to 20 min. Utricles were explanted to chilled Medium-199 containing 25 mM Hepes buffer and Hanks' salts (Gibco). The sensory epithelia were isolated, and the otolithic membranes were removed with fine forceps. The culture chambers contained small wells made from cover glasses and Polyallomer rings 9 mm in diameter attached with Silastic adhesive. They were coated with Cell-Tak (Collaborative Research) before one or two utricles were placed in each well with 50 μl of medium. The medium consisted of Medium-199 with Earle's salts, 26 mM sodium bicarbonate, 25 mM Hepes, 0.69 mM L-glutamine (Gibco), supplemented with 20% fetal bovine serum (FBS) (Gibco), penicillin (10 units/ml) and Fungizone (25 ng/ml). Cultures were maintained at 37°C in a 5% CO_2 environment. Guinea pigs become sexually mature at 4 to 8 weeks of age [J. E. Wagner and P. J. Manning, *The Biology of the Guinea Pig* (Academic Press, New York, 1976), p. 9]. Seven of the specimens were older than 8 weeks, and all were at least 6 weeks old. The labeling observed was comparable in utricles from older and younger specimens. All protocols were in accordance with the University of Virginia's guidelines for use of animals in research.

5. Cultures were incubated for 24 hours in Medium-199 with 20% FBS that contained 0.5 to 1.0 mM neomycin or 1.0 to 2.0 mM gentamicin.
6. After 24 hours, cultures were rinsed twice with Medium-199, and fresh medium without any aminoglycoside antibiotics was added. The aminoglycoside-free media contained either of two mitotic tracers: [^{3}H]methyl-thymidine (0.8 μCi/ml, 65 Ci/mmol) or 5-bromo-2′-deoxyuridine (BrdU, 3 μg/ml) in solution with 5-fluoro-2′-deoxyuridine (0.27 μg/ml) from Amersham.
7. The epithelia that had been cultured with BrdU were rinsed in Medium-199 and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for 30 min. Fixed epithelia were rinsed three times in phosphate-buffered saline (PBS), treated in 2 N HCl for 30 min, incubated in PBS with 0.5% Triton X-100 for 15 min, and incubated in PBS alone for an additional 15 min. The epithelia were then incubated overnight in a solution containing a monoclonal antibody to BrdU (Amersham) with 0.5% Triton X-100 at 4°C. After three rinses in PBS, specimens were incubated for 30 min with a secondary antibody conjugated to peroxidase-antiperoxidase. They were reacted with diaminobenzidine (0.5 mg/ml) in 0.05 tris buffer containing 0.01 M imidazole and 0.03% hydrogen peroxide, mounted in glycerol-PBS (9:1), and viewed as whole mounts.
8. The utricles that were cultured with [^{3}H]methyl-thymidine were fixed in 3% glutaraldehyde in 0.1 M PB (pH 7.4) and postfixed in 1% OsO_4. They were dehydrated in ethanol and embedded in methacrylate (Historesin, Leica). Serial sections were cut at 3-μm thickness, mounted on slides, and dipped in a 50% aqueous solution of nuclear track emulsion (Kodak NTB-2). Emulsion-coated slides were placed in light-tight boxes and stored at 4°C for 10 to 15 days. Slides were developed in Kodak D-19 and counterstained with thionine or toluidine blue.
9. Hair cells were counted in comparable regions through control and aminoglycoside-treated utricles that were maintained in culture for 5 days. Every third section through a 100-μm-wide band across the center of each sensory epithelium was counted. In two controls, the bands contained 458 and 615 hair cells, with 41.6 ± 5.1 and 55.9 ± 5.3 (mean ± SD) hair cells per section, respectively. In two utricles that had been treated for 24 hours with 1 mM neomycin and then cultured for 4 days in aminoglycoside-free medium, the bands contained 148 and 73 hair cells, with 13.5 ± 2.5 and 6.6 ± 2.9 hair cells per section, respectively.
10. All the utricles in this series were cultured for 24 hours in media that contained 1 mM neomycin. Then they were rinsed three times in fresh medium and mounted on cover glasses with the use of 20 to 25 μl of Matrigel (Collaborative Research). Cover glasses were placed in roller tubes that contained 2 ml of culture medium [Medium-199 supplemented with 20% FBS and containing [^{3}H]methyl-thymidine (0.8 μCi/ml)]. Cultures were maintained at 37°C and rolled through four revolutions per hour. At least half of the culture medium was replaced with fresh medium every 4 to 7 days. After 27 days in roller tube culture, the utricles were fixed in glutaraldehyde (in 0.1 M PB) and processed for autoradiography as described (*8*).
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12. Utricles were obtained from three human patients, two females (40 and 52 years of age) and one male (63 years of age), during surgical intervention for the removal of acoustic neuromas. Written informed consent was given before surgery. Utricles were placed in sterile 0.9% NaCl, quickly transferred to chilled Medium-199, and transported to culture facilities in a chilled ice chest. After dissection to expose the sensory epithelium, utricles were placed in culture wells containing 50 μl of medium. Sixty to ninety minutes elapsed between surgical removal and placement in culture. Utricles were incubated in 1 mM neomycin in Medium-199 with 20% FBS for 24 hours and then cultured for either 6 or 24 days in aminoglycoside-free media that contained [^{3}H]methyl-thymidine (0.8 μCi/ml). At 2-day intervals, half of the culture medium was replaced with fresh medium that contained [^{3}H]methyl-thymidine. The utricles were fixed with 3% glutaraldehyde in 0.1 M PB and processed for autoradiography as outlined above.
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19 August 1992; accepted 19 January 1993

