

## Multiple Functional Domains of AML1: PU.1 and C/EBP $\alpha$ Synergize with Different Regions of AML1

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**Control elements of many genes are regulated by multiple activators working in concert to confer the maximal level of expression, but the mechanism of such synergy is not completely understood. The promoter of the human macrophage colony-stimulating factor (M-CSF) receptor presents an excellent model with which we can study synergistic, tissue-specific activation for two reasons. First, myeloid-specific expression of the M-CSF receptor is regulated transcriptionally by three factors which are crucial for normal hematopoiesis: PU.1, AML1, and C/EBP $\alpha$ . Second, these proteins interact in such a way as to demonstrate at least two examples of synergistic activation. We have shown that AML1 and C/EBP $\alpha$  activate the M-CSF receptor promoter in a synergistic manner. As we report here, AML1 also synergizes, and interacts physically, with PU.1. Detailed analysis of the physical and functional interaction of AML1 with PU.1 and C/EBP $\alpha$  has revealed that the proteins contact one another through their DNA-binding domains and that AML1 exhibits cooperative DNA binding with C/EBP $\alpha$  but not with PU.1. This difference in DNA-binding abilities may explain, in part, the differences observed in synergistic activation. Furthermore, the activation domains of all three factors are required for synergistic activation, and the region of AML1 required for synergy with PU.1 is distinct from that required for synergy with C/EBP $\alpha$ . These observations present the possibility that synergistic activation is mediated by secondary proteins contacted through the activation domains of AML1, C/EBP $\alpha$ , and PU.1.**

In order to understand the mechanisms that control monocytic commitment and differentiation, we have investigated the tissue-specific regulation of the human macrophage colony-stimulating factor (M-CSF) receptor. We have previously identified three factors required for M-CSF receptor transcription in monocytic cell lines, PU.1, C/EBP $\alpha$  (CCAAT/enhancer-binding protein alpha), and AML1, and demonstrated that mutations in any of the three DNA-binding sites decreases promoter activity significantly in transient transfection studies (77–79). In addition, PU.1 transactivates the M-CSF receptor promoter, and although C/EBP $\alpha$  has little transactivation potential alone, it synergizes with AML1B to increase the activity of the promoter an average of 90-fold (78, 79). All three of these transcription factors play important roles in hematopoiesis.

AML1 (also known as CBF $\alpha$ 2 and PEBP2 $\alpha$ B) contains a domain that is highly similar to the DNA-binding domain of the *Drosophila runt* transcription factor, which mediates both DNA-binding and heterodimerization abilities (25). The heterodimerization partner of AML1, CBF $\beta$ , does not bind DNA directly but increases the affinity of AML1 for DNA (37, 51, 75). In addition to the M-CSF receptor, the target genes of the AML1-CBF $\beta$  heterodimer include granulocyte-macrophage colony-stimulating factor (GM-CSF), T-cell receptor (TCR) subunits, interleukin-3, osteocalcin, neutrophil elastase, and myeloperoxidase (2, 3, 6, 15, 18, 21, 50, 67, 70). In several cases AML1 functions in concert with neighboring factors. For ex-

ample, AML1 binds cooperatively with another member of the *ets* family, Ets-1, to the TCR $\alpha$ , TCR $\beta$ , and Moloney murine leukemia virus enhancers (18, 67). AML1 exhibits functional synergy with c-Myb in the absence of cooperative binding in the context of the TCR $\delta$  and myeloperoxidase enhancers (6, 21). Both AML1 and CBF $\beta$  are frequently involved in genetic rearrangements identified in human leukemias (12, 19, 35, 40, 47–49). Furthermore, mice which have homozygous disruptions of either gene, or are heterozygotes containing either the AML/ETO or CBF $\beta$ /MYH11 fusion genes, have strikingly similar phenotypes. All die in midgestation, exhibit multiple hemorrhages in the central nervous system, and have severely impaired hematopoiesis (7, 53, 61, 73, 74, 76). These data support the theory that AML1 function is critical for normal hematopoietic development.

C/EBP $\alpha$ , a basic region leucine zipper (bZip) transcription factor (31, 32), regulates not only a variety of hepatocyte and adipocyte genes which are important for energy homeostasis but several myeloid-specific genes as well (9, 10, 16, 17, 22, 24, 50). For example, in addition to the M-CSF receptor promoter, C/EBP $\alpha$  has also been shown to regulate the G-CSF receptor and GM-CSF receptor  $\alpha$  promoters (23, 66). Mice with a homozygous disruption of the C/EBP $\alpha$  gene die at birth from hypoglycemia (14, 72) and exhibit hematopoietic defects as well. Analysis of the fetal and newborn hematopoietic tissues revealed a profound absence of mature neutrophils. In addition, there were no neutrophils observed after transplantation of the fetal liver into an irradiated recipient, implying that the block in neutrophil development was intrinsic to the cell and not a defect in the environment (80). Therefore, it is clear that C/EBP $\alpha$  plays a critical role in normal granulocyte development.

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PU.1, the product of the *spi-1* oncogene and a member of the *ets* family, is upregulated during hematopoietic development and is specifically expressed in myeloid and B cells (8, 29, 55, 59, 71). The pivotal role that PU.1 plays in hematopoietic differentiation is established by the following observations. There are a number of genes that are regulated by PU.1 in both myeloid and B-cell lineages, including those encoding CSF receptors and immunoglobulin subunits (45, 57, 64, 69). Overexpression of PU.1 early in erythroid development blocks erythroblast differentiation (62), and addition of PU.1-binding oligonucleotides to human CD34<sup>+</sup> bone marrow cells decreases in vitro colony formation (71). In addition, mice with a disruption in both alleles of the PU.1 locus die in utero (63) or shortly after birth (36) and exhibit major defects in hematopoiesis, including a block in myeloid development. The DNA-binding *ets* domain shows sequence similarity with other members of the *ets* family and is contained within amino acids 171 to 267 of the C terminus (29). The activation domain of PU.1 is located within the N terminus and consists of several regions rich in either acidic amino acids or glutamines and a region from amino acids 118 to 160 which has a high number of prolines, glutamic acids, serines, and threonines (PEST domain) (28). PU.1 has been shown to interact with TATA-binding protein (TBP) and the retinoblastoma protein in vitro, requiring amino acids 1 to 75 (20). There are multiple examples where PU.1 functions in concert with other transcription factors, including NF-IL6 $\beta$  (C/EBP $\delta$ ) (44) and NF-EM5/PIP (11, 56, 58), c-Myb and C/EBP $\alpha$  (50), c-Fos and c-Jun (5, 56), and Ets-1 (13).

