MOLECULAR AND CELLULAR BIOLOGY, June 1995, p. 3090–3099 Vol. 15, No. 6
0270-7306/95/$04.00+0

c-Myb and Core-Binding Factor/PEBP2 Display Functional Synergy but Bind Independently to Adjacent Sites in the T-Cell Receptor δ Enhancer

CRISTINA HERNANDEZ-MUNAIN AND MICHAEL S. KRANGEL*

Department of Immunology, P.O. Box 3010, Duke University Medical Center, Durham, North Carolina 27710

Received 8 November 1994/Returned for modification 25 January 1995/Accepted 22 March 1995

A T-cell-specific transcriptional enhancer lies within the $J_\delta3$-C_δ intron of the human T-cell receptor δ gene. We have previously shown that a 30-bp element, denoted δE3, acts as the minimal TCR δ enhancer and that within δE3, adjacent and precisely spaced binding sites for core-binding factor (CBF/PEBP2) and c-Myb are essential for transcriptional activity. These data suggested that CBF/PEBP2 and c-Myb synergize to mediate transcriptional activity but did not establish the molecular basis for synergy. In this study, we have examined in detail the binding of CBF/PEBP2 and c-Myb to δE3. We found that CBF/PEBP2 and c-Myb could simultaneously occupy the core site and one of two overlapping Myb sites within δE3. However, equilibrium binding and kinetic dissociation experiments suggest that the two factors bind to δE3 independently, rather than cooperatively. This was found to be true by using isoforms of these factors present in extracts of transfected COS-7 cells, as well as the natural factors present in nuclear extracts of the Jurkat T-cell line. We further showed that CBF/PEBP2 and c-Myb provide unique transactivation functions, since the core-Myb combination cannot be substituted by dimerized core or Myb sites. We propose that spatially precise synergy between CBF/PEBP2 and c-Myb may result from the ability of the two factors to form a composite surface that makes unique and stereospecific contacts with one or more additional components of the transcriptional machinery.

Transcriptional regulation is a complex process that is mediated by the binding of *trans*-acting factors to *cis*-regulatory DNA sequences. Bound activator proteins can regulate transcription in a variety of ways, i.e., by direct contact with the basal transcriptional machinery, by modification of local chromatin structure so as to permit the binding of additional factors to DNA, by stabilization of the binding of additional factors to DNA through cooperative interactions, and by the recruitment of so-called coactivator proteins that do not themselves bind directly to DNA (16, 52, 65).

The genes encoding T-cell receptor (TCR) polypeptides consist of gene segments whose rearrangement and expression are controlled as a function of time and cell lineage during T-lymphocyte development (17, 53, 63). An extensive effort to understand the molecular basis for such regulation has defined important *cis*-regulatory elements such as promoters, enhancers, and silencers, as well as some of the *trans*-acting factors that interact with these sequences (32). A transcriptional enhancer within the $J_\delta3$-C_δ intron of the human TCR δ gene activates T-cell-lineage-specific gene transcription as assessed in transient transfection experiments (55) and is essential for temporal and T-cell-lineage-specific gene rearrangement as assessed with transgenic mice (30, 31). In transient transfection experiments, we have previously shown that a 30-bp element designated δE3 functions as the minimal TCR δ enhancer (57) and that adjacent core (TGTGGTTT) and Myb sites (CCGTTA) within δE3 are essential for enhancer activity (19, 57). We and others showed that core binding factor (CBF) and c-Myb bind specifically to their cognate sites within δE3 (19, 56, 68) and that c-Myb can transactivate gene expression as a consequence of binding (19). Since intact core and Myb sites are both required for enhancer function, we concluded that CBF and c-Myb must cooperate to mediate transcriptional activation. This functional cooperation depends on the relative positioning of core and Myb sites, suggesting that bound proteins either interact directly with each other or together interact in a stereospecific fashion with additional components of the transcription apparatus.

CBF, which is also known as polyomavirus enhancer-binding protein 2 (PEBP2), binds to the consensus core site PyGPyGGTPy (22, 36, 67). CBF/PEBP2 consists of two subunits: an α chain that mediates sequence-specific DNA binding and a noncovalently associated β chain that does not contact DNA but rather increases the intrinsic DNA binding affinity of the α subunit (49, 50, 68). Two different murine genes encode related CBF/PEBP2α proteins, αA and αB. The latter gene is the murine equivalent of the human *AML-1* gene (3, 4, 42), which is disrupted by translocations associated with myelogenous leukemia (14, 41, 42, 48). All of these proteins have a region highly homologous with the product of Runt, a *Drosophila* segmentation protein (25). The Runt homology region is sufficient for both specific binding to the core site and heterodimerization with CBF/PEBP2β (40, 49, 50, 68). CBF/PEBP2β displays no significant homology with other proteins and does not contain recognizable DNA-binding or dimerization motifs.

CBF/PEBP2 is an important regulator of gene expression in T cells and myeloid cells. For example, the core sites of the Moloney murine leukemia virus and SL3-3 murine leukemia virus enhancers are important determinants of the T-cell tropism and pathogenicity of these viruses (18, 62). Further, CBF/PEBP2 binds to the TCR δ (56, 68), γ (20), and β (51) enhancers, and mutagenesis and transactivation experiments indicate that such binding is important for the transcription of these genes (3, 20, 50, 51, 57). Similarly, there are functionally important CBF/PEBP2 binding sites in myeloid-specific genes

* Corresponding author. Phone: (919) 684-4985. Fax: (919) 684-8982.

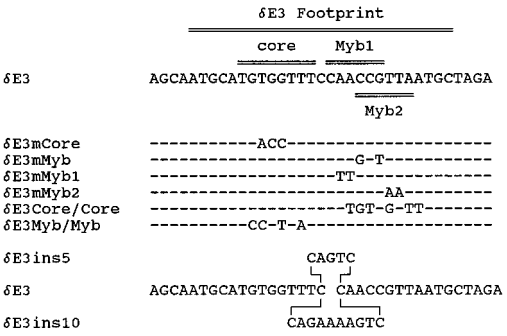

FIG. 1. Wild-type and mutant δE3 sites analyzed. The actual oligonucleotides used in this study include flanking *Bam*HI sites appended to facilitate cloning and radiolabelling. The extent of the δE3 binding site as defined by DNase I footprinting and the locations of the core, Myb1, and Myb2 sites are indicated.

such as those encoding myeloperoxidase and neutrophil elastase (47, 64).

Myb proteins activate transcription by specific binding to the nucleotide sequence PyAACG/TG (5, 27, 45, 69). The Myb family includes the viral oncoprotein v-Myb and the cellular proteins c-Myb, A-Myb, and B-Myb (28, 29, 46, 58). c-Myb is composed of three functional domains that are responsible for DNA binding, transcriptional activation, and negative regulation, respectively (59). A leucine zipper motif in the carboxy-terminal negative regulatory domain has been shown to mediate interactions with other proteins (23); however, the functional significance of these interactions is uncertain (12).

c-Myb clearly plays an important role in the differentiation and proliferation of hematopoietic cells. It is expressed at high levels in immature hematopoietic cells, is down-regulated during cell differentiation, and is transiently up-regulated in mature cells during cell proliferation (34, 60). Mice homozygous for a null c-*myb* allele die by days 14 to 15 of fetal development on account of severe anemia and other defects in hematopoiesis (43). Furthermore, overexpression of tissue-specific dominant negative Myb alleles in transgenic mice has established a specific role for Myb-binding sites in T-cell differentiation and proliferation (2). Nevertheless, only a few genes that are specifically expressed in hematopoietic cells are known to be regulated by c-Myb: CD4 and TCR δ in T cells (19, 61), *mim-1* and c-*fms* in myeloid cells (45, 54), and CD34 in stem cells (37).

Because CBF/PEBP2 and c-Myb play essential and synergistic roles in TCR δ enhancer function, we have analyzed the binding of these factors to their adjacent sites within the δE3 element in greater detail. We find, as predicted from the functional experiments, that CBF/PEBP2 and c-Myb can bind simultaneously to δE3. However, despite the precise spatial relationship of core and Myb sites revealed in previous functional experiments, we find that CBF/PEBP2 and c-Myb do not appear to bind cooperatively to δE3. Rather, they appear to bind independently, arguing that synergy between the two factors may result from their ability to make simultaneous and stereospecific contacts with additional components of the transcriptional machinery.

MATERIALS AND METHODS

Plasmids and oligonucleotides. δE3 and related binding sites used in this study are indicated in Fig. 1. Complementary oligonucleotides representing each binding site included appended *Bam*HI-compatible overhangs (not shown). The δE6 binding site has been described previously (19).

To generate plasmid pCMV4-c-Myb, a 2.7-kb fragment encoding murine c-Myb was excised from plasmid pRmb3SVneo (15) by digestion with *Hin*dIII and was then religated into *Hin*dIII-digested and phosphatase-treated pCMV4 (1). The cDNA encoding murine CBF/PEBP2αA was a generous gift from Y. Ito (Kyoto University, Kyoto, Japan). To generate plasmid pCMV4-CBF/PEBP2αA, a 2.7-kb fragment encoding a CBF/PEBP2 isoform with a molecular mass of 55.8 kDa (PEBP2αA1) was excised from pBluescript by digestion with *Xba*I, treatment with T4 polymerase, and redigestion with *Eco*RV and was then ligated into *Sma*I-digested and phosphatase-treated pCMV4 vector. The cDNA encoding murine CBF/PEBP2β was a generous gift from N. Speck (Dartmouth Medical School, Hanover, N.H.). To generate plasmid pCMV4-CBF/PEBP2β, a 0.6-kb fragment encoding a CBF/PEBP2β protein isoform with a molecular mass of 21.5 kDa (CBF/PEBP2β2) was obtained from the plasmid pBluescript SK^+ by digestion with *Pst*I and *Bam*HI and treatment with T4 polymerase and was then subcloned into *Sma*I-digested and phosphatase-treated pCMV4. The plasmid $V_δ1$-CAT and versions of this plasmid carrying the monomeric 35-bp δE3 site, the monomeric 35-bp δE3mCore site, and the monomeric 35-bp δE3mMyb site have been described previously (19, 57). Oligonucleotides δE3Core/Core and δE3Myb/Myb were treated with T4 polynucleotide kinase, annealed, and ligated into *Bam*HI-digested and phosphatase-treated $V_δ1$-CAT plasmid. The structures of these constructs were confirmed by DNA sequence analysis.