Effect of PU.1 Phosphorylation on Interaction with NF-EM5 and Transcriptional Activation

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PU.1 recruits the binding of a second B cell–restricted nuclear factor, NF-EM5, to a DNA site in the immunoglobulin κ 3′ enhancer. DNA binding by NF-EM5 requires a protein-protein interaction with PU.1 and specific DNA contacts. Dephosphorylated PU.1 bound to DNA but did not interact with NF-EM5. Analysis of serine-to-alanine mutations in PU.1 indicated that serine 148 (Ser^{148}) is required for protein-protein interaction. PU.1 produced in bacteria did not interact with NF-EM5. Phosphorylation of bacterially produced PU.1 by purified casein kinase II modified it to a form that interacted with NF-EM5 and that recruited NF-EM5 to bind to DNA. Phosphopeptide analysis of bacterially produced PU.1 suggested that Ser^{148} is phosphorylated by casein kinase II. This site is also phosphorylated in vivo. Expression of wild-type PU.1 increased expression of a reporter construct containing the PU.1 and NF-EM5 binding sites nearly sixfold, whereas the Ser^{148} mutant form only weakly activated transcription. These results demonstrate that phosphorylation of PU.1 at Ser^{148} is necessary for interaction with NF-EM5 and suggest that this phosphorylation can regulate transcriptional activity.

Modification by phosphorylation can influence either the binding to DNA or the functional activity of a number of transcription factors including SRF, Myb, Max, c-Jun, and cyclic adenosine monophosphate response element–binding protein (CREB) (*1*). Phosphorylation of IκB-like proteins can also control the nuclear localization of Rel-related proteins such as NF-κB (*2*). PU.1 is an Ets-related transcription factor (*3*) that is implicated in the genesis of erythroleukemia (*4*). PU.1 expression is restricted to macrophages and B cells. Many Ets family members are expressed in a tissue-restricted fashion and therefore may control the expression of tissue-specific genes (*5*). PU.1 recruits the binding of another B cell–restricted nuclear factor, NF-EM5, to a specific DNA sequence in the immunoglobulin κ 3′ enhancer (κE3′) (*6*). NF-EM5 requires a protein-protein interaction with PU.1 and specific protein-DNA interactions in order to bind to DNA. The DNA binding sites for PU.1 and NF-EM5 lie adjacent to one another in the κE3′ enhancer. These two sites appear to be important for κE3′ enhancer activity because mutation of either site reduces transcriptional activity (*6*). Recruitment of NF-EM5 to DNA requires a 43–amino acid

