MOLECULAR AND CELLULAR BIOLOGY, Jan. 1996, p. 390–397 Vol. 16, No. 1
0270-7306/96/$04.00+0

Activation of Transcription by PU.1 Requires both Acidic and Glutamine Domains

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Received 16 May 1995/Returned for modification 18 July 1995/Accepted 20 October 1995

The B-lymphocyte- and macrophage-specific transcription factor PU.1 is a member of the *ets* family of proteins. To understand how PU.1 functions as a transcription factor, we initiated a series of experiments to define its activation domain. Using deletion analysis, we showed that the activation domain of PU.1 is located in the amino-terminal half of the protein. Within this region, we identified three acidic subdomains and one glutamine-rich subdomain. The deletion of any of these subdomains resulted in a significant loss in the ability of PU.1 to transactivate in cotransfection studies. Amino acid substitution analysis showed that the activation of transcription by PU.1 requires acidic residues between amino acids 7 and 74 and a group of glutamine residues between amino acids 75 and 84. These data show that PU.1 contains two types of known activation domains and that both are required for maximal transactivation.

Transcription factors can regulate gene expression through a two-step process. The first step is the binding of a transcription factor to a specific DNA sequence element in a promoter and/or enhancer (18, 32). The second step is the interaction of the activation domain of this factor with the basal transcription machinery, resulting in an increase in the rate of transcription for a given gene (2). For most factors, these two functions reside in different parts of the protein (13, 18). Thus, to understand how individual transcription factors regulate gene expression, it is important to define the domains that mediate DNA binding and transactivation.

The B-lymphocyte- and macrophage-specific protein PU.1 is a member of the *ets* family of transcription factors (21). These factors have been defined on the basis of a region of identity with the v-*ets* oncogene. This region is approximately 90 amino acids in length and has been shown to encode a novel DNA binding domain. Several studies have shown that this region is sufficient for DNA binding and that most family members recognize a purine-rich DNA sequence centered on a core sequence of 5′-GGAA/T-3′. This led to the designation of these proteins as the ETS domain family (19).

Our initial studies of PU.1 showed that it binds to DNA in a sequence-specific manner and functions as a transacting factor in cotransfection studies (21). Thus, PU.1 could be classified as a transcription factor. Subsequently, the expression of an increasing number of genes have been shown to be regulated by PU.1. These include the kappa (30, 31) and lambda (7) light-chain, heavy-chain (27, 34), J chain (36), and B29 (28) genes in B cells and the CD11b (29), macrophage colony-stimulating factor receptor (37), scavenger receptor (26), FcγRIIIA (8), FcγR1b (6), and interleukin 1β (22) genes in macrophages. PU.1 regulates macrophage-specific expression of the equine infectious anemia virus retrovirus (1) and mast cell-specific expression of the interleukin 4 gene (15). PU.1 binds to sites located in the mouse β-globin intervening sequence 2, which may help regulate globin transcription during erythroid development (9).

To understand how PU.1 functions as a transcription factor, we initiated studies to define its functional domains. In this study, we performed detailed mapping and analysis of the PU.1 activation domain. In this report, we show that the activation domain of PU.1 is located in the amino-terminal half of the protein (between amino acids 7 and 118). Within this region, we have identified a large acidic subdomain and a small glutamine-rich subdomain. The acidic domain could be further divided into three regions. The deletion of any of these regions resulted in a dramatic decrease in the ability of PU.1 to transactivate in cotransfection studies. Substitution analysis showed that 10 acidic residues between amino acids 7 and 74 and a group of 5 glutamines between amino acids 74 and 84 are required for maximal activation of transcription by PU.1. These data show that PU.1 contains two types of known activation domains and that both are required for maximal activation of transcription.

MATERIALS AND METHODS

PU.1 constructions. All deletions and amino acid substitutions were created by PCR, with three exceptions. The clones with internal deletions of amino acids 33 to 100 and 74 to 100 were made by digestion of the full-length PU.1 cDNA with *Nsi*I and *Nco*I (33 to 100) or *Pst*I and *Nco*I (74 to 100), followed by self-ligation. The M5/M6/M2 amino acid substitution clone was made by ligating a *Pst*I fragment containing the M2 mutation and the 3′ half of this PU.1 clone into a PJ6 plasmid containing the 5′ end of PU.1 with the M5/M6 mutation. For these studies, all clones contained only the coding region of PU.1 (no 3′ untranslated sequence). PU.1 cDNA clones truncated after the coding region function as well as the full-length PU.1 cDNA clone 25.1 does in our cotransfection studies (see Fig. 1) (data not shown). The 5′ deletions were created by using 5′ primers with initiation codons placed at the designated amino acid positions. The internal deletions were created by overlap extension PCR (16). Briefly, a pair of complementary oligonucleotides spanning the region to be deleted were made. These oligonucleotides contain sequences from the 5′ end of the cDNA directly before the deletion and from the 3′ end directly after the deletion. In the first round of PCR, the top oligonucleotide is used with a primer to the 3′ end of the entire cDNA clone and the bottom oligonucleotide is used with a primer to the 5′ end of the entire cDNA clone. The two fragments generated have an overlapping sequence that is equivalent to the complementary oligonucleotides described above. These two fragments are mixed and added to a second PCR that uses only the 5′ and 3′ primers. The product of this reaction is agarose gel purified, ligated into Bluescript KS+ (Stratagene, Inc., La Jolla, Calif.), and sequenced to confirm that the correct deletion has been created. Amino acid substitutions were made