Preparation of COS-7 cellular extracts. COS-7 cells were maintained in Dulbecco's modified Eagle's medium (Cellgro-Mediatech, Herndon, Va.) supplemented with 10% fetal bovine serum (Atlanta Biological, Norcross, Ga.) and 5 mg of gentamicin (GIBCO-BRL, Gaithersburg, Md.) per ml. Expression plasmids were transfected by using the DEAE-dextran technique (10). Briefly, a 100-mm subconfluent dish of COS-7 cells was incubated with 10 μg of plasmid DNA and 100 μg of DEAE-dextran (molecular weight, 500,000; Pharmacia LKB, Piscataway, N.J.) in phosphate-buffered saline for 30 min at 37°C. Medium supplemented with 100 μM chloroquine (Sigma Chemical Co., St. Louis, Mo.) was added for a 3-h incubation and then replaced with fresh medium. After 48 h, transfected cells were lysed at 4°C in 1 ml of a low-stringency buffer containing 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.0), 250 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1 μg of pepstatin, 1 μg of aprotinin, and 1 μg of leupeptin per ml and were centrifuged at 12,000 × *g* for 15 min at 4°C.

EMSAs. Preparation of Jurkat cell nuclear extract, radiolabelling of binding-site probes with the Klenow fragment of DNA polymerase I and [α-^{32}P]dGTP (ICN Radiochemicals, Irvine, Calif.), and electrophoretic mobility shift assays (EMSAs) were performed as described previously (19, 57). In EMSAs using COS-7 cellular extracts, 0.2 to 3 μl of cellular extract, 1 μg of bovine serum albumin (BSA), and 0.5 μg of poly(dI-dC) carrier were used. The total amount of cellular extract in each sample was adjusted to 4 μl by addition of cellular extract from untransfected cells. In EMSAs using Jurkat cell nuclear extract, 4 μg of nuclear extract from Jurkat cells was incubated with 5 μg of BSA and 2 μg of poly(dI-dC) DNA carrier. A murine monoclonal antibody raised against the C-terminal region of murine c-Myb (UBI, Lake Placid, N.Y.) or a control immunoglobulin G1 monoclonal antibody were used in supershift experiments as described previously (19).

In order to assess complex dissociation rates by EMSA, binding reactions were first allowed to reach equilibrium by incubation for 30 min at 4°C. An initial aliquot of the binding reaction mixture was loaded onto a running gel, following which a 50× excess of unlabelled δE3 oligonucleotide was added to the remainder of the reaction, and successive aliquots of the reaction mixture were loaded onto the gel at defined times. Complexes in EMSA experiments were quantified with a PhosphorImager (Molecular Dynamics).

CAT assays. The human T-cell leukemia cell line Jurkat was grown in RPMI 1640 supplemented with 10% fetal bovine serum and penicillin-streptomycin (GIBCO-BRL). For chloramphenicol acetyltransferase (CAT) assays, cells were transfected with CsCl-purified plasmid DNA by using the DEAE-dextran method (11, 35). Acetylation of [^{14}C]chloramphenicol (Dupont-New England Nuclear) was assayed as described previously (55) and was quantified using a Betascope (Betagen, Waltham, Mass.).

RESULTS

CBF/PEBP2 and c-Myb bind simultaneously to the δE3 element. In order to study the binding of CBF/PEBP2 and c-Myb to δE3, we generated cellular extracts from COS-7 cells transfected with pCMV4-c-Myb, pCMV4-CBF/PEBP2αA, or pCMV4-CBF/PEBP2β expression vectors. We then tested these extracts alone and in combination by EMSAs to determine whether CBF/PEBP2 and c-Myb could bind simultaneously to a radiolabelled δE3 site probe (Fig. 2). When this probe was incubated with an extract from untransfected cells, protein-DNA complexes were barely detectable (Fig. 1, lane 1). However, a complex was readily detected by using an extract from cells transfected with CBF/PEBP2αA (Fig. 2, lane 2, α). The migration and yield of this complex were altered by inclusion of extract from cells transfected with CBF/PEBP2β

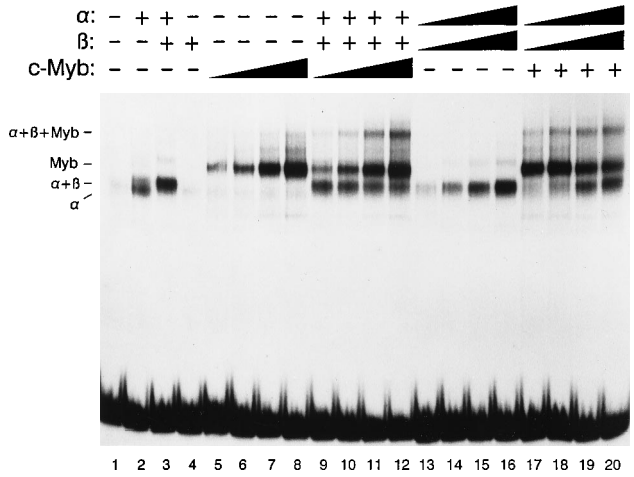

FIG. 2. Simultaneous binding of CBF/PEBP2 and c-Myb to δE3. A radiolabelled δE3 probe was incubated in the presence of combinations of extracts from COS-7 cells transfected with CBF/PEBP2αA (α), CBF/PEBP2β (β), and c-Myb (Myb), as noted. In lanes 2 to 4, the volume of CBF/PEBP2α extract was 0.2 μl and the volume of CBF/PEBP2β extract was 3 μl. In all other lanes, the ratio of α to β was maintained at 1:15, and the total volume was 2 μl (lanes 9 to 12) or varied from 0.5 to 3.0 μl (lanes 12 to 16 and 17 to 20). The volume of c-Myb extract was 1 μl (lanes 17 to 20) or varied from 0.25 to 2 μl (lanes 5 to 8 and 9 to 12). In all cases, the total volume of extract was adjusted to 4 μl with extract from untransfected cells. DNA-protein complexes were resolved by electrophoresis. Complexes containing CBF/PEBP2α (α), CBF/PEBP2α and CBF/PEBP2β (α+β), c-Myb (Myb), and all three components (α+β+Myb) are indicated.

(lane 3, α+β), even though cellular extract containing CBF/PEBP2β alone did not have any detectable DNA-binding activity (lane 4). These data are consistent with previous results from other laboratories using core sites from Moloney murine leukemia virus or polyomavirus (49, 68). Note also that in addition to the major α+β complex formed in the presence of CBF/PEBP2α and CBF/PEBP2β extracts, there is at least one additional low-abundance complex with a slower electrophoretic mobility (lane 3). The nature of this complex is uncertain.

Incubation of the δE3 probe with increasing amounts of cellular extracts containing c-Myb resulted in the dose-dependent formation of a complex (designated Myb) with an electrophoretic mobility slower than that of the α+β complex (Fig. 2, lanes 5 to 8). Two additional minor complexes with still slower electrophoretic mobilities could also be detected, particularly when high levels of c-Myb-containing cellular extracts were used; the natures of these complexes are unclear. To test whether CBF/PEBP2 and c-Myb could bind simultaneously to δE3, binding reactions were performed with fixed amounts of cellular extracts containing CBF/PEBP2α and CBF/PEBP2β and increasing amounts of cellular extracts containing c-Myb (Fig. 2, lanes 9 to 12). A new complex was formed in a dose-dependent fashion. It displayed a mobility slower than those of the major complexes generated by either the combination of CBF/PEBP2α and -β or c-Myb when tested separately (lanes 9 to 12), and it was detected in much higher yield than the minor comigrating complex formed by c-Myb alone. This higher-order complex (designated α+β+Myb) presumably reflects the binding of CBF/PEBP2 and c-Myb to the same DNA molecule. Similar results were obtained in the reciprocal experiment; incubation of the δE3 probe with increasing amounts of cellular extracts containing CBF/PEBP2α and CBF/PEBP2β resulted in the dose-dependent formation of an α+β complex (lanes 13 to 16), whereas similar titration in the presence of a constant amount of extract containing c-Myb resulted in the dose-dependent formation of an apparently identical higher-order complex (lanes 17 to 20).