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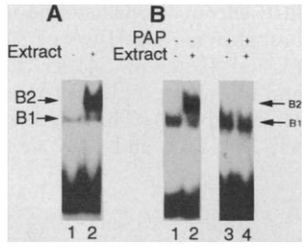

Fig. 1. Effect of phosphatase treatment of PU.1 on its interaction with NF-EM5. (**A**) PU.1 prepared by in vitro translation was assayed by EMSA (*15*) with a κE3′ enhancer probe (GATCCCTTTGAGGAACTGAAAACAGAACCTAGATC) either alone (lane 1) or in the presence of NF-EM5 from a nuclear extract from Ag8 plasmacytoma cells (lane 2) (*6*). (**B**) In vitro–translated PU.1 (6 μl) was treated with PAP in a total volume of 20 μl of a solution containing 100 mM KCl, 20 mM Hepes (pH 7.9), 0.2 mM EDTA, 0.5 mM DTT, and 2 U of PAP (Sigma) at 30°C for 30 min and was then assayed by EMSA alone or in the presence of NF-EM5 as described. The presence or absence of phosphatase or nuclear extract is indicated at the top of each lane.

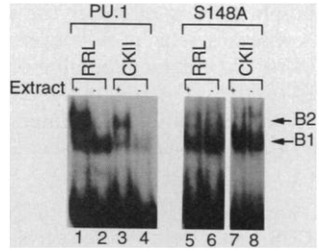

Fig. 4. Effect of a mutation in PU.1 on its interaction with NF-EM5. S148A was expressed in bacteria from the pET11d vector and purified to homogeneity (*16*). The bacterially expressed protein was treated with RRL or with purified CKII (*17*) and assayed by EMSA as described (Figs. 1 and 2).

segment in PU.1 spanning amino acid residues 118 to 160. This region contains three serine residues that lie in two consensus sequences (*7*) for casein kinase II (residues 132 to 137 and residues 148 to 153). We therefore determined whether the phosphorylation status of PU.1 controlled its interaction with NF-EM5, the binding of these factors to DNA, and the subsequent transcriptional activity in the cell.

PU.1 protein was prepared by in vitro transcription and translation of the cloned cDNA. In vitro–translated PU.1 protein interacts with NF-EM5 and recruits it to bind to DNA (6). When a κE3′ enhancer probe spanning residues 445 to 469 (*8*) is used in electrophoretic mobility shift assays (EMSAs) in the presence of PU.1 and NF-EM5, two bound complexes are observed (6). The faster migrating complex (B1) represents PU.1 bound to DNA and the slower migrating complex (B2) represents PU.1 and NF-EM5 bound to the DNA (Fig. 1A). To determine whether phosphorylation affects PU.1 interaction with NF-EM5, we treated the PU.1 protein prepared by in vitro translation with potato acid phosphatase (PAP). The untreated and PAP-treated PU.1 proteins were then assayed for their ability to bind to DNA and to interact with NF-EM5. Both the untreated and treated samples bound to the κE3′ enhancer PU.1 site when assayed by EMSA (Fig. 1B). The untreated PU.1 protein recruited NF-EM5 [supplied by an Ag8 plasmacytoma nuclear extract, which contains NF-EM5 but lacks PU.1 (6)] to bind to DNA. However, no binding of NF-EM5 was observed in the presence of the PAP-treated PU.1. No specific binding to this probe was observed when the Ag8 extract was assayed alone (6). These results suggest that phosphorylation of PU.1 is necessary for PU.1 interaction with NF-EM5. If that is true, PU.1 produced in bacteria should not interact with NF-EM5 because bacteria do not have protein kinases analogous to those in eukaryotes.

