Physical Interaction of the Activator Protein-1 Factors c-Fos and c-Jun with Cbfa1 for Collagenase-3 Promoter Activation*

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Previously, we determined that the activator protein-1 (AP-1) binding site and the runt domain (RD)-binding site and their binding proteins, c-Fos-c-Jun and Cbfa, regulate the collagenase-3 promoter in parathyroid hormone-treated and differentiating osteoblasts. Here we show that Cbfa1 and c-Fos-c-Jun appear to cooperatively bind the RD- and AP-1-binding sites and form ternary structures in vitro. Both in vitro and in vivo co-immunoprecipitation and yeast two-hybrid studies further demonstrate interaction between Cbfa1 with c-Fos and c-Jun in the absence of phosphorylation and without binding to DNA. Additionally, only the runt domain of Cbfa1 was required for interaction with c-Jun and c-Fos. In mammalian cells, overexpression of Cbfa1 enhanced c-Jun activation of AP-1-binding site promoter activity, demonstrating functional interaction. Finally, insertion of base pairs that disrupted the helical phasing between the AP-1- and RD-binding sites also inhibited collagenase-3 promoter activation. Thus, we provide direct evidence that Cbfa1 and c-Fos-c-Jun physically interact and cooperatively bind the AP-1- and RD-binding sites in the collagenase-3 promoter. Moreover, the AP-1- and RD-binding sites appear to be organized in a specific required helical arrangement that facilitates transcription factor interaction and enables promoter activation.

Collagenase-3 is a member of the matrix metalloproteinase family of matrix-degrading enzymes and is synthesized by osteoblasts in response to a variety of bone resorption agents including parathyroid hormone (PTH)1 (1, 2). Matrix degradation by collagenase-3 has been implicated in several bone and cartilage remodeling events including bone remodeling, endochondral bone formation, bone repair, and PTH-induced bone resorption (3–7). Recently, it has been shown that homozygous mutation of the collagenase cleavage site in the Col1a1 gene of mutant mice results in diminished PTH-induced bone resorption, diminished PTH-induced calcemic responses, and thicker bones (8). Collagenase-3, like most other matrix metalloproteinases, is substantially regulated at the level of transcription (1, 9). Earlier, we and others (10–12) had shown that the AP-1- and RD-binding sites located within 148 base pairs upstream of the rat collagenase-3 transcriptional start site are required for basal expression and PTH activation of the collagenase-3 gene and appear to act in a cooperative fashion. We also demonstrated increased binding of c-Fos and Cbfa proteins to the AP-1-binding site in response to PTH with no change in the abundance of Cbfa (core-binding factor alpha)-related proteins binding to the RD-binding site. More recently, we have shown that PTH induces protein kinase A-dependent phosphorylation and transactivation of Cbfa1 and leads to stimulation of collagenase-3 promoter activity in osteoblastic osteosarcoma (UMR 106-01) cells (13).

Classically, AP-1 dimers bind to AP-1-binding sites and consist of two subunits formed either by heterodimerization of a fos family protein (c-Fos, Fos B, Fra-1, Fra-2) with a Jun family protein (c-Jun, JunB, and JunD) or homodimerization of the Jun family members (14, 15). Similarly, the RD-binding site is bound by members of the Cbfa family, which is comprised of a variety of proteins including the Drosophila Run and Lozenge proteins, and the human and murine core-binding factor α proteins (Cbfa1, Cbfa2, and Cbfa3, now renamed Runx-2, Runx-1, and Runx-3) (16–21). Cbfa proteins contain a DNA-binding domain called the runt domain and are capable of binding to DNA as monomers, but they can also heterodimerize with Myc-related nuclear localization signal, and a glutamine-alanine-rich domain unique to Cbfa1 (28). Although much is known about the functional implications of Cbfa1 expression, little is known about its mechanism of action as a transcriptional initiator in osteoblasts.