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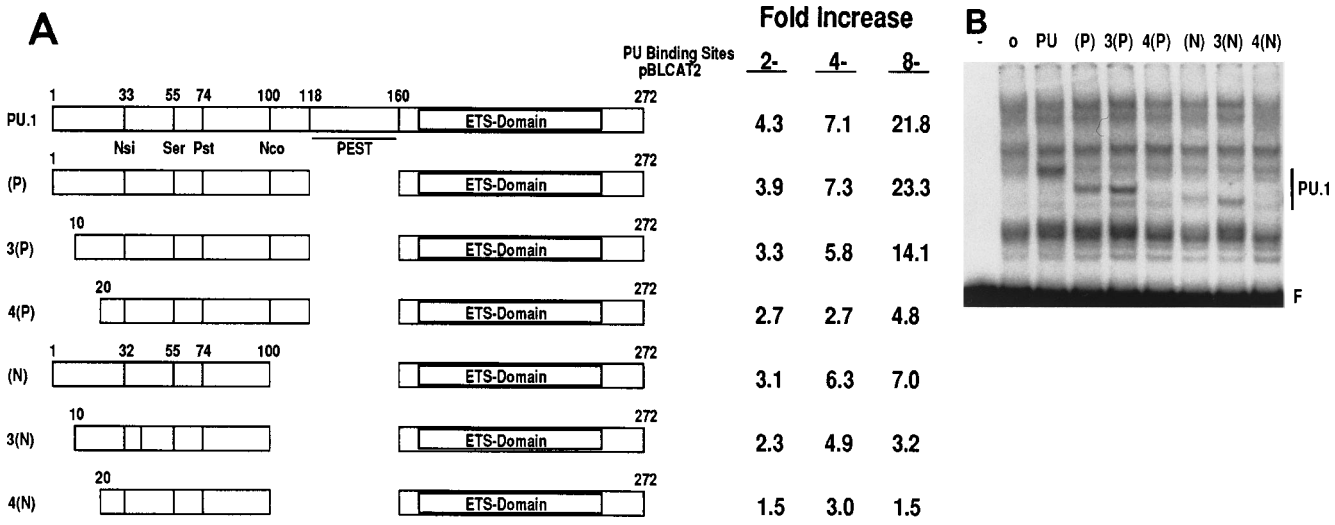

FIG. 1. Mapping the minimal activation domain of PU.1. (A) A combination of 5′ amino-terminal and 3′ internal deletions were used to map the minimal activation domain of PU.1. The top diagram represents the 272-amino-acid coding sequence of PU.1. The ETS domain is the DNA binding domain for PU.1. The numbers above each diagram represent the amino acids in PU.1 used as boundaries for the internal deletion studies of this report (see Fig. 3). The PEST domain (between amino acids 118 and 160) is shown. Nsi, Pst, and Nco, restriction enzyme sites used for internal deletions. Ser, designation used to map the locations of several serine residues in an acidic subdomain. Thin-layer chromatography plates were quantitated by using an AMBIS Radioanalytic Imaging System. The fold increase above the level of CAT reporter plasmid alone is shown for each set of DNA binding sites (PU binding sites) in the reporter plasmid pBLCAT2. Each mutant PU.1 clone was tested in at least six experiments. (B) Band shift analysis with nuclear extracts from HeLa cells transfected with each PU.1 clone. Lane - and F, free probe; PU.1, full-length PU.1 proteins; lane o, nuclear extracts from HeLa cells transfected only with the reporter plasmid.

by overlap extension PCR, except the internal primers have nucleotide base substitutions that code for the new amino acids. The individual clones were transferred to the expression vector PJ6 (25) and used in these cotransfection studies. PU.1 binding sites were cloned into the chloramphenicol acetyltransferase (CAT) reporter vector pBLCAT2 (24).

Transfection assays. Transfections were carried out with HeLa cells by lipofection, as previously described (21). Briefly, 3 μg of the PJ6 expression vector containing a PU.1 clone; 2 μg of the pBLCAT2 reporter plasmid containing PU.1 DNA binding sites; 3 μg of the β-galactosidase expression plasmid pCH110 (Pharmacia, Inc., Piscateway, N.J.), which is used as an internal control for transfection efficiency; and Bluescript KS+ to a final total of 20 μg of DNA were mixed in 2.5 ml of Dulbecco modified Eagle medium (DMEM) without fetal calf serum. Lipofectin (Life Technologies, Inc., Gaithersburg, Md.) (45 μg per each plate transfected) was mixed with 1.5 ml of DMEM without fetal calf serum. After a 15-min incubation at room temperature, the lipid and DNA samples were mixed. Cells plated the previous day at 10^6/10-cm-diameter dish were washed two times with DMEM without fetal calf serum, and the DNA-lipid mixture was added to each plate. After an 8-h incubation, 10 ml of DMEM with 5% Fetalclone (HyClone Laboratories, Inc., Logan, Utah) was added to each plate. At 24 h posttransfection, the medium was replaced with 15 ml of DMEM with 5% Fetalclone. Cells were harvested at 48 to 56 h posttransfection, washed in 1 ml of phosphate-buffered saline (PBS), and resuspended in 140 μl of 250 mM Tris (pH 7.8). Extracts were prepared by three freeze-thaw cycles. CAT enzyme activity was measured by using standard protocols (11). The amount of extract from each sample used to measure CAT enzyme activity was normalized on the basis of β-galactosidase activity. Thin-layer chromatography plates were quantitated by using an AMBIS Radioanalytic Imaging System (AMBIS Systems, Inc., San Diego, Calif.).