We are interested in determining the events that control myeloid differentiation so that we can better understand the aberrant differentiation that is exhibited in the leukemic state. For example, it is not clear how the fusion gene, AML/ETO, and other genomic abnormalities associated with myeloid leukemia contribute to the changes in differentiation and proliferation of the myeloid lineage. Alternative theories include inhibition of normal AML1 function (15, 27, 38) or increased activation by AML1 (60), or even direct activation by AML/ETO itself (39), resulting in the dysregulation of genes such as those encoding GM-CSF, the M-CSF receptor, or Bcl-2. Therefore, we have investigated the mechanism by which the transcription factors regulating the M-CSF receptor promoter interact in an effort to reveal the next layer of complexity in myeloid-specific transcriptional activation. Here we show that, in addition to C/EBP $\alpha$ , AML1B interacts with PU.1 to synergistically activate the M-CSF receptor promoter but requires different regions contained within the C terminus for each function.

#### MATERIALS AND METHODS

**Cell culture conditions and transfection.** HeLa cells (ATCC CCL 2; American Type Culture Collection), CV-1 cells (ATCC CCL 70; American Type Culture Collection), and COS-7 cells (ATCC CRL 165; American Type Culture Collection) were maintained in Dulbecco modified Eagle medium with 10% calf serum and 2 mM L-glutamine (GIBCO), and  $3 \times 10^5$  to  $5 \times 10^5$  cells were plated in 100-mm-diameter tissue culture plates 24 h before transfection. Except for immunoprecipitation experiments, transfections were performed by the calcium phosphate method with 5  $\mu$ g of the reporter construct and 1  $\mu$ g of each expression construct or empty vector, with the total amount of DNA brought to 20  $\mu$ g with sheared salmon sperm DNA. The medium was changed 14 h after transfection, and luciferase assays were performed as described previously (54) 24 to 36 h later. Luciferase values were normalized for transfection efficiency by cotransfecting a plasmid expressing the human growth hormone gene driven by the Rous sarcoma virus promoter and assaying the supernatant of the cultures with the human growth hormone radioimmunoassay from Nichols Institute Diagnostics (San Juan Capistrano, Calif.) according to the manufacturer's instructions.

**Immunoprecipitation.** COS-7 cells in 60-mm-diameter plates were transfected with Lipofectamine Plus (GIBCO) according to the manufacturer's recommen-

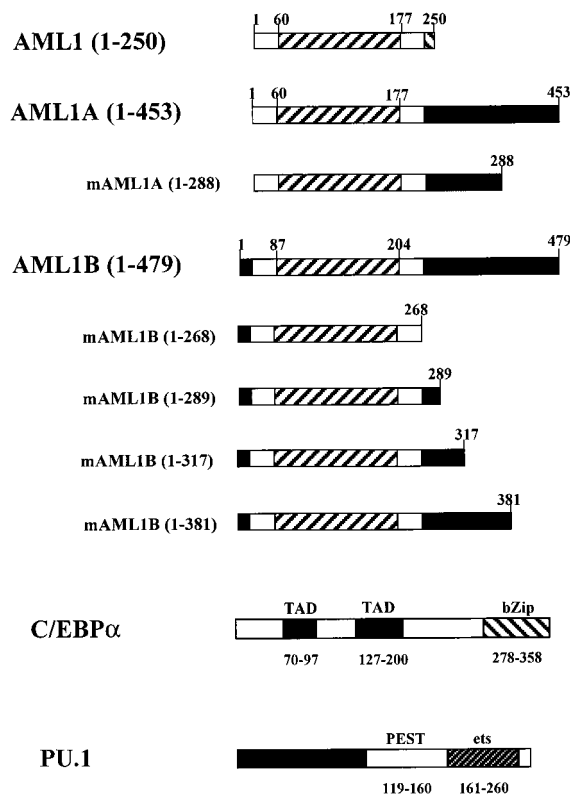


FIG. 1. General structures of three alternatively spliced forms of AML1 and mutations of AML1, PU.1, and C/EBP $\alpha$ . The diagram shows the three forms of AML1 and the mutations of AML1A and AML1B, which were used to investigate the physical and functional interaction with PU.1 and C/EBP $\alpha$ . AML1 (250 amino acids), AML1A (453 amino acids), and AML1B (479 amino acids) (38) correspond to AML1a, AML1b, and AML1c as published by Miyoshi et al. (41). AML1 contains 250 amino acids and is identical to AML1B from amino acid 5 to 242 (32 to 268 of AML1B). The  *runt*  domain is indicated between amino acids 60 and 177 of AML1 and AML1A and amino acids 87 and 204 of AML1B. AML1A has 453 amino acids and contains the N terminus of AML1 and the C terminus of AML1B. mAML1A(1-288) is terminated at amino acid 288 of AML1A. Mutants of AML1B include C-terminal truncations at amino acids 268, 289, 317, and 381 [mAML1B(1-268) and mAML1B(1-289), -(1-317), and -(1-381), respectively]. The bZip region of C/EBP $\alpha$ , which is responsible for both the DNA-binding and dimerization properties, is located in the C terminus, while the transactivation domains (TAD) are between amino acids 70 and 97 and amino acids 127 and 200 (17, 31, 32, 46). The activation domain of PU.1 resides within the N terminus, while the DNA-binding *ets* domain is at the C terminus (28, 29).

dations with 2  $\mu$ g of AML1 expression plasmid. After 20 h, the cells were incubated for 1 h in Dulbecco modified Eagle medium without methionine or cysteine–10% fetal bovine serum and then labeled in the same medium with the addition of 100  $\mu$ Ci of Express (NEN) per ml for 3 h. The cells were washed three times with cold phosphate-buffered saline, scraped from the plates, and lysed in radioimmunoprecipitation assay buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 50 mM Tris [pH 7.5]). The lysate was precleared by incubation with 5  $\mu$ l of normal rabbit serum for 1 h on ice, followed by 30 min with Sepharose-linked protein A (Pharmacia). The supernatant was then incubated with 1  $\mu$ l of antiserum specific for the N terminus of AML1 and Sepharose-linked protein A for 16 h at 4°C, with rocking. The immunocomplexes were separated on an SDS–10% acrylamide gel.

