transfected into the terminally differentiated B-cell line, J558L (Eisenbeis et al. 1993). To determine whether the complex formed by *Pip* and PU.1 has transcriptional activating potential, we cotransfected expression plasmids containing *PU.1* and *Pip* cDNAs into

NIH-3T3 cells (which express neither protein) along with a chloramphenicol acetyltransferase (CAT) reporter construct containing the thymidine kinase (TK) promoter and a tetramer array of λB sites (Fig. 6). The λB sites function as cell type-specific activation elements, as they promote transcription in J558L B cells but not in NIH-3T3 fibroblasts. Whereas expression of *Pip* or PU.1 alone had no effect on CAT expression, the simultaneous expression of *Pip* and PU.1 in 3T3 cells was sufficient to drive transcription of the reporter. Coexpression of *Pip* with the S148A mutant of PU.1 did not lead to an appreciable increase in CAT expression, suggesting that the direct interaction between these two proteins is important in vivo as well as in vitro. These results suggest that *Pip* acts as a transcriptional activator in conjunction with PU.1, which is required for *Pip* to bind to the λB element. Interestingly, PU.1, which has been shown by others to be a transcriptional activator (Klemsz et al. 1990), cannot activate transcription alone through the λB tetramer.

Discussion

We have identified *Pip*, a novel transcription factor, which interacts with the Ets protein, PU.1, to form an activating complex on the λB motif of the E_{λ2-4} enhancer. *Pip* does not bind λB autonomously but relies on DNA-bound PU.1 to facilitate its binding to this DNA element. *Pip* is restricted in its expression to B and T cells, because PU.1 is not expressed in the T lineage, the *Pip*/PU.1 complex is B cell specific and may thus be an important determinant of the B cell specificity of the mu-

Figure 6. Functional analysis of the Pip protein. Ectopic expression of Pip and PU.1 in cultured NIH-3T3 fibroblasts with a CAT reporter gene whose expression is under the control of a tetramer of λ B elements. PU.1, Pip, and PU.1 S148A cDNAs are cloned into a eukaryotic expression vector containing the CMV promoter and enhancer. The reporter is the B4TKCAT construct (Eisenbeis et al. 1993). The indicated constructs were transiently cotransfected into NIH-3T3 cells. After 46 hr, cells were harvested and extracts were analyzed for CAT activity. The results are reported as an average of at least three independent transfections. The activity of the reporter alone has been set at 1.0.

rine immunoglobulin λ , as well as $\kappa 3'$, enhancers. The Pip/PU.1 complex is identical in every respect to the NF-EM5/PU.1 and JB2 complexes described previously.

Pip is a novel member of the IRF family, particularly related to ICSBP

The amino-terminal DNA-binding domain of the Pip protein displays a strikingly high degree of homology to the DNA-binding domains of the IRF family, including IRF-1, IRF-2, ICSBP, and ISGF3 γ (Fig. 3A). The Pip protein is 80% identical to ICSBP across this region (amino acids 24–130 of Pip). In addition, Pip and ICSBP share many contiguous segments of homology in their carboxy-terminal halves (see Fig. 3B). The two proteins are 48% homologous over a 160-amino-acid region of the Pip protein (Pip amino acids 254–413). Thus, Pip represents a new member of the IRF gene family that is related most closely to ICSBP.

The IRF-1, IRF-2, ICSBP, and ISGF3 γ proteins are all transcription factors implicated in mediating transcriptional responses of cells to interferon stimulation (for review, see Williams 1991; Pellegrini and Schindler 1993). ISGF3 γ is one of the subunits of the multimeric transcription factor ISGF3 (Darnell et al. 1994) whose nuclear localization is regulated by interferon signaling. Whereas ISGF3 and IRF-1 function as activators of transcription (Fujita et al. 1989; Kessler et al. 1990), ICSBP and IRF-2 function as transcriptional repressors (Harada et al. 1989; Weisz et al. 1992; Nelson et al. 1993).

DNA binding by Pip

The Pip protein is able to bind specifically to sequences essential for the activities of the murine λ 2-4 (this paper) and $E_{\kappa 3'}$ (C.F. Eisenbeis and A. Brass, unpubl.) enhancers. In each case, Pip binding requires that PU.1 be bound at its adjacent binding site. We have shown that the Pip DNA-binding domain, itself, can bind to these sites independently, suggesting the presence of an inhibitory domain within Pip that prevents DNA binding by the full-length protein. We postulate that interaction with PU.1 alters the conformation of Pip to unmask its DNA-binding domain and allow it to interact with the λ B sequence. The interaction between Pip and PU.1 is reminiscent of a similar interaction between the Ets family members Elk-1 and SAP-1, which interact with serum response factor (SRF) to form a ternary complex on the bipartite binding site of the serum response element (SRE) of the *c-fos* promoter (Hipskind et al. 1991; Dalton and Treisman 1992).