Band shift analysis. Band shift analysis was performed as previously described (21). The probe used was an oligonucleotide representing the consensus PU.1 DNA binding site previously described (20). Nuclear extracts were generated from individual 10-cm-diameter plates after transient transfection. Briefly, cells were washed once in PBS and the cell pellet was frozen in a microcentrifuge tube at −80°C. Cells were thawed and resuspended in 100 μl of TKM (50 mM Tris [pH 8.0], 50 mM KCl, 15 mM $MgCl_2$), with the subsequent addition of 100 μl of 60% sucrose and a final concentration of 0.1% Nonidet P-40. The cells were lysed by inversion of tubes 10 times and centrifuged for 1 min. The pelleted nuclei were resuspended in 30 μl of nuclear lysis buffer (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.9], 100 mM KCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10% glycerol) and rotated on ice for 20 min. The chromatin was pelleted by centrifugation for 10 min at 4°C. Nuclear extracts were quantitated by the Bio-Rad protein assay (Bio-Rad Laboratories, Inc., Hercules, Calif.). Five micrograms of nuclear extract from each transfected plate was used for band shift analysis. Extracts made identically from the mouse macrophage cell line RAW 264.7 were used as a positive control in these experiments (data not shown).

RESULTS

Mapping the PU.1 activation domain. To map the activation domain of PU.1, we made a series of deletions and amino acid substitutions of the PU.1 cDNA. These constructions contain the minimal coding region of PU.1 and lack the 3′ untranslated sequence. PU.1 cDNA clones were ligated into the expression plasmid PJ6, which contains the rat β-actin promoter (25). PU.1 DNA binding site oligonucleotides were ligated into the CAT reporter plasmid pBLCAT2 (24). For all experiments, HeLa cells were used because they do not express PU.1. Cotransfection of the minimal coding PU.1 cDNA clone and reporter plasmids containing two, four, or eight PU.1 DNA binding sites resulted in 3-, 7-, and 21-fold increases in the level of CAT enzyme activity, respectively (Fig. 1A). The fold increase was calculated as the level of activity seen above that of the CAT reporter plasmid alone. This is consistent with our previous results with the wild-type full-length PU.1 cDNA clone 25.1 (21) and shows that the lack of 3′ untranslated sequences does not prevent PU.1 from increasing the rate of transcription of the CAT reporter gene. For all subsequent experiments, the PU.1 clones did not contain the 3′ untranslated sequences.

To determine the 5′ and 3′ boundaries of the PU.1 activation domain, a series of amino-terminal truncations and internal deletions were created (Fig. 1A). The deletion of the PEST region, amino acids 118 to 160, resulted in no significant decrease in the ability of PU.1 to transactivate from the CAT reporter plasmids (Fig. 1A). The deletion of 10 or 20 amino acids from the 5′ end of the PEST deletion clone resulted in a decrease in the ability of PU.1 to transactivate from a CAT reporter plasmid. Comparison of the −20 mutant clone to the wild-type PU.1 protein showed that the level of activity was reduced from a 4.3- to 2.7-fold increase with two DNA binding sites, from a 7.1- to 2.7-fold increase with four DNA binding sites, and from a 21.8- to 4.8-fold increase with eight DNA binding sites. The results seen with the 5′ deletions were the

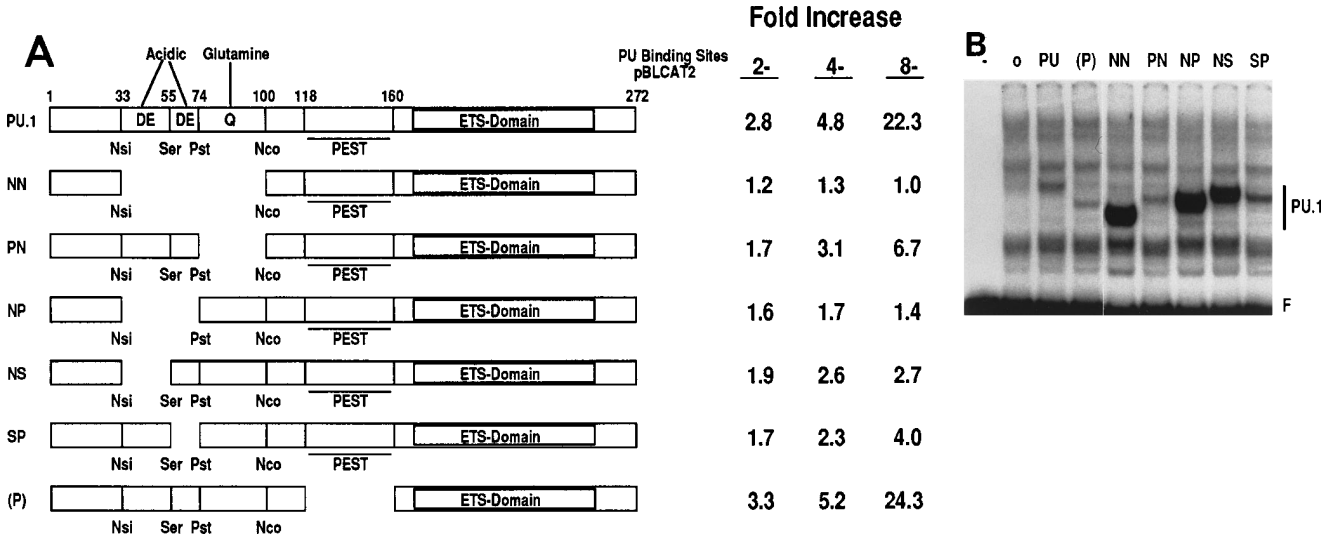

FIG. 2. Internal deletions of the PU.1 activation domain. A series of internal deletions were generated to determine whether the acidic and/or glutamine-rich subdomains are required for transactivation by PU.1. (A) Individual deletion mutants were tested with two, four, or eight DNA binding sites in cotransfection experiments. Each mutant PU.1 clone was tested in at least six independent experiments. (B) Band shift analysis to assess the levels of PU.1 proteins after transfection. The designations, abbreviations, and explanations of these deletions are the same as those in the legend to Fig. 1.