**Plasmids.** The M-CSF receptor promoter constructs in pXP2 were described previously: the wild-type promoter from bp –416 to +71, pM-CSF-R-luc, and pM-CSF-R(mPU.1)-luc [referred to as pM-CSF-R(m40)-luc] (78), pM-CSF-R(mAML1)-luc [referred to as pM-CSF-R(MB)-luc], and pM-CSF-R(DD)-luc, with a deletion of bp –86 to –37 (79). The expression constructs for murine PU.1 and mutants of PU.1 were gifts from M. J. Klemsz and R. A. Maki (29, 58). C/EBP $\alpha$  expression constructs in the vector pMSV have been analyzed and described in detail elsewhere (17). AML1B and CBF $\beta$  were described previously (38). pCMV5-AML1 (Fig. 1) was constructed by subcloning the 576-bp *ApaI*-*BamHI* fragment of the AML1 cDNA in pBluescript-KS (42) into pCMV5-AML1/ETO (38) digested with the same restriction enzymes, which replaced the





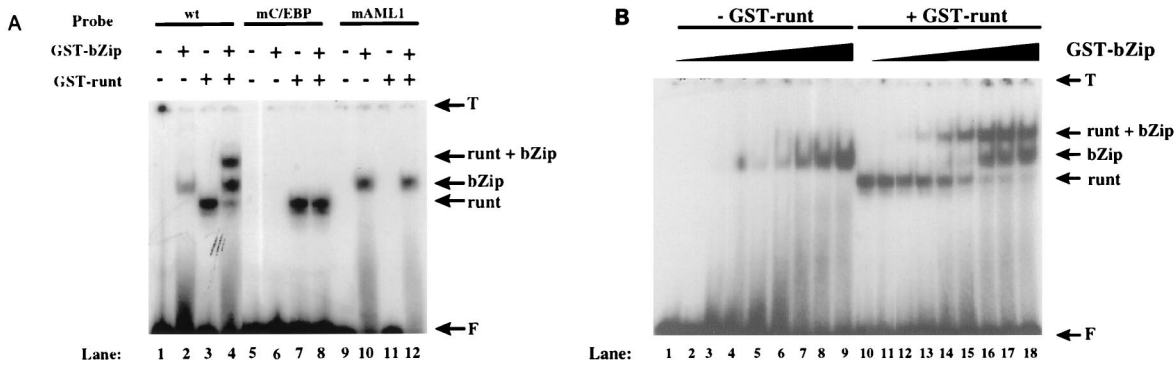


FIG. 3. AML1 forms a ternary complex on DNA with C/EBP $\alpha$  and exhibits cooperative DNA binding. (A) A radiolabeled oligonucleotide containing sites for AML1 and C/EBP (bp -88 to -59) (wt) or a probe mutated in the C/EBP binding site (mC/EBP) (lanes 5 to 8) or AML1 binding site (mAML1) (lanes 9 to 12) was incubated in the absence of protein (lanes 1, 5, and 9) or in the presence of 500 ng of GST-bZip (lanes 2, 6, and 10), 500 ng of GST-runt (lanes 3, 7, and 11), or both (lanes 4, 8, and 12). The migration of the protein-DNA complexes is indicated on the right, as are the top of the gel (T) and the free probe (F). (B) The -88 to -59 probe used for panel A was incubated with increasing concentrations of GST-bZip, ranging from 6 to 1,710 nM, in the absence (lanes 1 to 9) and in the presence (lanes 10 to 18) of a saturating amount of GST-runt. The migration of the protein-DNA complexes is indicated on the right, as are the top of the gel (T) and the free probe (F).

rabbit serum (lanes 10 and 9, respectively). These results show that AML1 and PU.1 can form a ternary complex with DNA. The incomplete self competition was observed when the high protein concentrations necessary to generate the higher-order complex were used. When a radiolabeled oligonucleotide containing only a PU.1 site was used in a similar gel shift experiment, the ternary complex was not detectable (lanes 11 to 14). This result indicates that both DNA-binding sites are required for the formation of the ternary complex. To investigate whether there is cooperation in the formation of the ternary complex, titration experiments were performed as shown in Fig. 2B. We detect more DNA associated with PU.1 (lanes 1 to 9) than with the higher-order complex (lanes 10 to 18) formed in the presence of the AML1 runt domain. When the concentration of GST-runt was titrated in the absence and presence of GST-PU.1, we observed the same result (data not shown). Based on these experiments, the amount of probe shifted by the higher-order complex is less than that shifted by either GST-runt (data not shown) or GST-PU.1, indicating that AML1 and PU.1 do not bind cooperatively to DNA.

We then analyzed the formation of ternary complexes with DNA by the runt domain of AML1 and the bZip DNA-binding domain of C/EBP $\alpha$ . When a radiolabeled oligonucleotide containing the M-CSF receptor binding sites for AML1 and C/EBP $\alpha$  (bp -88 to -59) was used in a gel shift experiment (Fig. 3A, lanes 1 to 4, wt), we could detect shifted bands with both purified GST-bZip (lane 2) and GST-runt (lane 3). A ternary complex containing both proteins and the oligonucleotide was also observed (lane 4). These shifted bands could be competed with nonradiolabeled self oligonucleotide and also supershifted with specific antiserum (data not shown). However, oligonucleotide carrying mutations in either the C/EBP binding site (lanes 5 to 8) or the AML1 binding site (lanes 9 to 12) failed to form the ternary complex. This result demonstrates the requirement for both factor binding sites in the formation of the ternary complex. As with AML1 and PU.1, we performed a titration experiment to investigate differences in DNA binding between binary and ternary complexes. As shown in Fig. 3B, we detected more DNA associated with the higher-order complex formed in the presence of the AML1 runt domain (lanes 10 to 18) than with GST-bZip (lanes 1 to 9), providing evidence that AML1 and C/EBP $\alpha$  exhibit cooperative DNA binding. When titrations of GST-runt were incu-

bated in the presence and absence of GST-bZip, we observed the same result (data not shown).

In summary, these data demonstrate that AML1-C/EBP and AML1-PU.1 DNA-protein ternary complexes can be detected with purified proteins. Furthermore, the formation of the ternary complexes requires the presence of both DNA-binding sites. Finally, while the runt domain of AML1 binds cooperatively to DNA in the presence of the C/EBP $\alpha$  bZip domain, the ternary complex with PU.1 is formed in a noncooperative manner. These differences in DNA binding may affect the interaction of the transcription factors in the regulation of the M-CSF receptor promoter.