To investigate the nature of this higher-order complex, binding analyses were performed by using radiolabelled δE3 probes that carry mutations in the relevant binding sites (Fig. 3A). These mutants, δE3mCore (3-bp mutation) and δE3mMyb (2-bp mutation), were previously shown to abrogate the binding of CBF/PEBP2 and c-Myb, respectively (19, 56, 57). As

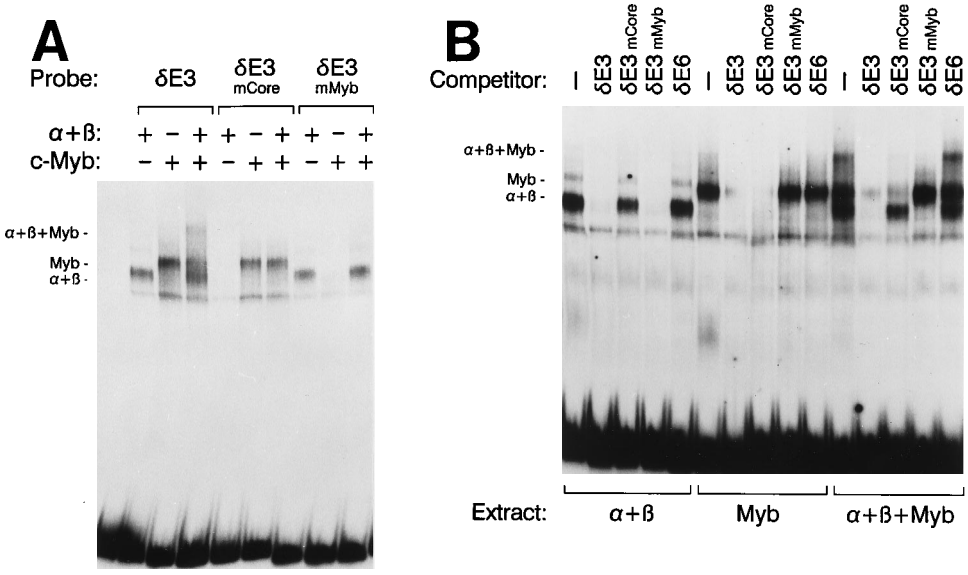

FIG. 3. Formation of higher-order complexes requires intact core and Myb sites. (A) Radiolabelled δE3, δE3mCore, and δE3mMyb probes incubated with 3 μl of a mixture of CBF/PEBP2α and CBF/PEBP2β extracts (1:15 ratio), and 1 μl of c-Myb extract, either separately or in combination as indicated; (B) a radiolabelled δE3 probe incubated with 3 μl of a mixture of CBF/PEBP2α and CBF/PEBP2β extracts (1:15 ratio), and 1 μl of c-Myb extract, either separately or in combination as indicated. The amount of total extract in each reaction mixture was adjusted to 4 μl with extract from untransfected cells. A 50-fold molar excess of competitor binding sites was included in the reaction mixtures as indicated.

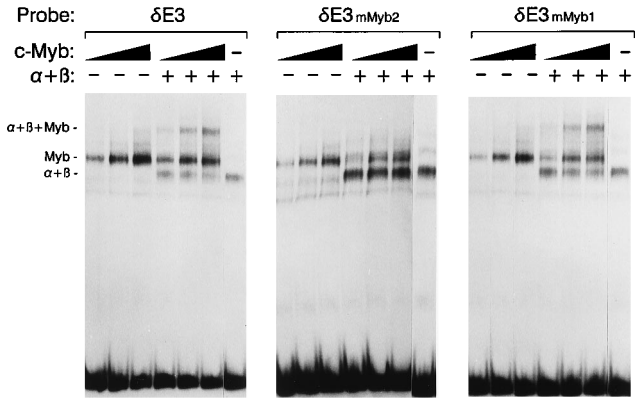

FIG. 4. Formation of higher-order complexes requires intact core and Myb2 sites. Radiolabelled δE3, δE3mMyb2, and δE3mMyb1 probes were incubated with 2 μl of a mixture of CBF/PEBP2α and CBF/PEBP2β extracts (1:15 ratio), and 0.5 to 2.0 μl of c-Myb extract, separately or in combination as indicated.

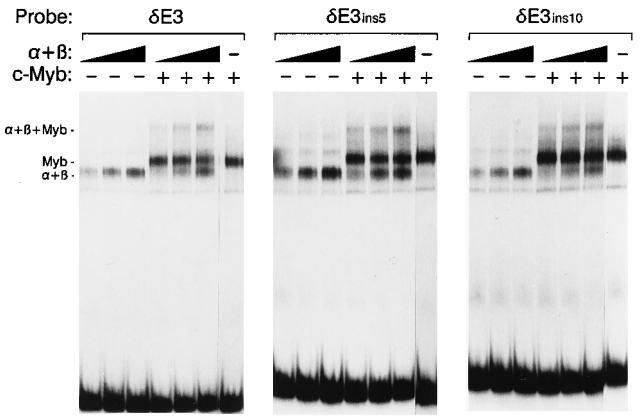

FIG. 5. Formation of higher-order complexes is independent of the spacing between core and Myb2 sites. Radiolabelled δE3, δE3ins5, and δE3ins10 probes were incubated with 1 to 3 μl of a mixture of CBF/PEBP2α and CBF/PEBP2β extracts (1:15 ratio), and 1 μl of c-Myb extract, separately or in combination as indicated.

expected, mutation of the core site eliminated CBF/PEBP2 binding but did not affect c-Myb binding, whereas mutation of the Myb site eliminated c-Myb binding but did not affect CBF/PEBP2 binding. However, both of these mutations eliminated formation of the higher-order (presumedly α+β+Myb) complex. Thus, formation of the higher-order complex requires that the δE3 core and Myb sites are both intact, arguing that the higher-order complex is composed of CBF/PEBP2 and c-Myb bound directly and simultaneously to the same δE3 binding site.

A similar conclusion was obtained when the specificities of these complexes were analyzed by competition of δE3 binding with a 50-fold molar excess of various unlabelled binding sites (Fig. 3B). As expected, the α+β complex is completely inhibited by an excess of δE3 or δE3mMyb binding sites but is only minimally inhibited by the same excess of δE3mCore binding site. Similarly, the Myb complex is completely inhibited by an excess of δE3 or δE3mCore binding sites but is not inhibited by an excess of δE3mMyb binding site. Of note, formation of the higher-order complex in the presence of cellular extracts containing CBF/PEBP2α, -β, and c-Myb is eliminated by an excess of the δE3, δE3mMyb, or δE3mCore binding sites but not by the unrelated δE6 binding site. These data therefore confirm that the higher-order complex results from simultaneous binding of CBF/PEBP2 and c-Myb to their cognate sites on the same molecule of δE3.

Simultaneous binding of CBF/PEBP2 and c-Myb to δE3 requires an intact Myb2 binding site. We have previously noted that two overlapping Myb sites (Myb1 and Myb2) are present within δE3. Both sites are destroyed by the δE3mMyb mutation. Although Myb can bind equivalently to the two sites, binding experiments demonstrated that they cannot both be occupied simultaneously. Since mutation of the Myb2 site eliminates enhancer activity, whereas mutation of the Myb1 site has minimal effects, our functional data argued that the Myb2 site is the primary site occupied in vivo (19). Therefore, we asked whether occupancy of the Myb2 site, but not the Myb1 site, was compatible with simultaneous binding of CBF/PEBP2 and c-Myb to δE3. To do so, we tested radiolabelled δE3mMyb1 and δE3mMyb2 sites in binding experiments in which increasing amounts of cellular extract containing c-Myb were titrated, in the absence or presence of constant amounts of cellular extracts containing CBF/PEBP2α and (Fig. 4). The higher-order complex (α+β+Myb) formed in the presence of all three proteins was detected when the δE3 or δE3mMyb1 binding site was used as a probe but was not detected when the δE3mMyb2 site was used. Similar results were obtained when binding was tested by titrating increasing amounts of cellular extract containing CBF/PEBP2α and -β into a fixed amount of cellular extract containing c-Myb (data not shown). These data indicate that CBF/PEBP2 and c-Myb cannot bind simultaneously to the core and Myb1 sites; rather, intact core and Myb2 sites are required for simultaneous binding of these proteins to DNA. This result correlates precisely with previous functional data and supports the idea that simultaneous binding of these factors is necessary for enhancer activity.

Simultaneous binding of CBF/PEBP2 and c-Myb is independent of the spacing between core and Myb sites. We have previously reported that δE3 mutants with an altered distance between the core and Myb2 sites, δE3ins5 (5-bp or half helical turn) and δE3ins10 (10-bp or full helical turn), are functionally inactive (19). These data suggest that CBF/PEBP2 and c-Myb are involved in essential stereospecific contacts with each other or with additional undefined proteins that are essential for enhancer activity. Contacts between CBF/PEBP2 and c-Myb might influence the propensity of these factors to load onto the same δE3 molecule, thus accounting for the functional effects of the insertion mutants. To examine this issue, we asked whether an α+β+Myb complex could be formed using δE3ins5 and δE3ins10 binding site probes (Fig. 5). Titration of graded amounts of cellular extracts containing CBF/PEBP2α and CBF/PEBP2β into a constant amount of cellular extract containing c-Myb revealed nearly identical higher-order complex formation with δE3, δE3ins5, and δE3ins10 probes. Similar results were obtained when binding reactions were performed by titration of increasing amounts of cellular extracts containing CBF/PEBP2α and CBF/PEBP2β into a constant amount of cellular extract containing c-Myb (data not shown). Thus, CBF/PEBP2 and c-Myb bind perfectly well, alone or in combination, independently of the distance between the core and Myb sites. These data suggest that inactivation of enhancer activity by these insertion mutants is not the result of diminished higher-order complex formation due to disrupted interactions between CBF/PEBP2 and c-Myb.