same as those with the wild-type protein with the PEST domain deletion (data not shown). Further deletion from the 5′ end resulted in almost a complete loss of transactivation from the reporter plasmids (data not shown). These data suggest that the removal of amino acids from the amino-terminal end of the protein reduces the ability of PU.1 to activate transcription. To further map the 3′ boundary of the PU.1 activation domain, a second internal deletion from amino acids 100 to 160 was tested in our cotransfection system. This deletion of an additional 18 amino acids resulted in a small decrease in transactivation by PU.1 when two or four DNA binding sites were present in the reporter plasmid. When eight DNA binding sites were present, however, the level of activity was reduced by approximately 70%. The deletion of 20 amino acids from the 5′ end in combination with the deletion of amino acids 100 to 160 resulted in a protein with minimal activity (Fig. 1A). These data suggest that the PU.1 activation domain is located between amino acids 1 and 118.

To confirm in our cotransfection system that the differences seen were the results of specific deletions or substitutions, we monitored the level of PU.1 protein produced from the DNA transfected into each plate of HeLa cells by using the same freeze-thaw extracts from the CAT assays in band shift analyses and normalizing the amount of extract used from each sample on the basis of β-galactosidase activity (data not shown). The results from this experiment suggested that equivalent amounts of mutant proteins were made after transfection. Since the freeze-thaw method results in the breakdown of the PU.1 protein, we also made nuclear extracts from transiently transfected HeLa cells for each mutant PU.1 protein. The results from this experiment showed that all deletion mutants are produced (Fig. 1B). The only mutant PU.1 clones that consistently produced approximately 50% of the wild-type PU.1 protein levels were those with 5′ deletions of 20 or more amino acids. Since we transfect an excess of expression plasmid, the amount of protein seen should still be able to transactivate. Further, the mutant 3(N) is produced at levels nearly equivalent to those of wild-type PU.1, yet its ability to activate transcription from our reporter plasmids has been significantly decreased. Extracts made from HeLa cells transfected with the reporter gene alone (Fig. 1B, lane o) show no specific PU.1 complexes on the band shift gel. These data suggest that the deletion of PU.1 from the 5′ end and internal deletions of amino acids 100 to 160 result in proteins that have lost the ability to transactivate in our cotransfection system, and the loss of activity is most likely due to the deletion of specific portions of the PU.1 protein.

Deletion of acidic or glutamine-rich subdomains. A series of internal deletions between amino acids 33 and 100 were generated to determine the importance of this region for transactivation by PU.1. The deletion of all 68 amino acids resulted in the complete loss of activation of transcription by PU.1 (Fig. 2A). This loss of transactivation is consistent whether two, four, or eight binding sites are present in the CAT reporter plasmid. An analysis of amino acids 33 to 100 in the PU.1 activation domain showed that we could divide this region into three subdomains. This is based on the presence of acidic residues in the two subdomains from amino acids 33 to 55 and 55 to 74 and glutamine residues in the subdomain between amino acids 74 and 100. Both acidic amino acids and glutamines have been implicated as critical features in the activation domains of other transcription factors (3, 13). The deletion of any of these three subdomains resulted in a significant decrease in transactivation by PU.1 (Fig. 2). As a control, the deletion of the PEST domain did not reduce the amount of transactivation by PU.1. These results suggest two possibilities. The first is that all three subdomains are required for maximal transcriptional activation by PU.1. The second is that the deletion of any portion of this region changes the structure of the activation domain of PU.1, which prevents PU.1 from functioning as a transcription factor.

Band shift analysis of nuclear extracts for each of these plasmids showed that the three deletion mutants, NN, NP, and NS, were produced at significantly higher levels than was wild-type PU.1 (Fig. 2B). This shows that the deletion of amino acids 33 to 55 results in very stable proteins that accumulate to higher levels than does wild-type PU.1. These mutants, however, do not transactivate efficiently in our cotransfection system.