**PU.1 and AML1B interact physically in vitro via the DNA-binding domain of each protein.** AML1 and PU.1, two transcription factors important for myeloid differentiation, bind to adjacent regions on the M-CSF receptor promoter and activate it (77, 78). To understand the mechanism of their function, we analyzed whether AML1 could physically interact with PU.1 as it does with C/EBP $\alpha$  to confer the maximal level of myeloid-specific regulation. Using the GST pull-down assay, we have established that radiolabeled, in vitro-translated AML1 interacts with a GST fusion protein containing full-length PU.1. Both the 250 (Fig. 4A, lanes 1 and 5)- and 479 (lanes 2 and 8)-amino-acid forms (AML1 and AML1B [Fig. 1]) are pulled down by GST-PU.1; however, the interaction with AML1B appears stronger. Furthermore, full length in vitro-translated PU.1 binds to a GST fusion protein containing the runt domain of AML1B (Fig. 4B, lane 4). To establish the specificity of the interaction, ethidium bromide was added to the binding reaction to prevent potential nonspecific interactions mediated by contaminating DNA (30). As shown in Fig. 4B, lane 9, addition of ethidium bromide decreased but did not abolish the interaction between the runt domain of AML1 and PU.1. We also observed a weak interaction between PU.1 and a GST fusion protein containing a domain (amino acids 213 to 395) within the C terminus of AML1B (Fig. 4B, lanes 5 and 10). Although this domain is critical for synergy between AML1 and PU.1 (see below), the strength of the interaction with PU.1 is only 17% of that observed between PU.1 and the runt domain. However, this relatively weak interaction may explain the different binding abilities of AML1 and AML1B with GST-PU.1 (Fig. 4A). We have also established that the interaction between PU.1 and the runt domain of AML1 localizes to the

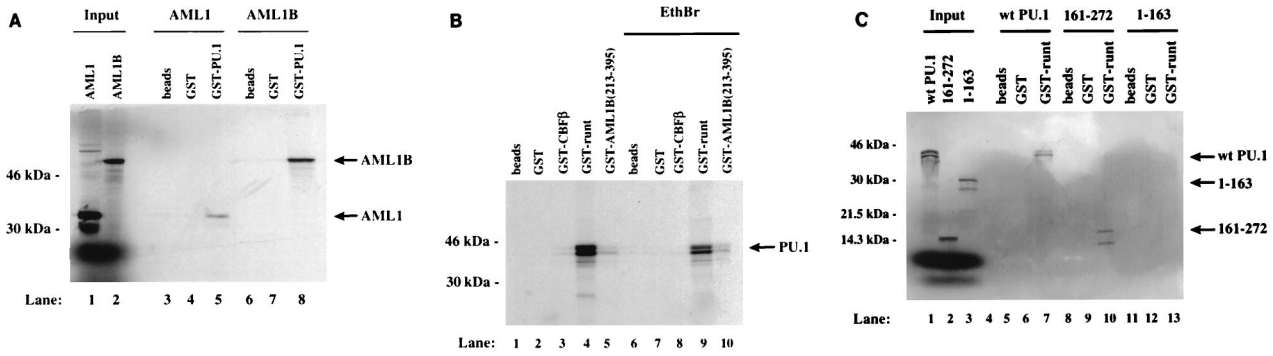


FIG. 4. The physical interaction between PU.1 and AML1 is localized to the DNA-binding domains. (A) AML1 (lanes 1 and 3 to 5) and AML1B (lanes 2 and 6 to 8) were transcribed and translated in vitro with [<sup>35</sup>S]methionine and incubated with glutathione-agarose (beads) (lanes 3 and 6), GST (lanes 4 and 7), or GST-PU.1 (lanes 5 and 8). Positions of molecular mass markers and the migration of the bound proteins are indicated to the left and right, respectively. (B) PU.1 was transcribed and translated in vitro with [<sup>35</sup>S]methionine and incubated with glutathione-agarose (beads) (lanes 1 and 6) or the following proteins immobilized on agarose: GST (lanes 2 and 7), GST-C/EBPβ (lanes 3 and 8), GST-runt (lanes 4 and 9), and GST-AML1B(213-395) (lanes 5 and 10). Ethidium bromide (EthBr; 50 μg/μl) was added to the reactions in lanes 6 to 10. Positions of molecular mass markers are shown to the left, and the migration of PU.1 is indicated to the right. (C) Full-length PU.1 (lanes 1 and 5 to 7), amino acids 161 to 272 of PU.1 containing the *ets* domain (lanes 2 and 8 to 10), and amino acids 1 to 163 of PU.1 (lanes 3 and 11 to 13) were transcribed and translated in vitro with [<sup>35</sup>S]methionine and incubated with glutathione-agarose (beads) (lanes 5, 8, and 11), immobilized GST (lanes 6, 9, and 12), or GST-runt (lanes 7, 10, and 13). Positions of the molecular mass markers, run in lane 4, are shown to the left, and the migration of the wild-type (wt) and mutant forms of PU.1 is indicated to the right.

DNA-binding *ets* domain of PU.1. In Fig. 4C, full-length PU.1 (lanes 1 and 7) and amino acids 161 to 272 containing the *ets* domain of PU.1 (lanes 2 and 10), but not the activation domain of PU.1 (amino acids 1 to 161 [lanes 3 and 13]), interact specifically with the GST fusion protein containing the AML1 runt domain. The lower band of the doublet observed in lane 9 is also present in the input lane 2 but is partially obscured by the free <sup>35</sup>S. In summary, these data demonstrate that AML1 and PU.1 interact physically and that this interaction occurs primarily through their DNA-binding domains.

**PU.1 and AML1 synergize to activate the M-CSF receptor promoter, a function which is dependent on regions within the activation domains of PU.1 and AML1B.** PU.1 and AML1 both activate the M-CSF receptor promoter (78, 79) and interact physically through their DNA-binding domains but do not bind cooperatively to DNA. We next investigated whether PU.1 and AML1 could synergize to activate the M-CSF receptor promoter. Transfections of HeLa cells, which contain no endogenous PU.1 and little detectable AML1, showed that while PU.1 activates the promoter 2-fold and AML1B 3.5-fold, together they synergize to increase the activity of the promoter 12-fold (Fig. 5A). Although the synergy between PU.1 and AML1B is weak relative to that observed with AML1B and C/EBPα, it is more than an additive effect. We calculated the fold synergy by dividing the activation of the promoter in the presence of both factors by the expected additive result. PU.1 and AML1B exhibit twofold synergy, or two times as much activation as an additive effect. However, this synergy is absent with reporter constructs bearing mutations in either the PU.1 or AML1 binding site [pM-CSF-R(mPU.1)-luc or pM-CSF-R(mAML1)-luc, respectively] or a deletion of bp -86 to -37 [pM-CSF-R(DD)-luc], containing the binding sites for C/EBP, AML1, and PU.1, indicating that DNA binding is required for both factors. Interestingly, we are unable to reproduce the synergy between AML1B and PU.1 in the CV-1 cell line, and although the synergy between AML1B and C/EBPα is observed in both HeLa and CV-1 cells, we saw no more than an additive increase in activity when PU.1 was included with AML1B and C/EBPα (data not shown).