CBF/PEBP2 and c-Myb do not bind cooperatively to δE3. Because the inclusion of all species in binding reactions with δE3 failed to drive the vast majority of CBF/PEBP2 complexes or c-Myb complexes into higher-order complexes (Fig. 2, 4,

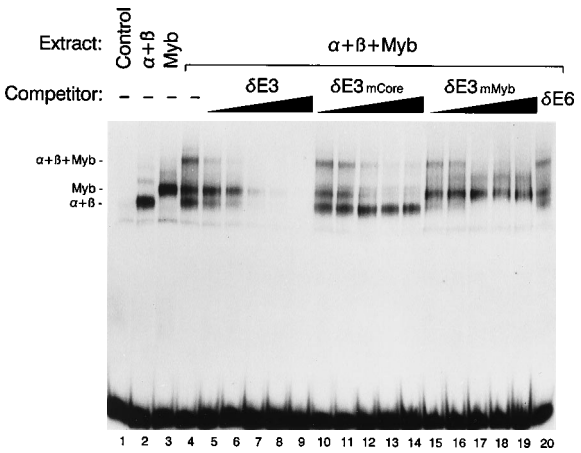

FIG. 6. CBF/PEBP2 and c-Myb do not bind cooperatively to δE3. Radiolabelled δE3 probe was incubated with 3 μl of a mixture of CBF/PEBP2α and CBF/PEBP2β extracts (1:15 ratio), and 1 μl of c-Myb extract, separately or in combination as indicated. The reaction mixtures included unlabelled δE3, δE3mCore, and δE3mMyb competitors at 2.5, 5, 25, 50, and 100× molar excess, or unlabelled δE6 competitor at 100× molar excess, as indicated.

and 5), our data suggested that CBF/PEBP2 and c-Myb do not bind with strong cooperativity to δE3. Nevertheless, careful quantification of the α+β, Myb, and α+β+Myb complexes in these titration experiments revealed that the α+β+Myb complex was slightly (2.1-fold) more abundant than would be expected from calculation of the products of the relative abundances of the α+β and Myb complexes. We sought to further address the issue of cooperativity in competition experiments that tested equilibrium binding of CBF/PEBP2 and c-Myb to δE3 sites with individual or paired binding sites for these factors (Fig. 6). A radiolabelled δE3 probe was incubated with extracts containing CBF/PEBP2α, -β, and c-Myb, in the absence or presence of graded amounts of an unlabelled δE3, δE3mCore, or δE3mMyb competitor. Unlabelled δE3 was an efficient competitor of all complexes formed with the radiolabelled δE3 probe (Fig. 6, lanes 4 to 9). Unlabelled δE3mCore inhibited formation of the Myb and α+β+Myb complexes, but not of the α+β complex (lanes 10 to 14). Importantly, the isolated Myb site in δE3mCore was as efficient a competitor for the α+β+Myb complex as it was for the Myb complex, arguing that Myb does not bind significantly more avidly in the higher-order complex than in the Myb complex. Similarly, unlabelled δE3mMyb inhibited formation of the α+β and α+β+Myb complexes, but not of the Myb complex (Fig. 6, lanes 15 to 19), and this isolated core site was as efficient a competitor for the α+β+Myb complex as it was for the α+β complex. Thus, CBF/PEBP2 does not bind significantly more avidly in the higher-order complex than in the α+β complex. These data suggest that CBF/PEBP2 and c-Myb bind independently, rather than cooperatively, to adjacent sites within δE3.

Identification of the complexes containing CBF/PEBP2 and c-Myb present in the T-cell line Jurkat. The above conclusions were based on the binding of specific isoforms of CBF/PEBP2 and Myb that were overexpressed in COS-7 cells, rather than the isoforms of these proteins that might naturally be found in T cells. Thus, it was important to confirm our conclusions using T-cell nuclear extracts. We previously detected CBF/PEBP2 in Jurkat cell nuclear extracts as a complex designated δE3A that formed with a radiolabelled δE3 probe (56, 57). However, we were unable to detect c-Myb in Jurkat cell nuclear extract by virtue of its binding to the same δE3 probe, on account of the complexity of protein binding to this probe. Rather, we could detect c-Myb binding only to a shorter version of δE3 that excluded the core site, thus eliminating the possibility of detecting higher-order complexes (19). Therefore, we attempted to reexamine Jurkat cell nuclear protein binding to an intact δE3 site as detailed below.

The typical pattern of Jurkat cell proteins bound to δE3 is shown in Fig. 7A. Two specific complexes detected in previous experiments, δE3A and δE3C, were efficiently inhibited by a 50× excess of unlabelled δE3 competitor. Using the δE3mCore oligonucleotide as a competitor, δE3C was partially inhibited but δE3A was unaffected (57). One component of δE3C is an E-box-binding protein that interacts with a site that partially overlaps the core site and that is disrupted in the δE3mCore mutation, thereby explaining the reduced competition by δE3mCore compared with δE3. However, previous data also suggested that a second component of the δE3C is a comigrating c-Myb-containing complex (19). Accordingly, unlabelled δE3mMyb served as an efficient competitor of δE3A but an inefficient competitor of δE3C. Notably, in this experiment an additional complex was detected that had slower electrophoretic mobility than δE3C and that was efficiently inhibited by all three oligonucleotides used as competitors. The electrophoretic mobility and specificity of this complex are similar to those of the higher-order complex formed using combined COS-7 cell extracts.

To more precisely identify Jurkat cell nuclear factors that bound to δE3, we performed parallel EMSAs that directly compared transfected COS-7 and Jurkat cell extracts (Fig. 7B). Some of the binding reactions were carried out in the presence of an anti-c-Myb antibody or a control antibody of the same isotype. The anti-c-Myb antibody was previously shown to supershift the complexes containing c-Myb (19). As expected, the α+β complex formed using transfected COS-7 extracts was unaffected by anti-c-Myb antibody (Fig. 7B, lanes 1 to 3), whereas the Myb-containing complex formed by using transfected COS-7 extract was supershifted by this antibody (Myb+Ab) but not by the control antibody (lanes 4 to 6). When COS-7 extracts containing CBF/PEBP2α, -β, and c-Myb were combined, the anti-c-Myb antibody supershifted two different complexes: one that contains c-Myb (Myb+Ab) and one that contains c-Myb and CBF/PEBP2 together (α+β+Myb+Ab) (lanes 7 to 9). As expected, the Jurkat cell δE3A and δE3C complexes displayed electrophoretic mobilities similar to those of the COS-7 α+β and Myb complexes, respectively (Fig. 7B, lane 10). Inclusion of anti-c-Myb antibody in binding reaction mixtures containing Jurkat cell nuclear extract clearly supershifted one complex so that it displayed an electrophoretic mobility identical to that of the α+β+Myb+Ab complex observed with COS-7 extract (compare lane 11 with lane 8). The complex banding pattern and poor resolution in this experiment did not allow us to clearly identify a c-Myb-containing complex in Jurkat cells by virtue of supershifting (but see below). Nevertheless, this result suggested that a higher-order complex containing CBF/PEBP2(α+β) and c-Myb was indeed formed by using Jurkat cell nuclear extract.

CBF/PEBP2 and c-Myb from Jurkat cells do not bind cooperatively to δE3. To further evaluate the specificity of the putative higher-order complex formed with Jurkat cell nuclear extract and to evaluate whether this complex results from cooperative binding of CBF/PEBP2 and c-Myb to DNA, a competition experiment was performed (Fig. 8). This experiment was again performed with the use of the anti-c-Myb antibody to isolate c-Myb-containing complexes from other comigrating complexes and to allow their detection. The resolution of this experiment allowed discrimination of two new complexes that

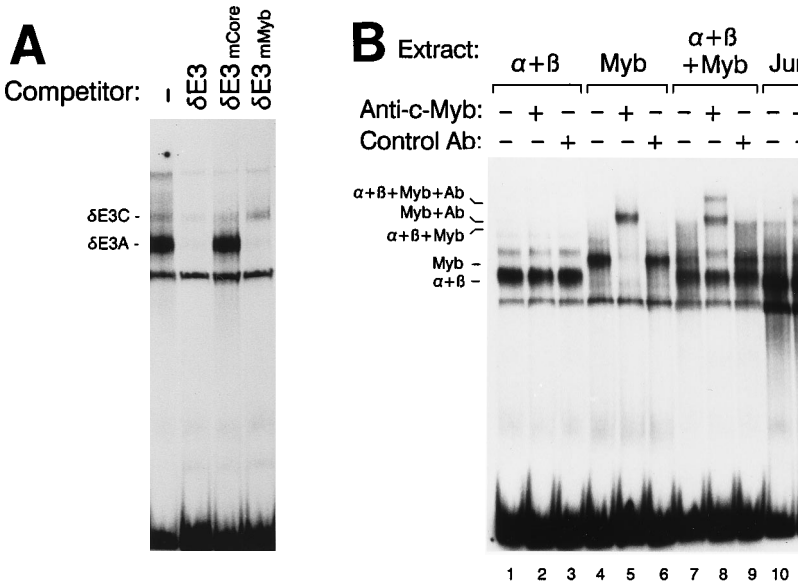

FIG. 7. Detection of higher-order complexes in Jurkat cell nuclear extract. (A) A radiolabelled δE3 probe incubated with 4 μg of Jurkat cell nuclear extract in the absence or presence of a 50× molar excess of the indicated unlabelled binding sites (the δE3A and δE3C are marked); (B) a radiolabelled δE3 probe incubated with 3 μl of a mixture of CBF/PEBP2α and CBF/PEBP2β extracts (1:15 ratio) and 1 μl of c-Myb extract, separately or in combination, or with 4 μg of Jurkat cell nuclear extract, as indicated. Binding reactions were performed without antibody (lanes 1, 4, 7, and 10), in the presence of 0.2 μg of anti-c-Myb antibody (lanes 2, 5, 8, and 11), or in the presence of 0.2 μg of control antibody (lanes 3, 6, 9, and 12). Supershifted complexes thought to contain c-Myb (Myb+Ab) and CBF/PEBP2+c-Myb (α+β+Myb+Ab) are marked.