Interaction between Pip and PU.1

The binding of Pip to the λ B element is dependent on direct interaction between the Pip and PU.1 proteins and requires the presence of a serine residue at position 148 of the PU.1 protein. Phosphorylation of S148 occurs both in vivo and during translation of PU.1 in the reticulocyte lysate system and is required for the interaction between PU.1 and NF-EM5 (Pongubala et al. 1993). The fact that this phosphorylation is also required for the PU.1/Pip interaction further supports the contention that Pip is NF-EM5. This phosphorylation event could represent a regulated step in the control of immunoglobulin κ and λ light-chain gene expression. Interestingly, λ B/PU.1 can also form a trimolecular complex with ICSBP, which also requires phosphorylation of S148 of PU.1.

The carboxy-terminal region of the Pip protein, which appears to be required for interaction with PU.1 (C.F. Eisenbeis, unpubl.) is highly conserved in the ICSBP protein (Fig. 3B), representing a possible shared interaction domain. Recently, it has been discovered that the activity of ICSBP may also be regulated by association with related IRF family members IRF-1 and IRF-2 to form complexes that bind strongly to the α , β interferon (IFN- α , β)-stimulated response element (ISRE) (Bovolenta et al. 1994). It will be interesting to determine whether Pip can also interact with IRF-1 and IRF-2.

Transcriptional activation by PU.1/Pip

PU.1 has been shown to be a transcriptional activator (Klemsz et al. 1990) which possesses a transcriptional activation domain in the amino-terminal 75 amino acids (Hagemeyer et al. 1993). However, when expressed alone in NIH-3T3 cells, PU.1 is incapable of stimulating the expression of a cotransfected CAT reporter whose transcription is driven by a tetramer of λ B elements (Fig. 6). Perhaps the affinity of PU.1 for the λ B site is lower than that for other sites, and an array of four sites is insufficient to achieve an activating potential. In this scenario, the Pip-dependent activation of the λ B element by PU.1 could be attributable to cooperative binding (Fig. 4; Eisenbeis et al. 1993). In addition, Pip may contain a transcriptional activation domain that enhances the activation potential of the complex.

The Pip protein contains a glutamine-rich domain at its carboxyl terminus and a proline-rich domain in the center of the protein sequence. Glutamine- and proline-rich sequences have been shown to function as transcriptional activation domains (Courey and Tjian 1988; Mermoud et al. 1989). In the Pip protein an 87-amino-acid region (151–237) contains 20% proline residues, and a 66-amino-acid region (354–419) contains 15% glutamine residues. Within these domains, the proline and glutamine residues are clustered, so that the P-rich domain consists of three P-rich modules (151–162, 33% P; 172–200, 24% P; 225–237, 38% P), and the Q-rich domain consists of two Q-rich modules (354–369, 25% Q; 395–419, 20% Q). Recent reports suggest that some transcriptional activation domains have a modular structure composed of 10- to 20-amino-acid subdomains with little transactivational activity, which cooperate to form a highly functional activation domain when present in combination (Seipel et al. 1992; Sutherland et al. 1992). In addition, analysis of Oct-2 and the Fos:Jun heterodimer has demonstrated that different types of activation domains may exist within a single transcription factor and that these domains cooperate to provide a greater activation potential (Sutherland et al. 1992; Tanaka et al. 1994).

The role of IFNs in immunoglobulin gene expression

The relationship of Pip to ICSBP and the IRF family has interesting implications for the regulation of immunoglobulin light-chain gene expression. The IRF family members are regulated by extracellular actions of IFNs and the consequently activated signaling pathways. Whereas IFNs have been shown to play important roles in the regulation of the murine immune system (Sen and Lengyel 1992), it is not known at the present time whether the expression of Pip, like ICSBP, is regulated by IFNs.

It is intriguing that the λ B and κ 3' composite elements are highly related to the ISRE. The ISRE consensus sequence is A/GNGAAANNGAAACT (Darnell et al. 1994). This is identical to the λ B site, except at the highlighted positions (Fig. 1C). The λ B and κ 3' elements may

therefore function as ISREs, and expression of κ - and λ -genes may be positively regulated by IFN- α , β signaling.