The PU.1 cDNA sequence was placed into the pET11d vector in a manner such that the entire PU.1 coding sequence was fused in frame with six histidine residues at the NH_2-terminal end. The PU.1 protein was synthesized in bacteria, purified to homogeneity by chromatography over a nickel chelate resin (which binds to the six histidines fused to the PU.1 sequence), and assayed for DNA binding and interaction with NF-EM5 by EMSA. The bacterially produced PU.1 protein bound to the κE3′ enhancer sequence (Fig. 2) but did not interact with NF-EM5 to produce the B2 complex (the faint upper band in lane 1 does not comigrate with the B2 complex). After incubation of the bacterially made PU.1 protein with a rabbit reticulocyte translation lysate (RRL), the protein did interact with NF-EM5. Because reticulocyte lysates contain casein kinase II (CKII) activity (9) and because the PU.1 sequences between residues 118 to 160 (which are required for interaction with NF-EM5) contain two CKII consensus sequences, we tested whether CKII could substitute for the reticulocyte lysate. Bacterially produced PU.1 phosphorylated by CKII interacted with NF-EM5 and recruited NF-EM5 to bind to DNA (Fig. 2). Inclusion of the CKII inhibitor heparin in the kinase assay inhibited the modification of PU.1 that caused it to interact with NF-EM5. When the inhibitor was added after CKII treatment, PU.1 did interact with NF-EM5 (*10*). To further ensure that CKII was modifying only PU.1 and not NF-EM5, the bacterially produced PU.1 phosphorylated by CKII was purified by DNA affinity chromatography (*11*). This purified PU.1 sample interacted with NF-EM5 (Fig. 2). These results indicate that CKII can modify PU.1 to a form capable of interacting with NF-EM5.

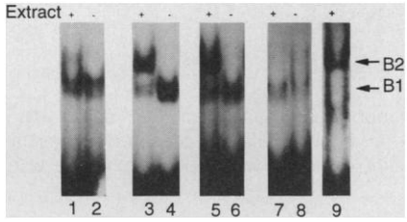

Fig. 2. Effect of phosphorylation on the binding of bacterially synthesized PU.1 to NF-EM5. The PU.1 coding sequence fused to six histidine residues at the NH_2 end was expressed in bacteria and was purified to homogeneity by nickel chelate chromatography (*16*). The bacterially expressed PU.1 protein was either left unmodified (lanes 1 and 2), incubated with an equal volume of RRL for 30 min at 30°C (lanes 3 and 4), treated with purified CKII (lanes 5 and 6) (*17*), treated with CKII in the presence of heparin (lanes 7 and 8), or treated with CKII and then purified by DNA affinity chromatography (lane 9). A portion (200 ng) of each sample was then assayed by EMSA either alone or in the presence of NF-EM5 supplied by 8 μg of Ag8 plasmacytoma nuclear extract.

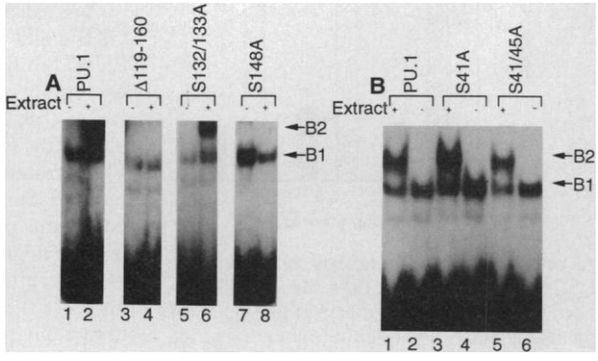

Fig. 3. Effect of mutations in PU.1 on its interaction with NF-EM5. Serine-to-alanine mutations were prepared at PU.1 residues 41, residues 41 and 45, residues 132 and 133, or residue 148 (*18*). Construction of the Δ119–160 construct was as described (*6*). Proteins were prepared by in vitro translation from (**A**) clones Δ119–160, S132/133A, and S148A or (**B**) clones S41A and S41/45A and assayed by EMSA as described in Fig. 1.