were supershifted in the presence of anti-c-Myb antibody, one presumably containing c-Myb alone (Myb+Ab) and another one presumably containing CBF/PEBP2 and c-Myb together (α+β+Myb+Ab) (compare lanes 1 and 2 of Fig. 8). Competition with an excess of unlabelled δE3 inhibited formation of the δE3A or α+β complex (lane 9), as well as both supershifted complexes, Myb+Ab and α+β+Myb+Ab, whereas

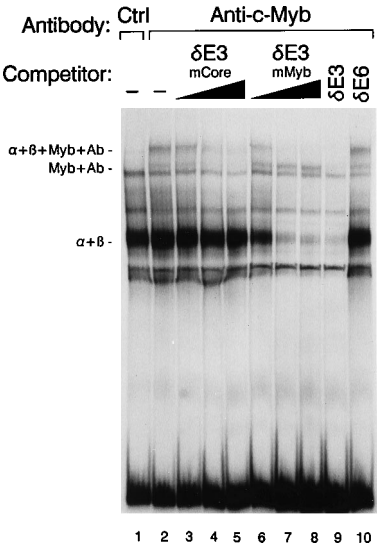

FIG. 8. CBF/PEBP2 and c-Myb in Jurkat cell nuclear extract bind independently to δE3. A radiolabelled δE3 probe was incubated with 4 μg of nuclear extract from Jurkat cells in the presence of 0.2 μg of a control antibody (lane 1) or 0.2 μg of anti-c-Myb antibody (lanes 2 to 10), in the presence of unlabelled δE3mCore or δE3mMyb competitor at 5, 25, and 50× molar excess, or in the presence of unlabelled δE3 or δE6 competitor at 50× molar excess. Supershifted complexes thought to contain c-Myb (Myb+Ab) or CBF/PEBP2+c-Myb (α+β+Myb+Ab) are marked.

competition with the same excess of the unrelated δE6 oligonucleotide had no effect, thus confirming the specificities of these complexes (lane 10). Moreover, unlabelled δE3mCore served as an efficient competitor of supershifted complexes (Myb+Ab and α+β+Myb+Ab) but not of the α+β complex (lanes 3 to 5), whereas unlabelled δE3mMyb served as an efficient competitor of the α+β complex and the α+β+Myb+Ab complex but not of the Myb+Ab complex (lanes 6 to 8). These competition results confirm the presence of CBF/PEBP2 and c-Myb in the supershifted complex with slower electrophoretic mobility and the presence of c-Myb alone in the supershifted complex with higher electrophoretic mobility. Importantly, δE3mCore was equally efficient as a competitor of the α+β+Myb+Ab and Myb+Ab complexes, and δE3mMyb was equally efficient as a competitor of the α+β+Myb+Ab and α+β complexes. These data argue that although CBF/PEBP2 and c-Myb from Jurkat cells can bind simultaneously to δE3, they do not bind cooperatively. These data are consistent with those obtained by using transfected COS-7 cell extracts and eliminate the possibility that an additional or modified factor(s) present in Jurkat cells and absent in COS-7 cells is important for cooperative binding of CBF/PEBP2 and c-Myb onto an isolated δE3 binding site.

To further characterize the binding properties of CBF/PEBP2 and c-Myb in both COS-7 and Jurkat cell extracts, we directly measured the rates at which the various proteins and protein complexes dissociate from δE3 (Fig. 9). The binding reactions were allowed to reach equilibrium for 30 min at 4°C, and an aliquot was loaded onto a running gel at $t = 0$. A 50× molar excess of unlabelled δE3 oligonucleotide was then added to each reaction mixture, and successive aliquots of the mixture were loaded onto the gel at specific times. Consistent with previous experiments (68), CBF/PEBP2α had almost completely dissociated from the DNA within 90 s, whereas α+β displayed a significantly decreased rate of dissociation. However, the dissociation rates of α+β, c-Myb, and α+β+Myb

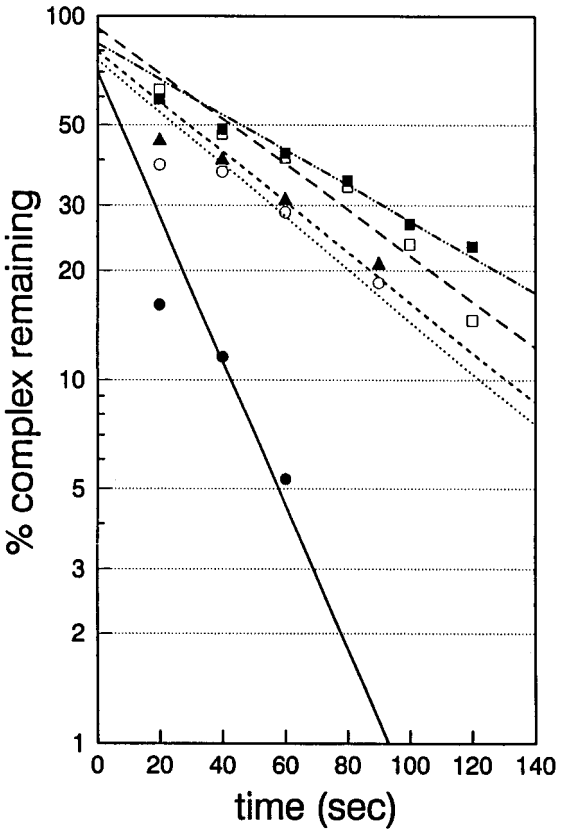

FIG. 9. Independent dissociation of CBF/PEBP2 and c-Myb from δE3. A radiolabelled δE3 probe was incubated with CBF/PEBP2α extract to measure α (●); CBF/PEBP2α and CBF/PEBP2β extracts at a 1:15 ratio to measure α+β (▲); c-Myb extract to measure Myb (○); a combination of CBF/PEBP2α, CBF/PEBP2β, and c-Myb extracts in the presence of 0.2 μg of anti-c-Myb antibody to measure α+β+Myb (□); or nuclear extract from Jurkat cells in the presence of 0.2 μg of anti-c-Myb antibody to measure α+β+Myb (■). Following the addition of an excess of unlabelled δE3, complex dissociation as a function of time was assessed by loading aliquots onto a running gel. Following electrophoresis, DNA-protein complexes were quantified with a PhosphorImager.

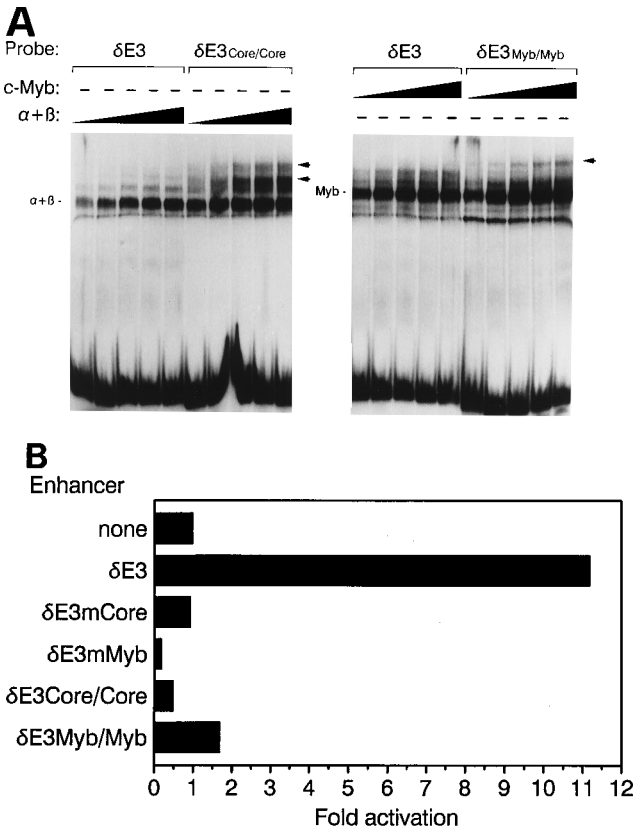

FIG. 10. Paired core sites or Myb sites cannot substitute for the core-Myb combination. (A) Radiolabelled δE3, δE3Core/Core, and δE3Myb/Myb binding sites incubated with 1 to 5 μl of a mixture of CBF/PEBP2α and CBF/PEBP2β extracts (1:15 ratio) or 1 to 5 μl of c-Myb extract, as indicated. Arrowheads denote new complexes formed with the δE3Core/Core and δE3Myb/Myb probes that do not form with the δE3 probe. (B) The indicated sites tested as monomers cloned upstream of the $V_\delta 1$ promoter in the enhancer-dependent test construct $V_\delta 1$-CAT. Constructs were transfected in triplicate into Jurkat cells, and values for percent chloramphenicol acetylation were averaged and normalized to the activity of $V_\delta 1$-CAT.

complexes in both COS-7 and Jurkat cell extracts were all very similar. Therefore, consistent with the results of equilibrium competition experiments, we found no kinetic evidence for cooperative binding.