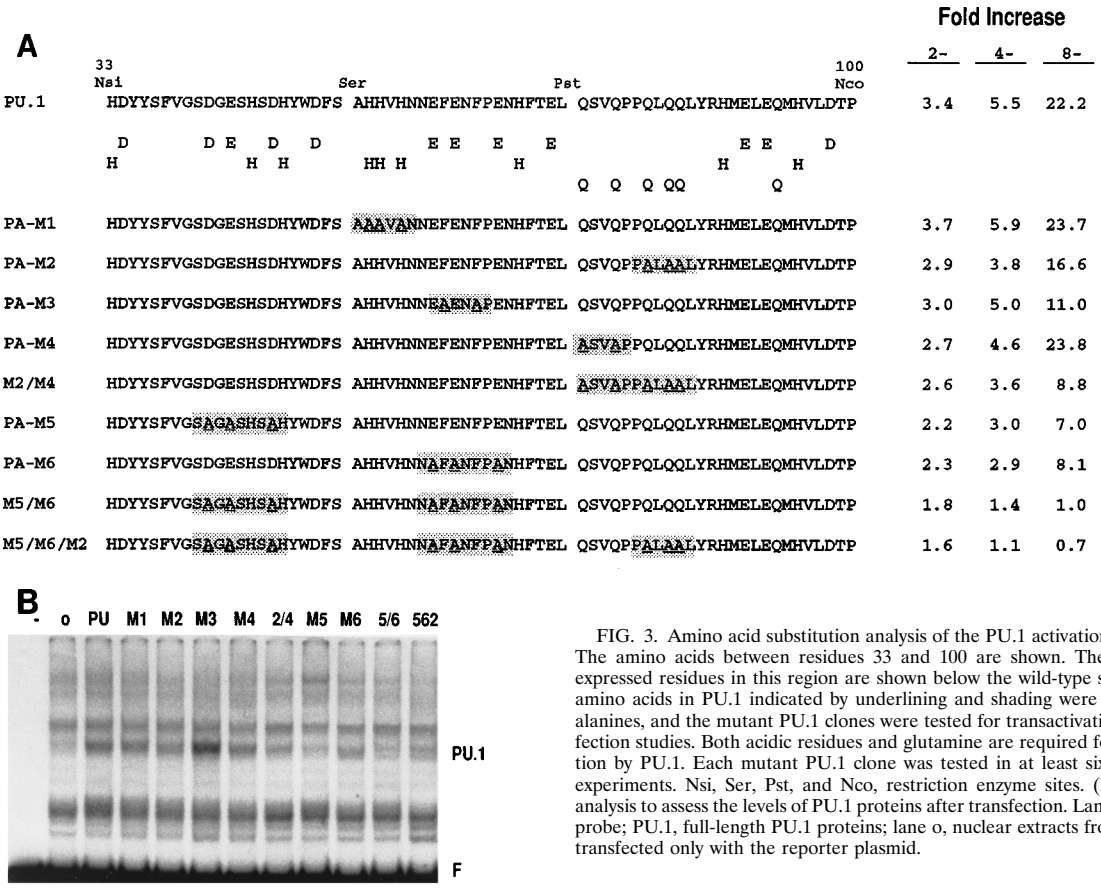

FIG. 3. Amino acid substitution analysis of the PU.1 activation domain. (A) The amino acids between residues 33 and 100 are shown. The three highly expressed residues in this region are shown below the wild-type sequence. The amino acids in PU.1 indicated by underlining and shading were replaced with alanines, and the mutant PU.1 clones were tested for transactivation in cotransfection studies. Both acidic residues and glutamine are required for transactivation by PU.1. Each mutant PU.1 clone was tested in at least six independent experiments. Nsi, Ser, Pst, and Nco, restriction enzyme sites. (B) Band shift analysis to assess the levels of PU.1 proteins after transfection. Lane - and F, free probe; PU.1, full-length PU.1 proteins; lane o, nuclear extracts from HeLa cells transfected only with the reporter plasmid.

Acidic and glutamine residues are required for transactivation. Between residues 33 and 100 of PU.1, four amino acids are present at higher-than-normal frequencies. This region contains 12 aspartic acid and glutamic acid residues (18%), 9 histidines (13%), and 6 glutamines (9%). All six glutamines are located in the third subdomain, between amino acids 74 and 100 (23%). The acidic residues are arranged as the repeat D/E-X-E-X-X-X-D/E in each of the first two subdomains. The histidines are located randomly over the entire region. To determine if any of these residues are required for transactivation, a series of amino acid substitutions in PU.1 were generated and the mutant clones were tested by cotransfection. The replacement of three histidine residues (PA-M1) within the second subdomain had no effect on transactivation by PU.1 (Fig. 3A). The replacement of five glutamine residues in the third subdomain (M2/M4) resulted in a decrease in transactivation by PU.1. The level of activity was reduced from a 3.4- to 2.6-fold increase when two DNA binding sites were present, from a 5.5- to 3.6-fold increase when four DNA binding sites were present, and a 22.2- to 8.8-fold increase when eight DNA binding sites were present in the CAT reporter plasmid. Substitutions for three acidic amino acids in either of the first two subdomains resulted in a similar decrease in activity. Substitutions for these six acidic residues reduced the levels of activity to 1.8-, 1.4-, and 1.0-fold increases when two, four, and eight DNA binding sites were present, respectively. The combination of acidic and glutamine substitutions (M5/M6/M2) was the least active of all PU.1 constructions. Interestingly, the amino acid substitutions had the greatest effect on transcriptional activation by PU.1 as the number of binding sites increased from two to eight. As shown, the combination of acidic and glutamine substitutions in mutant M5/M6/M2 showed some activity when two binding sites were next to the CAT reporter plasmid, no activity when four sites were present, and below-baseline activity when eight sites were present. Aromatic and hydrophobic residues have been shown to be important for transactivation by the prototype acidic activation domain of VP16 (4, 33). Substitutions for two phenylalanine residues (PA-M3), however, only partially reduced the ability of PU.1 to transactivate when eight DNA binding sites were present in the reporter plasmid. The results from these experiments show that three acidic residues in each of the first two subdomains and five glutamine residues in the third subdomain are required for maximal activation of transcription by PU.1.

Band shift analysis showed nearly equivalent levels of all mutant proteins after transfection, compared with that of wild-type PU.1 (Fig. 3B). Thus, the loss of activity is due to the specific amino acid substitutions and not to the amount of protein produced.

Acidic residues in the 5′ end of PU.1 are required for transactivation. The internal deletion of amino acids 33 to 100, as well as substitutions for several acidic residues and glutamines in this region, resulted in PU.1 proteins that could not transactivate in our cotransfection system. Our data, however, also showed that the deletion of more than 20 amino acids from the amino terminus resulted in PU.1 proteins that lost the ability to activate transcription. Therefore, we generated a series of mutant proteins to study the importance of amino acids 1 to 32 in the activation of transcription by PU.1.