If phosphorylation of one of the serines in the consensus sequences between residues 118 to 160 enhances binding of PU.1 to NF-EM5, then mutation of the serine in this site should inhibit the interaction, whereas other serine mutations should have little effect. We made serine-to-alanine mutations in residue 41 (clone S41A), residues 41 and 45 (clone S41/45A), residues 132 and 133 (clone S132/133A), or residue 148 (clone S148A). These serine residues (41, 45, 132, 133, and 148) are sites of phosphorylation in vivo (although it is not clear whether serines 132 and 133 are both phosphorylated) (*12*). Proteins were prepared by in vitro translation of each mutant cDNA. Proteins were also prepared from the unmutated PU.1 sequence and from a clone in which amino acids 119 to 160 are deleted (clone Δ119–160). As expected, the unmutated PU.1 protein bound to the κE3′ enhancer probe and recruited NF-EM5 to the DNA (Fig. 3A). The Δ119–160 clone bound to DNA but did not interact with NF-EM5. Mutant S132/133A bound to DNA and still interacted with NF-EM5, albeit to a lesser extent than the wild-type protein. On the contrary, clone S148A bound to DNA but did not interact with NF-EM5. Mutation of serines 41 and 45 did not affect the interaction of PU.1 with NF-EM5 (Fig. 3B). These results indicate that Ser^{148} is required for a protein-protein interaction between PU.1 and NF-EM5.

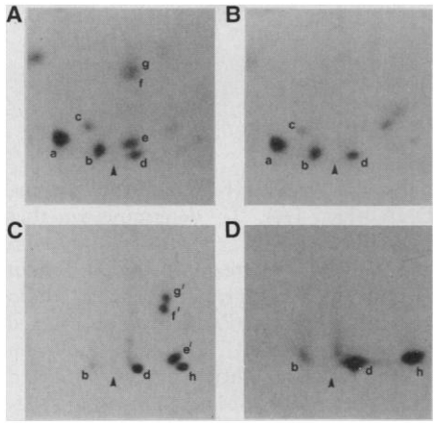

Fig. 5. Phosphorylation of Ser^{148} by CKII. (**A** and **B**) COS-1 cells were transfected with wild-type and S148A expression plasmids (*3*). After 3 days, cells were labeled with [^{32}P]orthophosphate (2.5 mCi/ml) in phosphate-free Dulbecco's modified Eagle's medium supplemented with bovine serum albumin (0.4 mg/ml) for 4 hours. PU.1 proteins were isolated from cell lysates with anti-PU.1 serum, separated on an SDS-polyacrylamide gel, and transferred to PVDF membranes. The 43.5-kD protein bands were excised and processed for phosphopeptide analysis with chymotrypsin and staphylococcal V8 proteases (*19*). (**C** and **D**) Bacterial PU.1 protein was isolated, phosphorylated with CKII (*20*), and processed for phosphopeptide analysis (*19*). (A and C) Wild-type protein. (B and D) S148A mutant PU.1 protein. The maps shown in (A) and (B) were derived from sample containing 140 cpm, and film was exposed to the gel with an intensifying screen at −80°C for 15 days; (C) 1000 cpm with film exposed for 27 hours; (D) 100 cpm with film exposed for 24 days. We overexposed the autoradiograph shown in (D) relative to that in (C) to permit detection of any peptides in the e, f, and g regions. Arrowheads indicate the electrophoretic origins.