We investigated the role of the activation domains of AML1 and PU.1 in synergistic activation by performing transient

transfections with a variety of mutants. The data presented in Fig. 5B illustrate that deletion of the PEST domain, which is important for interaction with NF-EM5 and activation of B-cell genes (58), has no effect on activation of the M-CSF receptor promoter or synergy with AML1B. However, deletion of amino acids 1 to 70 or 33 to 100 abrogates synergy, indicating that amino acids 33 to 70 of the PU.1 activation domain are important for this function. EMSA analysis of COS cell extracts transfected with the PU.1 expression constructs demonstrated that the mutated proteins were produced in comparable amounts and are capable of binding to DNA (data not shown).

Transfections with AML1B mutants truncated in the carboxy terminus indicate that this region is necessary for synergy with PU.1 (Fig. 5C). While termination of AML1B at amino acid 381 does not affect synergistic activation of the promoter, termination at amino acid 317 clearly does. Production of the AML1 proteins was demonstrated by nonquantitative immunoprecipitation from transiently transfected COS-7 cells (Fig. 5D). Furthermore, the activation of the reporter construct by the AML1B mutants indicates that they are expressed, are transported to the nucleus, and are capable of binding the promoter. For example, in HeLa cells AML1B(1-289) and -(1-317) activate the M-CSF receptor promoter 3.5- and 3.8-fold, respectively, relative to the expression vector, pCMV5, and both of these proteins are capable of synergizing with C/EBPα (Fig. 6A). These data demonstrate that AML1 synergizes with PU.1 to activate the M-CSF receptor promoter and that this activity requires DNA-binding sites for both factors and regions contained within the activation domains of AML1 and PU.1.

**Synergy between AML1B and C/EBPα requires their trans-activation domains, and the region of AML1 critical for this synergy is distinct from that important for synergy with PU.1.** We have previously shown that AML1B and C/EBPα synergize to activate the M-CSF receptor promoter, that both DNA-binding sites are required for this function, and that the factors interact in vitro via their DNA-binding domains (*runt* and bZip domains, respectively) (79). Furthermore, in this report we have demonstrated that AML1 and C/EBPα form a ternary complex with DNA and exhibit cooperative DNA binding. To