PU.1 contains a methionine at position 7, and the nucleotide

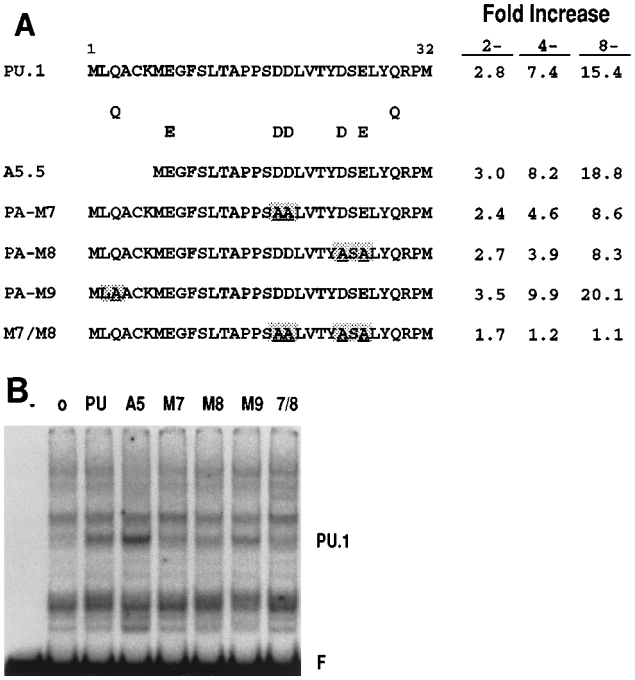

FIG. 4. Analysis of the 5′ end of the PU.1 activation domain. Deletion and amino acid substitution analyses were used to determine the importance of residues in the 5′ end (residues 1 to 32) of the PU.1 activation domain. (A) A deletion of the first six amino acids has no effect on transactivation by PU.1 (A5.5). The acidic residues in this region of PU.1 indicated by underlining and shading were necessary for transactivation by PU.1 in cotransfection studies. Each mutant PU.1 clone was tested in at least six independent experiments. (B) Band shift analysis to assess the levels of PU.1 proteins after transfection. Lane - and F, free probe; PU.1, full-length PU.1 proteins; lane o, nuclear extracts from HeLa cells transfected only with the reporter plasmid.

sequence more closely resembles a consensus Kozak sequence than at position 1 (23). We generated a PU.1 deletion mutant (A5.5) with amino acids 1 to 6 missing to test whether PU.1 protein could be produced from the second methionine and still transactivate transcription. As shown, this deletion mutant functioned as well as the wild-type PU.1 protein did whether 2, 4, or 8 DNA binding sites were present in the reporter plasmid (Fig. 4A). This places the PU.1 activation domain between amino acids 7 and 118.

We next focused on whether acidic residues and glutamines were important in the region between amino acids 1 and 32. PU.1 contains five acidic residues and two glutamines in this region. A substitution for a glutamine at position 2 (PA-M9) had no effect on transactivation by PU.1 (Fig. 4A). Substitutions for two different pairs of acidic residues (PA-M7 and -M8) resulted in a decrease in transactivation by PU.1. The drop in activity was most dramatic when the number of PU.1 DNA binding sites increased from 2 to 8. Substitutions for all four acidic residues resulted in a mutant PU.1 protein that had minimal activity in our cotransfection system. These results show that acidic residues located between amino acids 7 and 32 are required for maximal transactivation by PU.1. In addition, our results show that the PU.1 activation domain can be divided into an acidic domain between amino acids 7 and 74 and a glutamine domain between amino acids 74 and 84. Residues located in both domains are necessary for maximal transactivation by PU.1. Band shift analysis showed similar levels of these mutant proteins, compared with that of wild-type PU.1; this supports the conclusion that the loss in activity is due to the specific amino acid substitutions (Fig. 4B).

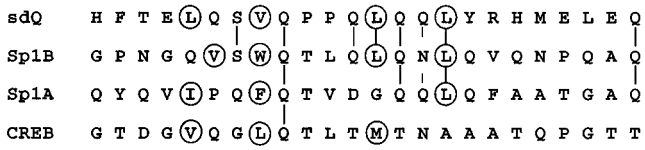

FIG. 5. Comparison of the glutamine subdomain of PU.1 (sdQ) with similar domains from Sp1 and CREB. Alignment of the amino acids in sdQ with those of the transcription activation domains of Sp1 and CREB. Sp1A and Sp1B, the two activation domains of Sp1. Conserved hydrophobic residues are circled. Identical amino acids are connected by vertical lines.

Hydrophobic residues in the glutamine domain are not required for transactivation. The glutamine group in the PU.1 activation domain is much smaller than the prototype glutamine-rich activation domain of Sp1 (3). Therefore, it is not surprising that substitutions for these five glutamines had only a partial effect on the ability of PU.1 to activate transcription. Interestingly, the glutamine group in PU.1 shows similarities to the glutamine subdomains in Sp1 that interact with $TAF_{II}110$ (10, 17). Transactivation by Sp1 and some other transcription factors require coactivators to communicate with the basal transcription machinery (10). These adapters, which are part of the TFIID multiprotein complex, create a protein-protein bridge between the transactivator and the TATA-binding protein (TBP). Recently, *Drosophila* $TAF_{II}110$ was cloned and shown to interact with Sp1 (10, 17). An analysis of Sp1 activation domain B showed that it contains a region of alternating glutamine and hydrophobic residues that interacts with $TAF_{II}110$. A mutation of the hydrophobic residues in Sp1 activation domain B abolished both interaction with $TAF_{II}110$