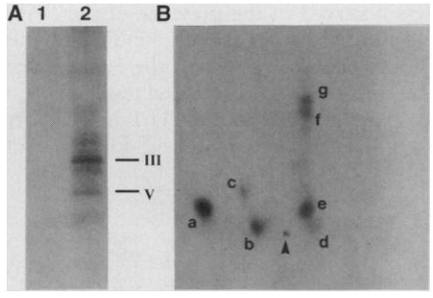

Fig. 6. Multiple PU.1 phosphorylation sites in B cells. (**A**) S107 plasmacytoma cells were labeled with carrier-free [^{32}P]orthophosphate (2.5 mCi/ml) in phosphate-free RPMI-1640 medium supplemented with bovine serum albumin (0.4 mg/ml) for 3.3 hours. PU.1 protein was isolated from cell lysates with anti-PU.1 serum and fractionated by SDS–polyacrylamide gel electrophoresis, and the separated proteins were transferred to PVDF membranes (*19*). Roman numerals indicate the form of the PU.1 protein determined from their mobilities versus the proteins expressed in a rabbit reticulocyte lysate: III, 43.5 kD; and V, 38 kD (*21*). Lane 1, normal rabbit serum; lane 2, anti-PU.1 serum. (**B**) The peptide map for the 43.5-kD form III of PU.1 isolated from S107 cells was prepared as described in Fig. 5. The map was developed from 110 cpm of sample and film was exposed to the gel with an intensifying screen at −80°C for 28 days.

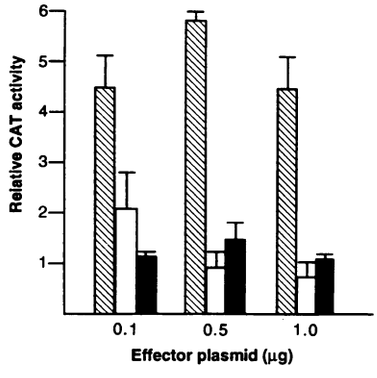

Fig. 7. Interaction of PU.1 and NF-EM5 to enhance transcriptional activity in vivo. Expression plasmids CMV-PU.1 or CMV-S148A were each co-transfected with reporter plasmids PU.1-EM5CAT (*6*) or PU.1CAT (*6*) into S194 plasmacytoma cells by the DEAE-dextran procedure (*22*). Transfections contained reporter plasmid (2 μg), effector plasmid (0.1, 0.5, or 1.0 μg), and β-galactosidase–expressing plasmid (1 μg), which we used to normalize transfection efficiencies. The total DNA content was maintained at 6 μg by the addition of pUC18 (Gibco BRL, Gaithersburg, Maryland) DNA. Cells were harvested after 44 hours, and CAT activities (*23*) were determined by liquid scintillation counting of the acetylated and nonacetylated compounds resolved by thin-layer chromatography. We normalized values to the amount of CAT activity of each reporter in the absence of effector plasmid to obtain relative CAT activity. Hatched bars represent the CMV-PU.1 effector and the PU.1-EM5CAT reporter, open bars the CMV-S148A effector and the PU.1-EM5CAT reporter, and filled bars the CMV-PU.1 effector and the PU.1CAT reporter. Results show the average of two independent transfections, and error bars show the range of the two values.

To confirm the role of Ser^{148} in the CKII-mediated modification of PU.1, we performed the following experiments. The S148A mutant protein was synthesized in bacteria and purified to homogeneity. This protein bound to the κE3′ probe, but incubation of the bacterially produced S148A protein with either a reticulocyte lysate or with purified CKII did not cause the protein to interact with NF-EM5 (Fig. 4).

These data suggest that Ser^{148} is phosphorylated. To confirm this, we expressed the wild-type and S148A proteins in transfected COS cells metabolically labeled with ^{32}P. Phosphoamino acid analysis demonstrated that PU.1 contains phosphoserine but no phosphothreonine or phosphotyrosine (*12*). Phosphopeptide analysis indicated that the peptide map derived from the wild-type PU.1 protein contains several phosphopeptides (Fig. 5A). The map derived from S148A lacks three peptides (labeled e, f, and g) found in the map of the wild-type PU.1 protein (Fig. 5B). Peptides e, f, and g are likely generated by cleavage with the staphylcoccal V8 endopeptidase at a variety of acidic residues near the labeled serine residue (*13*). These results suggest that PU.1 expressed in COS cells is phosphorylated at Ser^{148}.