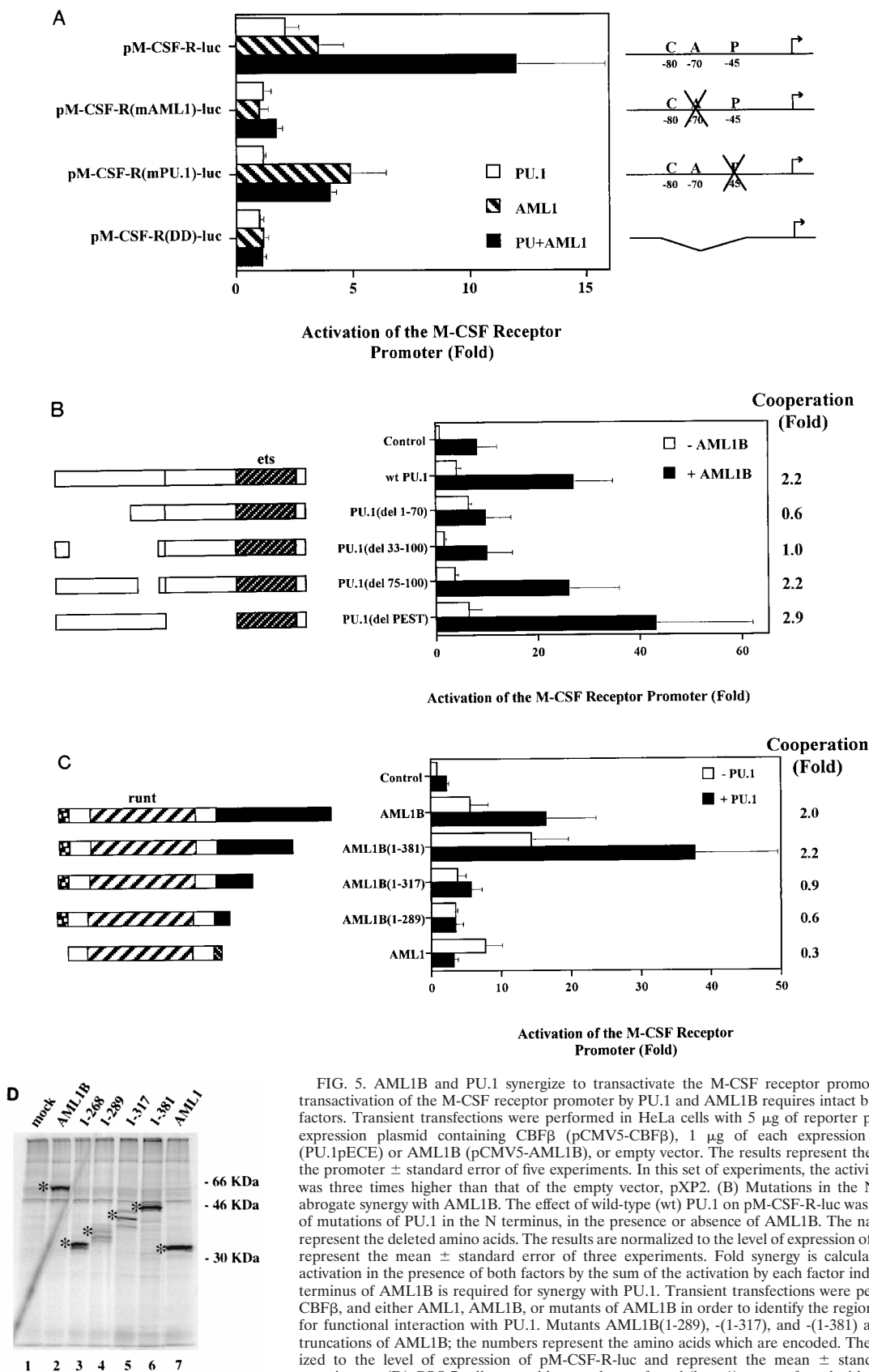


FIG. 5. AML1B and PU.1 synergize to transactivate the M-CSF receptor promoter. (A) Synergistic transactivation of the M-CSF receptor promoter by PU.1 and AML1B requires intact binding sites for both factors. Transient transfections were performed in HeLa cells with 5  $\mu$ g of reporter plasmid and 1  $\mu$ g of expression plasmid containing CBF $\beta$  (pCMV5-CBF $\beta$ ), 1  $\mu$ g of each expression plasmid for PU.1 (PU.1pECE) or AML1B (pCMV5-AML1B), or empty vector. The results represent the mean activation of the promoter  $\pm$  standard error of five experiments. In this set of experiments, the activity of pM-CSF-R-luc was three times higher than that of the empty vector, pXP2. (B) Mutations in the N terminus of PU.1 abrogate synergy with AML1B. The effect of wild-type (wt) PU.1 on pM-CSF-R-luc was compared to effects of mutations of PU.1 in the N terminus, in the presence or absence of AML1B. The names of the mutants represent the deleted amino acids. The results are normalized to the level of expression of pM-CSF-R-luc and represent the mean  $\pm$  standard error of three experiments. Fold synergy is calculated by dividing the activation in the presence of both factors by the sum of the activation by each factor individually. (C) The C terminus of AML1B is required for synergy with PU.1. Transient transfections were performed with PU.1, CBF $\beta$ , and either AML1, AML1B, or mutants of AML1B in order to identify the region of AML1 required for functional interaction with PU.1. Mutants AML1B(1-289), -(1-317), and -(1-381) are carboxy-terminal truncations of AML1B; the numbers represent the amino acids which are encoded. The results are normalized to the level of expression of pM-CSF-R-luc and represent the mean  $\pm$  standard error of three experiments. (D) COS-7 cells were either mock transfected (lane 1) or transfected with Lipofectamine Plus, 2  $\mu$ g of expression plasmid encoding AML1B (lane 2), or AML1B truncated at amino acid 268 (1-268, lane 3), 289 (1-289, lane 4), 317 (1-317, lane 5), or 381 (1-381, lane 6) or with AML1 (lane 7). After 20 h, the cells were labeled with [ $^{35}$ S]methionine and [ $^{35}$ S]cysteine and immunoprecipitated, and the AML1 proteins were resolved by SDS-PAGE. Bands corresponding to the expected molecular masses are indicated with asterisks, and the migration of the molecular mass markers is indicated to the right.

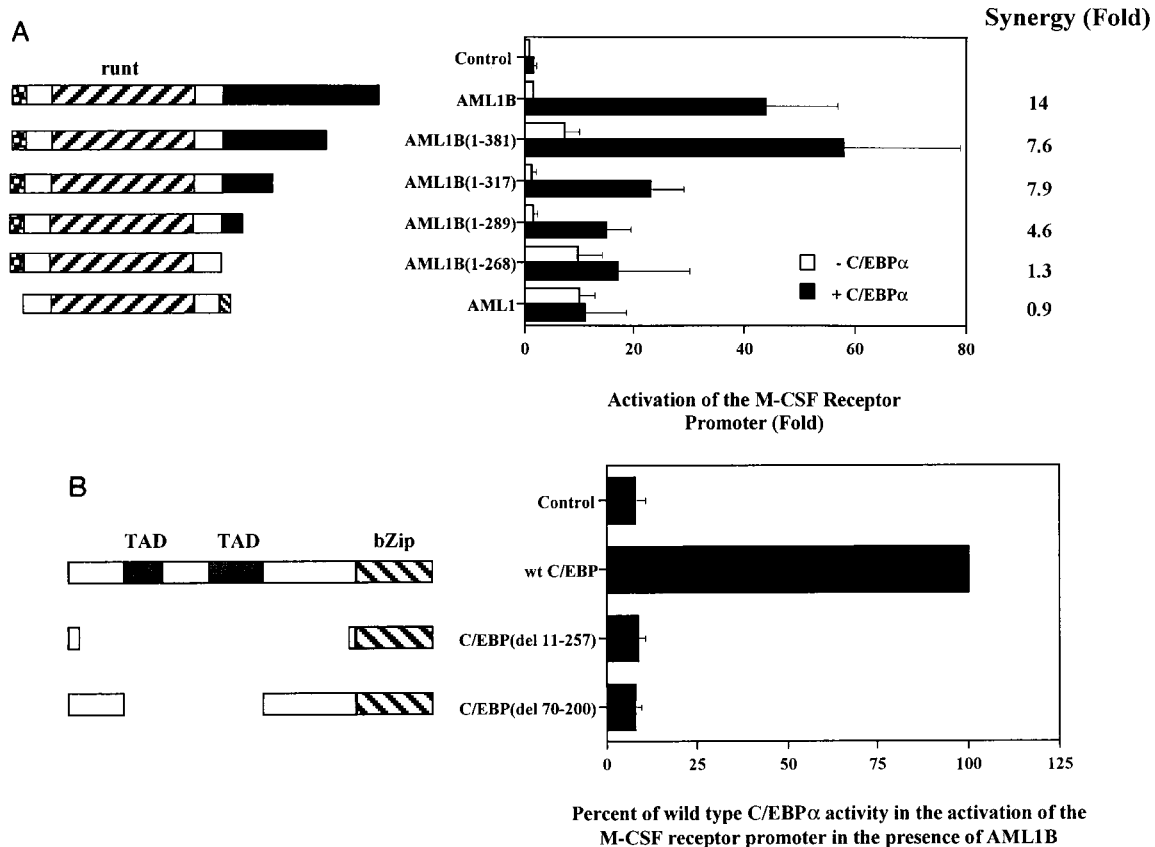


FIG. 6. The C terminus of AML1 and the transactivation domains of C/EBP $\alpha$  are required for synergistic activation of the M-CSF receptor promoter. (A) The C terminus of AML1B is important for synergy with C/EBP $\alpha$ . CV-1 cells were transfected with 5  $\mu$ g of pM-CSF-R-luc and 1  $\mu$ g each of expression vectors for AML1, AML1B, and C-terminal truncations of AML1B, in the presence and absence of C/EBP $\alpha$ . Transfection results are normalized to the level of expression of pM-CSF-R-luc in the presence of control plasmid and represent the means  $\pm$  standard error of three experiments. Fold synergy is calculated by dividing the activation in the presence of both factors by the sum of the activation by each factor individually. (B) The transactivation domains (TAD) of C/EBP $\alpha$  are required for synergy with AML1B. The results of transfections in CV-1 cells are expressed as percentages of the activity of wild-type (wt) C/EBP $\alpha$ , which alone activates the M-CSF receptor promoter 1.7-fold, and represent the means  $\pm$  standard error of three experiments. The mutations indicate the amino acids which are deleted.

clarify the mechanism of synergistic activation, we proceeded to investigate whether mutations outside of the DNA-binding domains would affect the ability of either factor to synergize with the other.