IFN- γ , on the other hand, stimulates the expression of ICSBP, which is most closely related to Pip. ICSBP antagonizes activation by IRF-1 and ISGF3. Because ICSBP can also interact with PU.1 it may be a negative regulator of the λ and κ 3' enhancers.

The expression of immunoglobulin light-chain genes is regulated at several levels. In late pre-B cells, the κ and λ light-chain loci become accessible to the action of the *V(D)J* recombinase (Doglio et al. 1994). Germ-line transcripts of κ and λ genes can be detected at the time of rearrangement and are presumed to precede the joining events. It is not known, however, how the activation of these loci is controlled. The proportion of rearranged κ genes greatly exceeds that of λ genes, and this discrepancy may be attributable to differential activation of the two loci. κ genes are under the control of the κ -intron enhancer ($E_{\kappa i}$) and the κ 3' enhancer ($E_{\kappa 3'}$), and only the latter is dependent on PU.1–NF–EM5/Pip. At the time of κ -gene rearrangement, only the intron enhancer appears to be active (Chen et al. 1994; Klug et al. 1994). The λ locus, on the other hand, is regulated only by enhancers dependent on the PU.1–NF–EM5/Pip complex. Thus, it is conceivable that at the early stage of B-cell development when the $E_{\kappa i}$ is active, and $E_{\kappa 3'}$ is inactive, the λ enhancers are also inactive. This would allow preferential accessibility of the κ locus to the *V(D)J* recombinase, whereas λ remains inaccessible. It will be interesting to determine the roles of Pip/ICSBP and their control by interferons in the differential expression of κ and λ genes.

Materials and methods *λ gt11 library construction and screening*

RNA was isolated from J558L, a murine myeloma cell line, by the guanidinium isothiocyanate method. Poly(A)⁺ RNA was generated by two rounds of chromatography on an oligo(dT)–cellulose column (Pharmacia). Using this poly(A)⁺ RNA, a custom cDNA library was made in the λ gt11 vector by Stratagene. cDNA was synthesized by priming the reverse transcription reaction with both oligo(dT) and random primers. Primary plaques (3.9×10^6) were obtained, and $\sim 1 \times 10^6$ of these were used to amplify the library. By use of a tetramer of λ B sites, $\sim 1.2 \times 10^6$ plaques were screened from the amplified library (Singh et al. 1988). The probe was isolated as a restriction fragment from a plasmid into which four directly repeated copies of the λ B site had been cloned and labeled by filling in the overhanging ends with Klenow fragment in the presence of 100 μ Ci each of [³²P]dATP and [³²P]dCTP. Putative positive clones were purified through at least five rounds of plaque purification. Specific binding affinity for the λ B probe was determined by probing each purified phage stock with a tetramer of λ A sites in a similar *in situ*-binding assay. High titer stocks of recombinant phage were generated by the plate lysis method (Sambrook et al. 1989).

Generation of lysogen extract

E. coli lysogens harboring recombinant λ gt11 phage were isolated as described (Singh 1991). After inducing for expression of

β -gal fusion proteins, lysogenized cells were used to prepare soluble extracts for DNA-binding assays.

In vitro transcription and translation

Plasmids containing cDNA inserts were linearized 3' of the coding sequence. Capped mRNA was synthesized by *in vitro* transcription of 1 μ g of linearized plasmid in the presence of 10 mM 7mG(5')ppp(5')G (New England Biolabs) and T3 or T7 RNA polymerase (Promega). After treatment with RNase-free DNase to remove the template, phenol extraction, ethanol precipitation, and resuspension in 20 μ l of water, 2 μ l of the RNA suspension was used to program a 50- μ l rabbit reticulocyte lysate (Promega) *in vitro* translation reaction, which was incubated at 30°C for 1 hr. In some cases, parallel reactions were prepared in which the proteins were labeled by inclusion of [³⁵S]methionine (NEN) in the reaction mix. When proteins were labeled with ³⁵S they were analyzed by SDS-PAGE and autoradiography.

EMSA and methylation interference

EMSAs were performed as described (Singh et al. 1986; Eisenbeis et al. 1993). Probes were generated by digestion of plasmid DNAs containing the sequence of interest and labeled by filling in the ends with Klenow in the presence of [³²P]dCTP. Methylation interference analysis was performed as described (Baldwin 1988; Eisenbeis et al. 1993). Lysogen extract from the λ 13 cDNA clone was incubated with methylated λ B site probe and electrophoresed on a 4% polyacrylamide gel to isolate free and complexed probe. Chemical cleavage products were electrophoresed on 5% polyacrylamide/7 M urea gels. Autoradiography was performed on dried gels.