We also examined whether purified CKII could phosphorylate bacterially made PU.1. Similar to the data in COS cells, the bacterially produced S148A mutant protein lacked three peptides (e′, f′, and g′) detected in the map of the bacterial, wild-type PU.1 protein (Fig. 5, C and D). The three putative Ser^{148}-containing peptides detected in the phosphorylated, bacterial PU.1 protein migrated in a manner similar to that of the peptides isolated from the mammalian protein in the chromatography step but always migrated more rapidly in the electrophoresis step (*14*). These results show

that mutation of Ser^{148}, which lies in a CKII consensus sequence, results in the loss of phosphopeptides e, f, and g from in vivo–labeled PU.1, as well as peptides e′, f′, and g′ from CKII-treated bacterial PU.1. Whereas sequence data for peptides e, f, and g would definitively identify Ser^{148} as the phosphorylated residue, the simplest interpretation of the data is that Ser^{148} is phosphorylated in vivo and that it can be phosphorylated by CKII.

To determine the phosphorylation state of PU.1 in B lymphocytes, we immunoprecipitated PU.1 from murine S107 plasmacytoma cells metabolically labeled with [^{32}P]orthophosphate (Fig. 6A). The map (Fig. 6B) derived for the 43.5-kD PU.1 protein (Fig. 6A, III) included peptides e, f, and g that are identified as diagnostic for phosphorylation at Ser^{148}. Thus, we conclude that PU.1 is phosphorylated on Ser^{148} in B lymphocytes.

Mutation of the PU.1 or NF-EM5 binding sites in the κE3′ enhancer reduces transcriptional activity (6). If PU.1 and NF-EM5 interact in vivo to contribute to enhancer activity, the Ser^{148} mutant form of PU.1 (clone S148A) should be a poor activator of transcription as compared with unmutated PU.1 because the former cannot recruit NF-EM5 to the DNA. The cDNA sequences encoding PU.1 and S148A were expressed from the cytomegalovirus (CMV) promoter and enhancer (constructs CMV-PU.1 and CMV-S148A) in transfection assays in S194 plasmacytoma cells. These constructs were co-transfected with a reporter construct containing the PU.1 and NF-EM5 binding sites linked to the thymidine kinase promoter and the chloramphenicol acetyltransferase (CAT) gene (construct PU.1-EM5CAT). Whereas the unmutated PU.1 cDNA activated expression nearly sixfold, the S148A mutant activated expression only twofold at low doses and did not activate expression at higher plasmid concentrations (Fig. 7). Co-transfection of cells with a CMV plasmid containing the PU.1 sequence in the noncoding orientation did not activate transcription of the CAT gene (*10*). These results suggest that both PU.1 and NF-EM5 contribute to enhancer activity. The difference in transactivation activity of the PU.1 and S148A proteins is unlikely to result from differences in protein stability because the proteins are comparably expressed in transfected COS and HeLa cells (*12*). To confirm the requirement for both PU.1 and NF-EM5 binding for efficient transactivation, we co-transfected cells with CMV-PU.1 and a reporter plasmid that contains a mutated NF-EM5 site but has an intact PU.1 site (construct PU.1CAT). PU.1 transactivated this reporter construct only 1.5-fold.

Our results demonstrate that the phosphorylation status of Ser^{148} in PU.1 controls its ability to interact with NF-EM5 and to recruit NF-EM5 to bind to DNA. This phosphorylation-mediated interaction appears to control transcriptional activity because the binding of both PU.1 and NF-EM5 to the κE3′ enhancer sequence was required for efficient transcriptional activation. The cellular kinase responsible for phosphorylating Ser^{148} may be CKII because Ser^{148} lies in a consensus sequence for this kinase. It will be interesting to determine whether Ser^{148} is differentially phosphorylated during B cell development.