Figure 6A presents data that demonstrate the requirement for the C terminus of AML1B in synergistic activation. The 250-amino-acid isoform, AML1, which effectively terminates at residue 268 of AML1B, does not synergize with C/EBP $\alpha$ , nor does AML1B truncated at amino acid 268. Therefore, we can exclude the possibility that the different termini of AML1 play negative roles in the synergy with C/EBP $\alpha$ . However, in contrast to the experiments done in the presence of PU.1, AML1B truncated at either amino acid 317 or 289 retains the ability to synergize with C/EBP $\alpha$  (represented by the fold synergy), indicating that amino acids 268 to 317 are important for this activity. Due to the variability between experiments, there is no significant difference between the fold synergies calculated for AML1B, AML1B(1-381), and AML1B(1-317). We are also able to show that mutations which delete nearly the entire N terminus of C/EBP $\alpha$  (del 11-257, previously referred to as regions 1 to 9) or both of the transactivation domains (del 70-200, previously referred to as regions 3 to 7) (17) completely abrogate synergy with AML1B (Fig. 6B). The production of C/EBP $\alpha$  protein from these constructs has been demonstrated previously by Western blot analysis of extracts from transfected

HepG2 cells (17). The results of these experiments show that amino acids 268 to 317 of AML1B, and the transactivation domains of C/EBP $\alpha$ , are critical for synergy.

**Mutation of potential phosphorylation sites in AML1 does not interfere with synergistic activation of the M-CSF receptor promoter.** The activation of an AML1-responsive reporter by AML1A can be increased following phosphorylation by ERK on serines 249 and 266 (corresponding to serines 276 and 293 of AML1B) (68). We were interested in determining whether the interaction between AML1 and its neighboring factors might be influenced by this modification. Since the phosphorylation state of AML1 is unknown under the conditions in which we observe synergistic activation, we addressed the role of serines 249 and 266 in this context. The data in Fig. 7 demonstrate that AML1A, which contains the same N terminus as AML1 and the same C terminus as AML1B (Fig. 1), synergizes with both C/EBP $\alpha$  (Fig. 7A) and PU.1 (Fig. 7B). In addition, AML1A truncated at amino acid 288 (corresponding to amino acid 315 of AML1B) fails to synergize with PU.1, further supporting the observation that amino acids C terminal to residue 317 are necessary for this function (Fig. 7B). However, mutation of serines 249 and 266, converting those residues to alanine, has no effect on the ability of AML1A to synergize with either C/EBP $\alpha$  or PU.1, demonstrating that



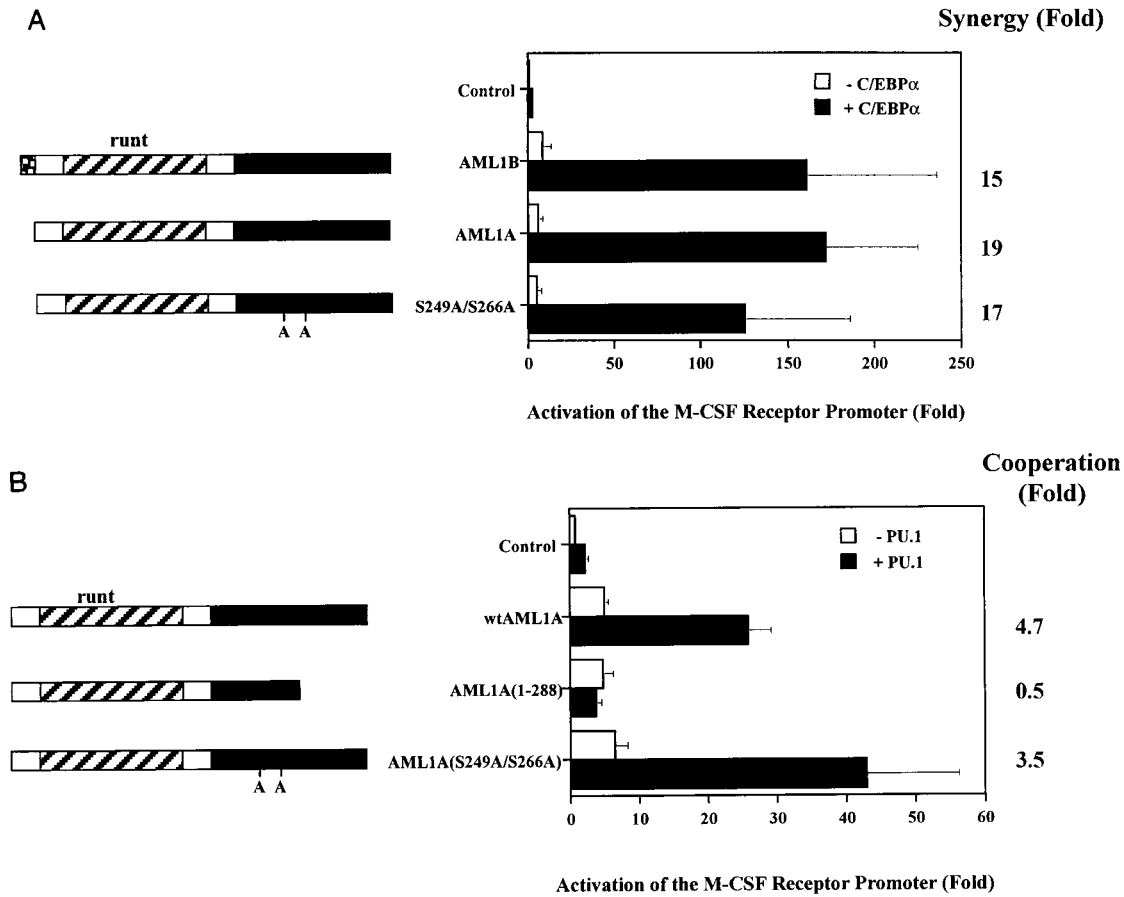


FIG. 7. Mutation of potential phosphorylation sites does not affect the ability of AML1 to synergize with either C/EBP $\alpha$  or PU.1. (A) Wild-type AML1A and mutant S249A/S266A both synergize with C/EBP $\alpha$ . Transfections of CV-1 cells with AML1B, AML1A, and AML1A mutated at serines 249 and 266 (S249A/S266A) were performed in the absence and presence of C/EBP $\alpha$ . The results represent the mean activation of the promoter  $\pm$  standard error of three experiments. Fold synergy is calculated by dividing the activation in the presence of both factors by the sum of the activation by each factor individually. (B) Wild-type (wt) AML1A and mutant S249A/S266A synergize with PU.1 to activate the M-CSF receptor promoter. HeLa cells were transfected with pM-CSF-R-luc and expression constructs for wild-type AML1A, a mutant terminated at amino acid 288 (1-288), and one with serine-to-alanine mutations at residues 249 and 266 (S249A/S266A), in the presence and absence of PU.1. The results represent the mean activation of the promoter  $\pm$  standard error of three experiments.

these potential phosphorylation sites are not required for synergistic activation by AML1.