The core-Myb combination is required for optimal δE3 enhancer activity. Since cooperative binding cannot explain the synergy between CBF/PEBP2 and c-Myb that is required for enhancer activity, we sought other explanations. One possibility is that δE3 function requires dimerization of functionally redundant transactivation domains provided by the two factors. Alternatively, the two factors might display functionally distinct transactivation domains, and synergy might result from the unique ability of this combination of transactivation domains to contact other proteins of the transcriptional apparatus. To distinguish between these possibilities, we generated mutant versions of the δE3 enhancer element with an additional core site precisely replacing the Myb site (Core/Core), or reciprocally, an additional Myb site precisely replacing the core site (Myb/Myb). We first asked whether the δE3Core/Core and δE3Myb/Myb binding sites could support the simultaneous loading of two CBF/PEBP2 molecules and two c-Myb molecules, respectively, as judged by EMSAs (Fig. 10A). Incubation of the δE3 probe with increasing amounts of cellular extracts containing CBF/PEBP2α and -β resulted in the formation of the major α+β complex, as well as one or more minor complexes with slower electrophoretic mobility, as noted previously. A similar titration using the δE3Core/Core probe revealed two new complexes (Fig. 10A, left panel, arrows) that presumably result from the loading of a second CBF/PEBP2 heterodimer onto the electrophoretically heterogeneous α+β complex. Similarly, titration of cellular extracts containing c-Myb resulted in the formation of a complex with the δE3Myb/Myb probe that was not observed with the δE3 probe (Fig. 10A, right panel, arrow). We then cloned the δE3Core/Core and δE3Myb/Myb binding sites into the enhancerless construct $V_\delta 1$-CAT and tested them for activity by transient transfection into Jurkat cells (Fig. 10B). Consistent with previous experiments (19, 57), a wild-type δE3 element induced transcriptional activity by 11.2-fold above the background activity of the enhancerless construct, whereas the δE3mCore and δE3mMyb mutants were transcriptionally inactive. Notably, δE3Core/Core was completely inactive in this assay, and δE3Myb/Myb displayed only very low activity. Thus, our data indicate that the specific combination of CBF/PEBP2 and c-Myb transactivation domains is required for optimal δE3 enhancer activity and suggest that this combination provides a

unique surface to contact other components of the transcriptional machinery.

DISCUSSION

Previous studies had shown that adjacent core and Myb sites within δE3 are both essential for transcriptional activity, suggesting that simultaneous binding of CBF/PEBP2 and c-Myb is required for δE3 enhancer function (19, 57). Further, these studies revealed that synergy between CBF/PEBP2 and c-Myb requires a precise spatial organization of core and Myb sites, since only one of two overlapping Myb sites (Myb2) was functional and the spacing between this site and the core site could not be varied. These data suggested that either CBF/PEBP2 and c-Myb interact directly with each other or that they together must interact in precise fashion with additional proteins to activate transcription. In this work, we analyzed in detail the binding of CBF/PEBP2 and c-Myb to δE3. We found that CBF/PEBP2 and c-Myb could simultaneously occupy the core and Myb2 sites within δE3 but not the core and Myb1 sites, thereby explaining the functional dichotomy between the two Myb sites. Further, we found that CBF/PEBP2 and c-Myb bind independently, rather than cooperatively, to the core and Myb2 sites. This was shown to be true by using isoforms of these factors present in extracts of transfected COS-7 cells, as well as the natural factors present in nuclear extracts of the T-cell line Jurkat. We further showed that CBF/PEBP2 and c-Myb provide unique transactivation functions, since the core-Myb combination cannot be effectively substituted by dimerized core or Myb sites. Therefore, we interpret the spatial constraints on synergy between CBF/PEBP2 and c-Myb to indicate that the two factors form a composite surface that makes unique, precise, and stereospecific contacts with other components of the transcriptional machinery.

The CBF/PEBP2αA, -αB, and -β genes produce multiple transcripts that encode different proteins with the potential for distinct transcriptional activities and distinct interactions with other transcription factors. To date, molecular cloning has identified two isoforms of αA (αA1 and αA2 [50]), two isoforms of αB (αB1 and αB2 [3, 4]), and two isoforms of β that can interact with α (β1 and β2 [49, 68]). We chose to express αA1 in COS-7 cells because, like the δE3A complex (57), αA1 is expressed specifically in T cells (50). Furthermore, αA1 is much more abundant and is more active than αA2 (50). Similarly, we chose to express β2 because it is the most abundant functional β isoform (49, 68). However, in a formal sense, the identity of the functionally relevant CBF/PEBP2 isoform that binds to δE3 in Jurkat cells has not been established. Importantly, our comparison of factors expressed in transfected COS cells with those naturally expressed in Jurkat cells argues strongly that the binding properties of the CBF/PEBP2 isoforms expressed in Jurkat cells are identical to the binding properties of the αA1 and β2 isoforms. Furthermore, our results argue that there are no additional proteins that are expressed in Jurkat cells but not in COS-7 cells that might coassemble with CBF/PEBP2 and c-Myb and modify their binding to an isolated δE3 binding site.

Our conclusion that CBF/PEBP2 and c-Myb bind to δE3 independently is based on several observations. First, we were unable to drive the vast majority of α+β complexes into the higher-order complex by inclusion of c-Myb or to drive the vast majority of Myb complexes into the higher-order complex by inclusion of CBF/PEBP2(α+β). Second, a cold competitor containing only Myb sites was equally efficient as a competitor of the Myb and α+β+Myb complexes, and an unlabelled competitor containing only a core site was equally efficient as a competitor of both the α+β and α+β+Myb complexes. Finally, the dissociation rates of the Myb, α+β, and α+β+Myb complexes were all found to be very similar. However, the amount of higher-order complex that could be recovered in our experiments was minimally greater (2.1-fold) than would be expected on the basis of theoretical calculations. Thus, although the cold competition experiments and kinetic dissociation experiments argue against cooperative binding between CBF/PEBP2 and c-Myb, we cannot exclude the possibility that a very low level of cooperativity indeed exists. Nevertheless, we note that minimally (2.5-fold) elevated levels of the higher-order complex are also detected by using functionally inactive δE3 binding sites with 5- and 10-bp insertions between the core and Myb sites, arguing that even if a low level of cooperativity exists, it cannot explain functional synergy between these factors.

Recently, Wotton et al. (70) demonstrated cooperative binding of recombinant Ets-1 and purified CBF/PEBP2 to the βE2 and βE3 elements of the TCR β enhancer. Cooperativity was primarily detected as a dramatic increase in the affinity of Ets-1 for its binding site in the presence of CBF/PEBP2. Interestingly, the cooperative binding of Ets-1 and CBF/PEBP2 was not dependent on the precise orientation and spacing of the cognate sites, suggesting substantial protein flexibility. This stands in striking contrast to our results, since we do not detect significant cooperativity in the binding of CBF/PEBP2 and c-Myb to δE3, even though a precise orientation of the binding sites is required for transcriptional activation. Thus, the mechanisms by which CBF/PEBP2 cooperates with Ets-1 to mediate TCR β enhancer activity and with c-Myb to mediate TCR δ enhancer activity may be very different.

The molecular mechanisms by which c-Myb transactivates gene expression are unclear. There are numerous examples of functional cooperation between c-Myb and other factors. For example, a fusion protein that contains both v-Myb and v-Ets sequences is required for the target cell specificity and transforming potential of the E26 virus (38, 39). Myb cooperates functionally with Ets-2 and C/EBP in transactivation of the *mim-1* promoter and with C/EBP in transactivation of the lysozyme promoter (7, 13, 44). BAS1, a Myb-related transcription factor in *Saccharomyces cerevisiae*, activates *HIS4* gene transcription only in combination with BAS2 (66). Transactivation by c-Myb without direct binding to DNA, presumably by interaction of c-Myb with other DNA-binding proteins, has also been reported elsewhere (24, 26, 27). However, in no case is there direct evidence that c-Myb binds cooperatively to DNA with another factor or otherwise directly contacts other transcriptional activators to mediate transactivation. Functional synergy between c-Myb and other activators may generally occur by a mechanism that does not involve cooperative binding to DNA.

An alternative model for synergy between adjacent bound factors that does not require cooperative binding has been proposed. In this model, bound activators may synergize not by directly interacting with each other but by simultaneously touching some part of the transcriptional machinery (8, 33). The two factors could be required both to contact a single transcription factor or coactivator or to contact two different components of the transcriptional machinery. In accordance with this, there is now direct evidence that different activators contact different TATA-binding-protein-associated factors (TAFs) in the TFIID complex (9). Moreover, it has been suggested that different TFIID complexes that differ in some TAFs may dictate requirements for specific activators (6, 21). With these results in mind, it is interesting to speculate about the molecular basis for CBF/PEBP2-c-Myb synergy in the ac-

tivation of TCR δ gene expression. We show in the present study that CBF/PEBP2 and c-Myb provide two distinct functions that are necessary for transactivation, since a pair of similarly situated Myb sites or core sites cannot substitute for the core-Myb combination. Previous experiments have shown that a δE3 monomer can activate the homologous $V_\delta 1$ promoter but cannot activate the heterologous c-*fos* promoter (19a, 57). Furthermore, although the $V_\delta 1$ promoter can be activated by a heterologous enhancer in COS cells, we have been unable to demonstrate activation of the $V_\delta 1$ promoter by δE3 in COS cells cotransfected with c-Myb, CBF/PEBP2α, and CBF/PEBP2β expression constructs (data not shown). Together, these data lead us to suggest that synergistic activation by c-Myb and CBF/PEBP2 in T cells involves simultaneous and specific contacts with one or more tissue-specific factors that are involved in $V_\delta 1$ promoter function, rather than contacts solely with the general transcriptional machinery.

It is interesting that core and Myb sites spaced and oriented precisely as those in δE3 are also found in the myeloperoxidase enhancer (64). However, functional collaboration between CBF/PEBP2 and c-Myb in the two systems seems to be very different. Myeloperoxidase enhancer activity depends primarily on the core site but not the Myb site, and CBF/PEBP2 seems to function independently of c-Myb in this system (47, 64). These differences may be due either to the specific requirements of the promoters tested in the two studies or to differences in the expression of CBF/PEBP2 isoforms or other factors in myeloid cells and T cells.