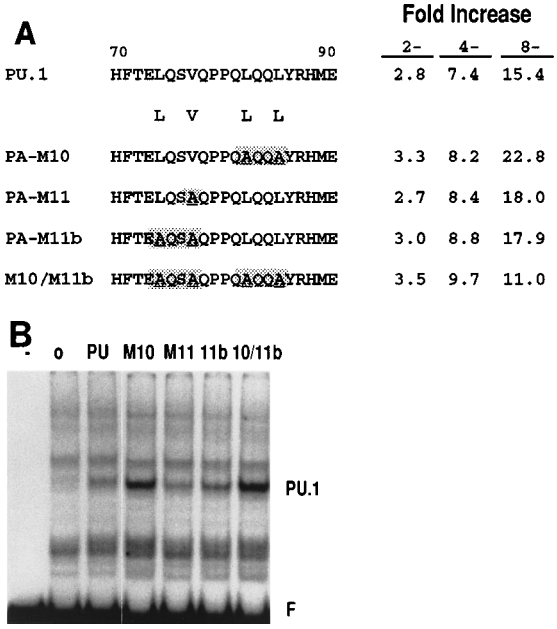

FIG. 6. Substitutions for hydrophobic residues in the PU.1 activation domain. Substitution analysis was used to determine the importance of hydrophobic residues surrounding the glutamine group in the PU.1 activation domain (residues 70 to 90). (A) Individual substitution mutants with two, four, or eight DNA binding sites were tested in cotransfection experiments. The hydrophobic residues indicated by underlining and shading were replaced with alanines. Each mutant PU.1 clone was tested in at least six independent experiments. (B) Band shift analysis to assess the levels of PU.1 proteins after transfection. Lane - and F, free probe; PU.1, full-length PU.1 proteins; lane o, nuclear extracts from HeLa cells transfected only with the reporter plasmid.

and the ability of this domain to transactivate (10). A comparison of the PU.1 glutamine group and the glutamine-rich hydrophobic patches of Sp1B, Sp1A, and CREB showed that the glutamine and hydrophobic residues in PU.1 could be aligned with their counterparts in these other domains (Fig. 5).

On the basis of the similarities between these domains in PU.1 and Sp1, it is possible that PU.1 interacts with the basal transcription machinery through $TAF_{II}110$. Since substitutions for hydrophobic residues in Sp1 subdomain B abolished both interaction with $TAF_{II}110$ and transactivation, we tested whether substitutions for the hydrophobic residues in the glutamine domain of PU.1 had an effect on its ability to transactivate in our cotransfection system. The results showed that substitutions for either pair of hydrophobic residues (PA-M10 and -M11b) had no effect on transactivation by PU.1 (Fig. 6A). Substitutions for these four hydrophobic residues had an effect only when eight DNA binding sites were present in the reporter plasmid. These data show that these hydrophobic residues are not critical for transactivation by PU.1. Band shift analysis showed similar levels of these mutant proteins, compared with that of wild-type PU.1 (Fig. 6B).

DISCUSSION

In this report, we have used deletion and amino acid substitution analyses to map the activation domain of the ETS domain transcription factor PU.1. These studies have shown that the activation domain of PU.1 is located between amino acids 7 and 118. The deletion of the PEST domain between amino acids 118 and 160, as well as the deletion of six amino acids from the amino-terminal end, had no effect on the ability of PU.1 to activate transcription. PU.1 contains a methionine at position 7, and the nucleotides more closely match a consensus Kozak sequence (23). This suggests that the translation of PU.1 may begin at either position 1 or 7 without any effect on the ability of PU.1 to function as a transcription factor. Further deletion of the amino terminus to amino acid position 10 or 20 resulted in a continuing decrease in transactivation by PU.1. The removal of 32 amino acids resulted in a PU.1 protein with minimal activity (data not shown). Internal deletions showed that the removal of amino acids 33 to 100 resulted in an inactive transcription factor. This 68-amino-acid region could be subdivided into two domains that had acidic residues and one domain that contained a cluster of glutamines. The deletion of any of these subdomains resulted in a decrease in the ability of PU.1 to activate transcription in our cotransfection system.

Analysis of the PU.1 activation domain showed that four amino acids were present at higher-than-normal frequencies; these included both acidic residues (aspartic acid and glutamic acid), glutamines, and histidines. Amino acid substitution analysis showed that 10 acidic residues between amino acids 7 and 74 are necessary for transactivation by PU.1. In addition, substitutions for five glutamines between amino acids 75 and 84 reduced the ability of PU.1 to transactivate by approximately 50%. Substitutions for hydrophobic residues throughout the activation domain had minimal effects on the function of PU.1, except when eight DNA binding sites were present in the reporter plasmid. These data suggest that PU.1 contains a single complex activation domain that requires both acidic residues and glutamines for maximal activation of transcription. Thus, the activation domain of PU.1 combines two previously described activation motifs.

Our approach to mapping the activation domain of PU.1 was twofold. The first consideration was to make all deletions and amino acid substitutions in the context of the overall structure of wild-type PU.1. The DNA binding domain of PU.1 is located in the carboxyl-terminal half of the protein, and we predicted that the activation domain would be located in the amino-terminal half of the protein. The first advantage to this approach was that the mapping of the activation domain was dependent on PU.1 binding to DNA with its own DNA binding domain. The second advantage was that the activation domain was kept on the 5′ side of the DNA binding domain, which is the structure of the wild-type PU.1 protein. Our mutant PU.1 proteins lost the ability to transactivate only when the deletions and amino acid substitutions removed critical residues or altered the structure of the activation domain. A second consideration was to test the deletion and amino acid substitution mutant clones of PU.1 with different numbers of DNA binding sites. As we increased the number of binding sites, the fold increase in activation was amplified. This provided a way to clearly see the effects of deletions or amino acid substitutions on the activation of transcription by PU.1. Regardless of whether two, four, or eight PU.1 DNA binding sites were present in the CAT reporter plasmid, the deletion and substitution mutations had similar effects on transactivation by PU.1. Therefore, our results support the idea that the residues we mapped as being critical for transactivation by PU.1 are important for PU.1 binding to a single site in a complex promoter or enhancer.

Our deletion results are consistent with those of three recent reports that suggested the activation domain of PU.1 was located within the N-terminal half of the protein (12, 22, 36). The first study (12) used a series of 3′ deletions of PU.1 fused to the DNA binding domain of the yeast protein GAL4. As this study showed, GAL4 fusion proteins containing either PU.1 amino acids 1 to 134 or amino acids 1 to 75 were able to transactivate from a reporter plasmid containing five GAL4 binding sites. Thus, both amino acids 1 to 75, which contain the acidic region, and amino acids 1 to 134 of PU.1 can function as independent activation domains when fused to a heterologous DNA binding domain. This study also showed that amino acids 1 to 75 of PU.1 can interact with TBP by using in vitro binding assays.