Subcloning and sequencing

The cDNA inserts from the λ gt11 clones were subcloned as *NcoI*-*NdeI* fragments into the *EcoRV* site of pBluescript II (SK+) to generate λ 13N/N, λ 15N/N, λ 37N/N, λ 37.5N/N, and λ 37.9N/N. In the case of λ 37.9, which contains two *NcoI* sites within its coding sequence, the full-length insert was isolated from a partial *NcoI*-*NdeI* digest. In addition, the Pip-coding sequence from λ 37.9N/N was subcloned as a *MluI*-*BclI* fragment into the *EcoRV* site of pBluescript II (SK+) to generate 9(M/B). The entire λ 37.9N/N cDNA insert was sequenced completely on both strands by generation of a set of nested exonuclease III deletions from each end of the insert (Slatko et al. 1991), followed by sequencing with T3 and T7 primers, dideoxynucleotide chain terminators, and Sequenase enzyme. The Pip ORF was subcloned into the pBSATG vector to generate 9ATG by engineering a *HindIII* site just upstream of the second amino acid of the Pip protein by polymerase chain reaction (PCR) using the 9ATG primer (5'-GGGGAAGCTTAACCTGGAGACGG-GCAG-3') in conjunction with the T7 primer and 9(M/B) as the template DNA. The Pip sequence was then isolated as a *HindIII* fragment, blunted by filling in with Klenow, and subcloned into *SmaI*-cut pBSATG (Baldwin et al. 1990). This construction places the Pip gene in the context of a strong translation initiation site and produces a protein *in vitro* that has the initial methionine and 8 amino acids provided by the vector and artificial restriction site, linked to the Pip ORF beginning with the second amino acid.

Northern analysis

Mouse tissue and cultured cell RNA was prepared by the guanidium thiocyanate method. Poly(A)⁺ RNAs were fractionated

on an oligo(dT)-cellulose column. For Northern analysis, 10 μ g of tissue RNA, 5 μ g of poly(A)⁺ RNA, or 10 μ g of cultured cell RNA was electrophoresed on a 1% agarose/formaldehyde gel and blotted onto Hybond N nylon membrane (Amersham). Blots were hybridized for ~16 hr at 40°C in 50% formamide, 5 $\times$ Denhardt's solution, 5 $\times$ SSC, 0.1% SDS, 10 μ g/ml of sonicated salmon sperm DNA, and ~10⁶ cpm/ml probe. After washing at high stringency the blots were subjected to autoradiography.

RPA

RPAs were performed using the RPA II kit (Ambion). The Pip antisense riboprobe was generated by digestion of the 9(M/B) Pip plasmid clone with *NheI* and transcription of the linearized plasmid *in vitro* using T7 RNA polymerase and 50 μ Ci of [³²P]CTP. A β -actin control probe was synthesized in a similar manner using T3 RNA polymerase and the control plasmid DNA provided with the kit. Riboprobes were gel purified on 5% polyacrylamide/7 M urea gels before use. Hybridization reactions contained 10 μ g of tissue RNA or 5 μ g of cultured cell RNA and 5 $\times$ 10⁴ cpm of each riboprobe, and were incubated for 18 hr at 45°C before digestion with RNase. Protected fragments were analyzed by electrophoresis on 5% polyacrylamide/7 M urea gels, followed by autoradiography with an intensifying screen at -70°C.

Transient transfection and CAT assay

The CAT reporter construct, B₄TKCAT has been described previously (Eisenbeis et al. 1993). To create Pip/CMV, PU.1/CMV, and S148A/CMV, the Pip, PU.1, and PU.1 S148A cDNAs were subcloned as *HindIII*-*XbaI* fragments into the *HindIII* and *XbaI* sites in the polylinker of pRc/CMV (Invitrogen), which places the cDNA inserts under the control of the CMV promoter. NIH-3T3 cells were transfected by the DEAE-dextran protocol (Selden 1987) using a total of 25 μ g of DNA. Transfection involved 5 μ g of reporter construct and 10 μ g of the expression constructs, with the balance made up with empty expression vector. After 46 hr at 37°C, cell lysates were prepared and CAT assays performed using 160 μ l of lysate as described previously (Eisenbeis et al. 1993). TLC plates were analyzed on a Molecular Dynamics PhosphorImager; the results shown here represent an average of at least three independent transfections.

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