Although we have not detected significant cooperativity in the binding of CBF/PEBP2 and c-Myb to δE3, it remains possible that these factors bind cooperatively in conjunction with other factors as part of a larger complex that assembles on an intact TCR δ enhancer or with the homologous $V_\delta 1$ promoter. It will certainly be important to investigate this possibility in future experiments. However, even if such cooperativity could be demonstrated, our experiments argue that there is a distinct and independent basis for functional synergy between CBF/PEBP2 and c-Myb. Further work is clearly necessary to elucidate the molecular targets of c-Myb and CBF/PEBP2 proteins so that synergy between these proteins can be better understood.

ACKNOWLEDGMENTS

We thank Yoshiaki Ito for the CBF/PEBP2αA1 plasmid, Nancy Speck for the CBF/PEBP2β plasmid, Carlos Suñé for his help with the PhosphorImager and dissociation experiments, and Stefan Doerre for helpful advice about preparing COS-7 cellular extracts. We also thank Carlos Suñé, Pilar Lauzurica, and Michael Ostrowski for their comments on the manuscript.

This work was support by NIH grant RO1 GM41052. M.S.K. is the recipient of a Faculty Research Award from the American Cancer Society (FRA-414).

REFERENCES

1. **Andersson, S., D. L. Davis, H. Dahlback, H. Jornvall, and D. W. Russell.** 1989. Cloning, structure, and expression of the mitochondrial cytochrome P-450 stero 26-hydrolase, a bile acid biosynthetic enzyme. J. Biol. Chem. **264:**8222–8229.
2. **Badiani, P., P. Corbella, D. Kioussis, J. Marvel, and K. Weston.** 1994. Dominant interfering alleles define a role for c-Myb in T-cell development. Genes Dev. **8:**770–782.
3. **Bae, S.-C., E. Ogawa, M. Maruyama, H. Oka, M. Satake, K. Shigesada, N. A. Jenkins, D. J. Gilbert, N. G. Copeland, and Y. Ito.** 1994. PEBP2αB/mouse AML1 consist of multiple isoforms that possess differential transactivation potentials. Mol. Cell. Biol. **14:**3242–3252.
4. **Bae, S. C., Y. Yamaguchi-Iwai, E. Ogawa, M. Maruyama, M. Inuzuka, H. Kagoshima, K. Shigesada, M. Satake, and Y. Ito.** 1993. Isolation of PEBP2αB cDNA representing the mouse homolog of human acute myeloid leukemia gene, AML1. Oncogene **8:**809–814.
5. **Biedenkapp, H., U. Borgmeyer, A. E. Sippel, and K.-H. Klempnauer.** 1988. Viral myb oncogene encodes a sequence-specific DNA-binding activity. Nature (London) **335:**835–837.
6. **Brou, C., S. Chaudhary, I. Davidson, Y. Lutz, J. M. Egly, L. Tora, and P. Chambon.** 1994. Distinct TFIID complexes mediate the effect of different transcriptional activators. EMBO J. **12:**489–499.
7. **Burk, O., S. Mink, M. Ringwald, and K.-H. Klempnauer.** 1993. Synergistic activation of the chicken mim-1 gene by v-myb and C/EBP transcription factors. EMBO J. **12:**2027–2038.
8. **Carey, M., Y.-S. Lin, M. R. Green, and M. Ptashne.** 1990. A mechanism for synergistic activation of mammalian gene by GAL4 derivatives. Nature (London) **345:**361–364.
9. **Chen, J.-L., L. D. Attardi, C. P. Verrijzer, K. Yokomori, and R. Tjian.** 1994. Assembly of recombinant TFIID reveals differential coactivator requirements for distinct transcriptional activators. Cell **79:**93–105.
10. **Cullen, B. R.** 1987. Use of eukaryotic expression technology in the functional analysis of cloned genes. Methods Enzymol. **152:**684–704.
11. **Dorsett, D. L., I. Keshet, and E. Winocour.** 1983. Quantitation of a simian virus 40 nonhomologous recombination pathway. J. Virol. **48:**218–228.
12. **Dubendorff, J. W., L. J. Whittaker, J. T. Eltman, and J. S. Lipsick.** 1992. Carboxy-terminal elements of c-Myb negatively regulate transcriptional activation in cis and in trans. Genes Dev. **6:**2524–2535.
13. **Dudek, H., R. V. Tantravahi, V. N. Rao, E. S. P. Reddy, and E. P. Reddy.** 1992. Myb and Ets proteins cooperate in transcriptional activation of the mim-1 promoter. Proc. Natl. Acad. Sci. USA **89:**1291–1295.
14. **Erickson, P., J. Gao, K. S. Chang, T. Look, E. Whisenant, S. Raimondi, R. Lasher, J. Trujillo, J. Rowley, and H. Drabkin.** 1992. Identification of breakpoints in t(8;21) acute myelogenous leukemia and isolation of a fusion transcript, AML-1/ETO with similarity to Drosophila segmentation gene, runt. Blood **80:**1825–1831.
15. **Evans, J. L., T. L. Moore, W. M. Kuehl, T. Bender, and J. P.-Y. Ting.** 1990. Functional analysis of c-Myb protein in T-lymphocytic cell lines shows that it *trans*-activates the c-*myc* promoter. Mol. Cell. Biol. **10:**5747–5752.
16. **Felsenfeld, G.** 1992. Chromatin as an essential part of the transcriptional mechanism. Nature (London) **355:**219–224.
17. **Fowlkes, B. J., and D. M. Pardoll.** 1989. Molecular and cellular events of T cell development. Adv. Immunol. **44:**207–264.
18. **Hallberg, B., J. Schmidt, A. Luz, F. S. Pedersen, and T. Grundstrom.** 1991. SL3-3 enhancer factor 1 transcriptional activators are required for tumor formation by SL3-3 murine leukemia virus. J. Virol. **65:**4177–4181.
19. **Hernandez-Munain, C., and M. S. Krangel.** 1994. Regulation of the T-cell receptor δ enhancer by functional cooperation between c-Myb and core-binding factors. Mol. Cell. Biol. **14:**473–483.

19a. **Hernandez-Munain, C., and M. S. Krangel.** Unpublished data.

20. **Hsiang, Y. H., D. Spencer, S. Wang, N. A. Speck, and D. H. Raulet.** 1993. The role of viral enhancer "core" motif-related sequences in regulating T cell receptor-γ and -δ gene expression. J. Immunol. **150:**3905–3916.
21. **Jacq, X., C. Brou, Y. Lutz, I. Davidson, P. Chambon, and L. Tora.** 1994. Human TAF30 is present in a distinct TFIID complex and is required for transcriptional activation by the estrogen receptor. Cell **79:**107–117.
22. **Kamachi, Y., E. Ogawa, M. Asano, S. Ishida, Y. Murakami, M. Satake, Y. Ito, and K. Shigesada.** 1990. Purification of a mouse nuclear factor that binds to both the A and B cores of the polyomavirus enhancer. J. Virol. **64:**4808–4819.
23. **Kanei-Ishii, C., E. M. MacMillan, T. Nomura, A. Sarai, R. G. Ramsay, S. Aimoto, S. Ishii, and T. J. Gonda.** 1992. Transactivation and transformation by Myb are negatively regulated by a leucine-zipper structure. Proc. Natl. Acad. Sci. USA **89:**3088–3092.
24. **Kanei-Ishii, C., T. Yasukawa, R. I. Morimoto, and S. Ishii.** 1994. c-Myb-induced trans-activation mediated by heat shock elements without sequence-specific DNA binding of c-Myb. J. Biol. Chem. **269:**15768–15775.
25. **Kania, M. A., A. S. Bonner, J. B. Duffy, and J. P. Gergen.** 1990. The Drosophila segmentation gene runt encodes a novel nuclear regulatory protein that is also expressed in the developing nervous system. Genes Dev. **4:**1701–1713.
26. **Kenney, S. C., E. Holley-Guthrie, E. B. Quinlivan, D. Gutsch, Q. Zhang, T. Bender, J. F. Giot, and A. Sergeant.** 1992. The cellular oncogene c-myb can interact synergistically with the Epstein-Barr virus BZLF1 transactivator in lymphoid cells. Mol. Cell. Biol. **12:**136–146.
27. **Klempnauer, K.-H., H. Arnold, and H. Biedenkapp.** 1989. Activation of transcription by v-myb: evidence for two different mechanisms. Genes Dev. **3:**1582–1589.
28. **Klempnauer, K.-H., T. J. Gonda, and J. M. Bishop.** 1982. Nucleotide sequence of the retroviral leukemia gene v-myb and its cellular progenitor c-myb: the architecture of a transduced oncogene. Cell **31:**453–463.
29. **Klempnauer, K.-H., G. Ramsay, J. M. Bishop, M. G. Moscovici, C. Moscovici, J. P. McGrath, and A. D. Levinson.** 1983. The product of the retroviral transforming gene v-myb is a truncated version of the protein encoded by the cellular oncogene c-myb. Cell **33:**345–355.
30. **Lauzurica, P., and M. S. Krangel.** 1994. Temporal and lineage-specific control of T cell receptor α/δ gene rearrangement by T cell receptor α and δ enhancers. J. Exp. Med. **179:**1913–1921.