## DISCUSSION

Defining the regions of PU.1, C/EBP $\alpha$ , and AML1 necessary for physical and functional interaction has led to an increased understanding of the mechanisms by which the M-CSF receptor promoter is regulated and how these factors interact to mediate transcriptional activation. We have demonstrated that the DNA-binding domain of AML1 can form higher-order complexes with the DNA-binding domain of either C/EBP $\alpha$  or PU.1 and that the formation of these complexes is dependent on DNA-binding sites for both AML1 and the neighboring transcription factor. In addition, we observed that a complex containing both the *runt* and bZip domains bound greater amounts of probe than either individual DNA-binding domain, indicating that cooperative DNA binding occurs between AML1 and C/EBP $\alpha$ . Alternatively, this effect was not observed between the *runt* domain and PU.1. Instead, less probe was associated with the ternary complex than with either PU.1 or the *runt* domain. However, since these experiments were performed with the DNA-binding domains of AML1 and C/EBP $\alpha$ , we cannot exclude the possibility that the native pro-

teins would exhibit different properties. In several studies, the physical interaction between transcription factors increases DNA-binding ability, providing an explanation for synergistic activation in the presence of two factors (11, 18, 56, 58, 67). For example, AML1 binds cooperatively with another member of the *ets* family, Ets-1, to the TCR $\alpha$ , TCR $\beta$ , and Moloney murine leukemia virus enhancers (18, 67). Cooperative DNA binding may contribute to the strong synergy between AML1B and C/EBP $\alpha$ .

We have shown that AML1 interacts physically with PU.1, and as with C/EBP $\alpha$ , this property maps to the DNA-binding domain of each protein. In addition, AML1 and PU.1 exhibit a relatively weak synergistic activation of the promoter. The effect seen in the presence of both PU.1 and AML1B is more than additive and therefore by definition synergistic. We have also shown that while the physical interaction between AML1B and either PU.1 or C/EBP $\alpha$  occurs between the DNA-binding domains, other regions are also necessary to achieve the observed activation. For example, while the 250-amino-acid form of AML1 contains the *runt* domain, there is no synergy observed with either PU.1 or C/EBP $\alpha$ . Furthermore, deletion of the activation domain of either PU.1 or C/EBP $\alpha$  abrogates synergy with AML1B. Therefore, while in the case of AML1 and C/EBP $\alpha$ , the physical interactions may contribute to co-



operative DNA binding, this is not sufficient for the strong synergy. Instead, we believe there are additional mechanisms controlling synergistic activation.

The carboxy terminus of AML1B contains at least two domains that serve disparate functions, each of which is responsible for synergy with C/EBP $\alpha$  and PU.1. This is demonstrated by the finding that amino acids 1 to 317, a region important for synergy between AML1 and c-Myb (6), are sufficient for synergy with C/EBP $\alpha$  but not PU.1. The existence of two functionally distinct domains implies that synergistic activation by AML1 is mediated by secondary proteins, or coactivators which bind specifically to one domain or the other. In support of this theory, we are able to show that TBP binds to a fusion protein containing the region of AML1B important for synergy with C/EBP $\alpha$  but not to a portion that contains the domain required for synergy with PU.1 (data not shown). Furthermore, each domain interacts with a different set of polypeptides from radiolabeled HeLa cells (data not shown), confirming that the C-terminal domains of AML1B make specific and distinct contacts, any of which may play a role in synergistic activation.

We hypothesize that PU.1, C/EBP $\alpha$ , and AML1 form a transcriptional unit, or primary complex, on the DNA and that this primary complex makes multiple and specific contacts with a second, perhaps ubiquitous, complex composed of coactivators. The role of the DNA-binding proteins is to confer tissue-specific, temporal regulation, while the coactivators serve to amplify the activation by increasing transcription efficiency. A similar mechanism has been described for the DNA-binding nuclear hormone receptors. Recent reports have revealed a complex mechanism, whereby nuclear hormone receptors are associated with both steroid receptor coactivators and with CBP, and the activation ability of the transcription factor is dependent on the efficient assembly of these complexes on the DNA (43). CBP is a ubiquitous adapter protein that mediates contacts between transcription factors and the basal transcription machinery (1, 26, 33) and is thought to stimulate transcription both by physical contact with the RNA polymerase II complex and through intrinsic histone acetylation activity (4, 33, 52). Kamei et al. have coined the term "integrator," postulating that CBP integrates diverse signals within the cell which culminate in the assembly of transcription factors and coactivators and translates them into transcriptional activation (26). Further definition of this mechanism and identification of the factors involved will increase our understanding of how transcription factors respond to signals from external stimuli or cell cycle regulators.

It is clear that AML1 serves disparate functions on various promoters. While ERK increases the transactivation abilities of AML1A on the TCR $\beta$  enhancer, mutation of the serines which are potentially phosphorylated by ERK decrease neither activation by AML1A nor synergy. In addition, while the fusion protein formed from the (8;21) translocation, AML/ETO, behaves as an inhibitor of AML1B function with respect to the GM-CSF promoter and the TCR $\beta$  enhancer (15, 38), it synergizes with AML1B to activate the M-CSF receptor promoter (60). Although AML1 is known to interact with Ets-1, which like PU.1 is a member of the *ets* family of transcription factors, in this situation it is not the conserved *ets* DNA-binding domain which makes contact with the *runt* domain of AML1, but rather amino acids 123 to 240 in the N terminus of Ets-1 (18). The functional variability of AML1 can be explained if it requires contacts with other factors to activate transcription and is accordingly dependent on the cell type as well as other DNA-binding sites. Clarification of these mechanisms may lead to an understanding of how transcription is regulated in response to external signals or changes in the cell cycle. For

instance, the ability for AML1 to synergize with PU.1 or C/EBP $\alpha$  or to coordinate the actions of all three transcription factors may depend on the activation or availability of coactivators and integrators. Therefore, in order to understand the mechanism by which AML1 and other factors that play pivotal roles in hematopoiesis function, it is important to explore and identify the contacts made in the course of transcriptional regulation.

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