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13. Ser^{148} is in a region of the PU 1 protein containing multiple glutamic acid residues (135-EEEGERQSPPLEVSDGEADGLE-156, abbreviations for the amino acid residues are: A, Ala; C, Cys, D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met, N, Asn; P, Pro; Q, Gln, R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr). Digestion by the staphylococcal V8 endopeptidase at pH 4.0 could generate alternative peptide products, depending on the initial cleavage site
14. Peptide maps derived from a mixture of digested protein phosphorylated in vivo and in vitro indicated that the peptides marked b and d in Fig. 5 migrated at the same respective positions. The cause for the anomalous migration of peptides e′, f′, and g′ during electrophoresis is under investigation.
15. H. R. Singh, R. Sen, D. Baltimore, P. A. Sharp, *Nature* **319**, 154 (1986).
16. To prepare the PU.1 bacterial expression vector, we inserted a Pvu II–Bam HI DNA fragment containing the entire PU.1 coding sequence, 4 bp of 5′ untranslated sequence, and 337 bp of 3′ untranslated sequence by blunt-end ligation in the blunted Hind III site of plasmid pET11d. To prepare the S148A bacterial expression vector, we inserted an Eco RI fragment containing the S148A cDNA coding sequence, 3 bp of 5′ untranslated sequence, and 16 bp of 3′ untranslated sequence in the Eco RI site of pET11d. Plasmids were transformed into bacterial strain BL21, and the integrity of each construct was verified by DNA sequencing. We induced protein expression by growing bacteria in a 500-ml culture in the presence of 1 mM isopropyl-1-thio-β-D-galactoside (IPTG) for 2 hours. Cells were centrifuged at 5000 rpm in a Sorvall GS3 rotor for 10 min and then lysed in 20 ml of buffer A [6 M guanidine HCl and 50 mM sodium phosphate (pH 8)] at 4°C overnight. The lysate was centrifuged at 15,000 rpm in a Sorvall SS34 rotor for 15 min and then loaded on a nickel chelate resin (Qiagen, Chatsworth, CA). The column was washed with three bed volumes of buffer A (pH 8) and then with three bed volumes of buffer A (pH 6). Proteins were eluted from the column with buffer A (pH 5) and dialyzed against four changes of phosphate-buffered saline with 5% glycerol (v/v).
17. In vitro CKII assays were performed at 30°C for 30 min in a reaction volume of 20 μl containing 1 μg of PU.1 or S148A, 20 mM tris (pH 7.9), 50 mM KCl, 8 mM $MgCl_2$, 2 mM adenosine triphosphate (ATP), 0.1 mM dithiothreitol (DTT), and 1 μl of purified CKII (0.04 mg/ml). EMSA was then performed with treated samples (4 μl). In some assays heparin (4 μg/ml) was included.
18. Plasmids containing the S41A, S41/45A, S132/133A, and S148A mutations were constructed by the polymerase chain reaction with oligonucleotides spanning the site to be mutated. The products of this reaction were isolated by agarose gel electrophoresis and inserted into the PU pECE plasmid (*3*) that had been digested with the appropriate enzymes. We determined the amino acid sequences of the inserted fragments to verify the DNA sequences.
19. J. A. Cooper, B. M. Sefton, T. Hunter, *Methods Enzymol.* **99**, 387 (1983).
20. For peptide maps, PU.1 protein expressed in bacteria was isolated by immune precipitation with antiserum to PU.1 (anti-PU.1 serum) and incubated with rat testes CKII (0.04 mg/ml) with 20 mM Hepes (pH 7.4), 10 mM $MgCl_2$, and [γ-^{32}P]ATP (4.5 mCi/ml) at 30°C for 25 min. Unlabeled ATP was added to a final concentration of 5 mM, and incubation was continued at 30°C for another 30 min.
21. C. Van Beveren *et al.*, in preparation.
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24. We thank R. Weinman, U. Kikkiawa, and T. Hunter for the purified CKII, T Kadesch for the pET11d plasmid, F. Rauscher for the CMV expression vector, and S. Schuetze and D. Kabat for the rabbit anti-PU 1 serum. Supported by NIH grants GM 42415 (to M.A.), AI 30656 (to R.M.), and CA 42909 (to C.V.B) and by the Lucille P Markey Charitable Trust (to M.A.)

1 June 1992, accepted 21 December 1992