31. **Lauzurica, P., and M. S. Krangel.** 1994. Enhancer-dependent and -independent steps in the rearrangement of a human T cell receptor δ transgene. J. Exp. Med. **179:**43–55.
32. **Leiden, J. M., and C. B. Thompson.** 1994. Transcriptional regulation of T-cell genes during T-cell development. Curr. Opin. Immunol. **6:**231–237.
33. **Lin, Y.-S., M. Carey, M. Ptashne, and M. R. Green.** 1990. How different eukaryotic transcriptional activators can cooperate promiscuously. Nature (London) **345:**359–361.
34. **Luscher, B., and R. N. Eisenman.** 1990. New light on Myc and Myb. Part II. Myb. Genes Dev. **4:**2235–2241.
35. **Luthman, H., and G. Magnusson.** 1983. High efficiency polyoma DNA transfection of chloroquine treated cells. Nucleic Acids Res. **11:**1295–1308.
36. **Melnikova, I. N., B. E. Crute, S. Wang, and N. A. Speck.** 1993. Sequence specificity of the core-binding factor. J. Virol. **67:**2408–2411.
37. **Melotti, P., D.-H. Ku, and B Calabretta.** 1994. Regulation of the expression of the hematopoietic stem cell antigen CD34: role of c-myb. J. Exp. Med. **179:**1023–1028.
38. **Metz, T., and T. Graf.** 1991. v-myb and v-ets transform chicken erythroid cells and cooperate both in trans and in cis to induce distinct differentiation phenotypes. Genes Dev. **5:**369–380.
39. **Metz, T., and T. Graf.** 1991. Fusion of the nuclear oncoproteins v-Myb and v-Ets is required for the leukemogenicity of E26 virus. Cell **66:**95–105.
40. **Meyers, S., J. M. Downing, and S. W. Hiebert.** 1993. Identification of AML-1 and the (8;21) translocation protein (AML-1/ETO) as sequence-specific DNA-binding proteins: the runt homology domain is required for DNA binding and protein-protein interactions. Mol. Cell. Biol. **13:**6336–6345.
41. **Mitani, K., S. Ogawa, T. Tanaka, H. Miyoshi, M. Kurokawa, H. Mano, Y. Yazaki, M. Ohki, and H. Hirai.** 1994. Generation of the AML1-EVI-1 fusion gene in the t(3;21)(q26;q22) causes blastic crisis in chronic myelocytic leukemia. EMBO J. **13:**504–510.
42. **Miyoshi, H., K. Shimizu, T. Kozu, N. Maseki, Y. Kaneko, and M. Ohki.** 1991. t(8;21) breakpoints on chromosome 21 in acute myeloid leukemia are clustered within a limited region of a single gene, AML1. Proc. Natl. Acad. Sci. USA **88:**10431–10434.
43. **Mucenski, M. L., K. McLain, A. B. Kier, S. H. Swerdlow, C. M. Schreiner, T. A. Miller, D. W. Pietryga, W. J. Scott, and S. S. Potter.** 1991. A functional c-myb gene is required for normal murine fetal hepatic hematopoiesis. Cell **65:**677–689.
44. **Ness, S. A., E. Kowenz-Leutz, T. Casini, T. Graf, and A. Leutz.** 1993. Myb and NF-M: combinatorial activators of myeloid genes in heterologous cell types. Genes Dev. **7:**749–759.
45. **Ness, S. A., A. Marknell, and T. Graf.** 1989. The v-myb oncogene product binds to and activates the promyelocyte-specific mim-1 gene. Cell **59:**1115–1125.
46. **Nomura, N., M. Takahashi, M. Matsui, S. Ishii, T. Date, S. Sasamoto, and R. Ishizaki.** 1988. Isolation of human cDNA clones of myb-related genes, A-myb and B-myb. Nucleic Acids Res. **16:**11075–11089.
47. **Nuchprayoon, I., S. Meyers, L. M. Scott, J. Suzow, S. Hiebert, and A. D. Friedman.** 1994. PEBP2/CBF, the murine homolog of the human myeloid AML1 and PEBP2β/CBFβ proto-oncoproteins, regulates the murine myeloperoxidase and neutrophil elastase genes in immature myeloid cells. Mol. Cell. Biol. **14:**5558–5568.
48. **Nucifora, G., C. R. Begy, P. Erickson, H. A. Drabkin, and J. D. Rowley.** 1993. The 3;21 translocation in myelodysplasia results in fusion transcript between the AML-1 gene and the gene for EAP, a highly conserved protein associated with the Epstein-Barr virus small RNA EBER 1. Proc. Natl. Acad. Sci. USA **90:**7784–7788.
49. **Ogawa, E., M. Inuzuka, M. Maruyama, M. Satake, M. Naito-Fujimoto, Y. Ito, and K. Shigesada.** 1993. Molecular cloning and characterization of PEBP2β, the heterodimeric partner of a novel Drosophila runt-related DNA binding protein PEBP2α. Virology **194:**314–331.
50. **Ogawa, E., M. Maruyama, H. Kagoshima, I. Inuzuka, J. Lu, M. Satake, K. Shigesada, and Y. Ito.** 1993. PEBP2/PEA2 represents a new family of transcription factor homologous to the products of the Drosophila runt and the human AML1. Proc. Natl. Acad. Sci. USA **90:**6859–6863.
51. **Prosser, H. M., D. Wotton, A. Gegonne, J. Ghysdael, S. Wang, N. A. Speck, and M. J. Owen.** 1992. A phorbol ester response element within the human T-cell receptor β-chain enhancer. Proc. Natl. Acad. Sci. USA **89:**9934–9938.
52. **Ptashne, M.** 1988. How eukaryotic transcriptional activators work. Nature (London) **335:**683–689.
53. **Raulet, D. H., D. M. Spencer, Y.-H. Hsiang, J. P. Goldman, M. Bix, N. S. Liao, M. Zijlstra, R. Jaenisch, and I. Correa.** 1991. Control of γδ T-cell development. Immunol. Rev. **120:**185–204.
54. **Reddi, M. A., B.-S. Yang, X. Yue, C. J. K. Barnett, I. L. Ross, M. J. Sweet, D. A. Hume, and M. C. Ostrowski.** 1994. Opposing actions of c-ets/PU.1 and c-myb protooncogene products in regulating the macrophage-specific promoters of the human and mouse colony-stimulating factor-1 receptor (c-fms) genes. J. Exp. Med. **180:**2309–2319.
55. **Redondo, J. M., S. Hata, C. Brocklehurst, and M. S. Krangel.** 1990. A T cell specific transcriptional enhancer within the human T cell receptor δ locus. Science **247:**1225–1229.
56. **Redondo, J. M., J. L. Pfohl, C. Hernandez-Munain, S. Wang, N. A. Speck, and M. S. Krangel.** 1992. Indistinguishable nuclear factor binding to functional core sites of the T-cell receptor δ and murine leukemia virus enhancers. Mol. Cell. Biol. **12:**4817–4823.
57. **Redondo, J. M., J. L. Pfohl, and M. S. Krangel.** 1991. Identification of an essential site for transcriptional activation within the human T cell receptor δ enhancer. Mol. Cell. Biol. **11:**5671–5680.
58. **Rosson, D., and E. P. Reddi.** 1986. Nucleotide sequence of chicken c-myb complementary DNA and implications for myb oncogene activation. Nature (London) **319:**604–606.
59. **Sakura, H., C. Kanei-Ishii, T. Nagase, H. Nakagoshi, T. J. Gonda, and S. Ishii.** 1989. Delineation of three functional domains of the transcriptional activator encoded by the c-myb protooncogene. Proc. Natl. Acad. Sci. USA **86:**5758–5762.
60. **Shen-Ong, G. L. C.** 1990. The myb oncogene. Biochim. Biophys. Acta **1032:** 39–52.
61. **Siu, G., A. L. Wurster, J. S. Lipsick, and S. M. Hedrick.** 1992. Expression of the CD4 gene requires a Myb transcription factor. Mol. Cell. Biol. **12:**1592–1604.
62. **Speck, N. A., B. Renjifo, E. Golemis, T. N. Fredrickson, J. W. Hartley, and N. Hopkins.** 1990. Mutation of the core or adjacent LVb elements of the Moloney murine leukemia virus enhancer alters disease specificity. Genes Dev. **4:**233–242.
63. **Strominger, J. L.** 1989. Developmental biology of T cell receptors. Science **244:**943–950.
64. **Suzow, J., and A. D. Friedman.** 1993. The murine myeloperoxidase promoter contains several functional elements, one of which binds a cell type-restricted transcription factor, myeloid nuclear factor 1 (MyNF1). Mol. Cell. Biol. **13:**2141–2151.
65. **Tanese, N., and R. Tjian.** 1994. Coactivators and TAFs: a new class of eukaryotic transcription factors that connect activators to the basal machinery. Cold Spring Harbor Symp. Quant. Biol. **58:**179–185.
66. **Tice-Baldwin, K., G. R. Fink, and K. T. Arndt.** 1989. BAS1 has a myb motif and activates HIS4 transcription only in combination with BAS2. Science **246:**931–935.
67. **Wang, S., and N. A. Speck.** 1992. Purification of core-binding factor, a protein that binds the conserved core site in murine leukemia virus enhancers. Mol. Cell. Biol. **12:**89–102.
68. **Wang, S., Q. Wang, B. E. Crute, I. N. Melnikova, S. R. Keller, and N. A. Speck.** 1993. Cloning and characterization of subunits of the T-cell receptor and murine leukemia virus enhancer core-binding factor. Mol. Cell. Biol. **13:**3324–3339.
69. **Weston, K., and J. M. Bishop.** 1989. Transcriptional activation by the v-myb oncogene and its cellular progenitor, c-myb. Cell **58:**85–93.
70. **Wotton, D., J. Ghysdael, S. Wang, N. A. Speck, and M. J. Owen.** 1994. Cooperative binding of Ets-1 and core binding factor to DNA. Mol. Cell. Biol. **14:**840–850.