In contrast, a second study (36) used 5′ deletion analysis to map the PU.1 activation domain to amino acids 70 to 100. In this study, the deletion of the first 70 amino acids of PU.1 had no effect on transactivation from a reporter plasmid containing four PU.1 binding sites from the J chain promoter upstream of a minimal γ-fibrinogen promoter. Further deletion of amino acids 70 to 100 completely inactivated PU.1. This result suggested that the activation domain of PU.1 is located after the first 70 amino acids. A third study (22) recently used deletion analysis to show that the regulation of interleukin 1β gene expression by PU.1 requires the amino-terminal half of the protein, suggesting that both the acidic and glutamine domains are required for transactivation.

Our detailed analysis of the PU.1 activation domain, using both deletion and amino acid substitution studies, has shown that the PU.1 activation domain extends from amino acids 7 to 118. Further, we have shown that 10 acidic residues between amino acids 7 and 74 and a cluster of five glutamines between amino acids 75 and 84 are required for maximal transactivation by PU.1. The substitution of alanines for the glutamine cluster reduces but does not eliminate transactivation by PU.1, suggesting that the acidic domain is dominant. One explanation for the differences between our results and those of the other three reports is that different portions of the PU.1 activation domain may function in specific circumstances. For instance, the glutamine domain may function in a B-cell- or J-chain-specific manner. This would open up several intriguing possibilities of mechanisms for the regulation of gene expression by

PU.1. Likewise, the acidic domains of PU.1 might function only in macrophages or when PU.1 is part of a transcription factor complex. The glutamine domain might function only in B cells or from sites located near TATA boxes. The PU.1 binding sites that are important for J chain and CD11b expression are located in the promoter near the TATA box (29, 36), whereas the site important for kappa light-chain expression is located in a distant 3′ enhancer (30). Thus, the acidic or glutamine domains in PU.1 may individually control the expression of each of these genes. Testing amino acid substitution clones of PU.1 in both B cells and macrophages and from several different promoters and enhancers will distinguish between these possibilities. Reports from two groups support the idea that different activation domains function either next to a TATA box or from a distant DNA binding site (5, 35). One of these studies showed that glutamine activation domains fused to the heterologous DNA binding of GAL4 were able to activate transcription only from sites next to a TATA box, a so-called proximal position (35). Acidic activation domains, however, were able to function equally well from both proximal and distal locations on the reporter plasmid. Distal sites are thought to mimic the properties of enhancers. Since PU.1 uses both acidic residues and glutamine, it may use different regions to activate transcription from proximal and distal sites.

As discussed above, the glutamine group in the third subdomain of PU.1 is much smaller than the glutamine-rich Sp1 activation domain. Since PU.1 also contains a region of acidic residues necessary for transactivation, it is not surprising that substitutions for these five glutamines had only a partial effect on the ability of PU.1 to activate transcription. Whether it actually interacts with an adapter protein such as $TAF_{II}110$ awaits additional experimentation. It will be interesting to determine if the glutamine group is always the most critical in B cells or from a subset of promoters that use PU.1 for expression.

The acidic region of the PU.1 activation domain shows both similarities and differences with the acidic activation domain of VP16. The net negative charge in the PU.1 activation domain appears to be only partially important for transactivation. The removal of six acidic residues between amino acids 33 and 74, or six negative charges, was more effective at decreasing the activation potential of PU.1 than was the removal of only three acidic residues in this region. The positions of these acidic residues, however, must also be important since the removal of four acidic residues between amino acids 7 and 32 inactivated PU.1 as well as the removal of the previous six acidic residues did. One testable hypothesis for our results is that one of these two acidic groups is critical for protein folding while the other interacts with TBP and activates transcription. A detailed analysis of VP16 has shown that net negative charge is important but insufficient to explain how it functions as an activation domain (4, 33). An aromatic phenylalanine residue and several hydrophobic residues are critical for transactivation by VP16 (33). The Epstein-Barr virus R protein appears to require a similar arrangement of hydrophobic residues to function as a transactivating factor (14). PU.1 contains two phenylalanine residues within the acidic repeat of the second subdomain, but substitutions of alanines for both of these had an effect only on the ability of PU.1 to activate transcription from a reporter plasmid containing eight DNA binding sites (Fig. 3A [mutant PA-M3]). This shows that the acidic region of PU.1 and the acidic activation domain of VP16 function in different ways to activate transcription. This may be due to the fact that PU.1 contains the glutamine group in the third subdomain. A previous study has shown that amino acids 1 to 75 in PU.1 can interact with TBP (12). A possibility is that one set of acidic residues, either the four between amino acids 7 and 32 or the six between amino acids 33 and 74, are necessary for interaction with TBP. The other set may then be used for actual recruitment of additional basal factors required for transcription initiation.

This report shows that the activation domain of PU.1 is located in the amino-terminal half of the protein. This region contains acidic and glutamine-rich domains, both of which are required for transactivation by PU.1. Amino acid substitution analyses showed that 10 acidic residues and 5 glutamines are important for the activation of transcription. It will be interesting to determine the role of these amino acids in the ability of PU.1 to regulate gene expression in B cells and macrophages.

ACKNOWLEDGMENTS

We thank Abigail Farr for critically reading the manuscript.

This work was partially supported by a pilot grant from the Indiana University Cancer Center (M.J.K.) and by the Project Development Program, Research and Sponsored Programs, Indiana University at Indianapolis (M.J.K.).

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