The close proximity of the AP-1- and RD-binding sites and their cooperative involvement in the activation of the collagenase-3 promoter by PTH suggest that proteins binding to these sites may also physically interact. Protein–protein interactions between these transcription factors could enable the generation of higher order nucleoprotein complexes that enhance transcription. Numerous studies demonstrate the ability of nuclear proteins to bind each other and to mediate cooperative

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§ The abbreviations used are: PTH, parathyroid hormone; AP-1, activator protein-1; RD, runt domain; AD, activation domain; BD, binding domain.
DNA binding and promoter activation when their respective binding sites are juxtaposed (29–31). Recent work by another group in fact does demonstrate interaction between c-Fos-c-Jun and Cbfα family members (32). Here we provide additional evidence of cooperative DNA binding and direct protein-protein interaction between c-Fos-c-Jun and Cbfα1 both in vitro and in vivo. Furthermore, we demonstrate that insertion of a specific number of nucleotides, which leads to a disruption of helical phasing between the AP-1- and RD-binding sites, also reduces the activation of the collagenase-3 promoter.

EXPERIMENTAL PROCEDURES

Materials—ECL reagents and radionucleotides were obtained from Amersham Biosciences, Inc. Polyclinidine diphosphode membrane (Immuno-Blot) was purchased from Bio-Rad. Tissue culture media and reagents were purchased from Life Technologies, Inc. Horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse antibodies and c-Jun and c-Fos polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-c-Myc antibody was obtained from CLONTECH (Palo Alto, CA), and monoclonal anti-c-Fos and anti-c-Jun antibodies were purchased from Oncogene (Cambridge, MA) and BD Pharmingen (San Diego, CA) respectively. Rabbit IgG was purchased from Sigma, and protein A/G-agarose was obtained from Calbiochem (La Jolla, CA).

Plasmids—The –148 promoter construct was previously generated by subcloning the 148 base pairs upstream of the rat collagenase-3 transcription start site into the pSV0CAT chloramphenicol acetyltransferase reporter plasmid (Promega, Madison, WI) (11). The –148 + 3 and the –148 + 10 plasmids were generated by modifying the –148 reporter construct using the ExSite PCR-based site-directed mutagenesis kit (Stratagene). Three nucleotides (CTT) (–148 + 3) or 10 nucleotides (CTAGCTAGCT) (–148 + 10) were inserted at position –91 upstream of the transcriptional start site between the AP-1- and RD-binding sites. Human cDNA clones for Cbfα2 and Cbfβ were kindly provided by Dr. Scott Hieter. The cDNAs for rat c-fos and c-jun in pGEM-4 vectors were kindly provided by Dr. Tom Curran (33, 34). The pCMV-c-Jun and pCMV-c-Fos expression vectors were generated by subcloning c-jun and c-fos cDNAs into a vector driven by a cytomegalovirus promoter as previously described (9). Cbfα1 was also ligated into the pCMV-Myc vector (CLONTECH) in frame with the Myc epitope to yield the pCMV-Cbfα1-Myc vector. The cDNAs coding for Type II Cbfα1 (beginning with the codon for methionine 69), the runt domain of Cbfα1 (amino acids 108–203 of mouse Type II Cbfα1), c-Fos, and c-jun were amplified from the above plasmids using PCR or were digested by restriction enzymes and ligated into the pAS2-1 and pACT-2 yeast two-hybrid vectors (CLONTECH). The resulting plasmids contained coding sequences for c-Jun, c-Fos, and Cbfα1 or the runt domain of Cbfα1 fused to either the GAL4 binding domain in the pAS2-1 vector or to the GAL4 activation domain in the pACT-2 vector. Additionally, using the same pAS2-1 site in the rat c-jun sequence and in the pACT-2 vector, the 5′ end of c-jun was deleted, yielding a construct coding for the leucine zipper region and carboxyl terminus of c-Jun but no activation domain (junZIP) (amino acids 176–334 of rat c-Jun) (35). Likewise, the 5′ region of c-jun, which codes for the amino region and activation domain but lacks the leucine zipper region and carboxyl terminus, was amplified by PCR (amino acids 1–253 of rat c-jun) and was ligated into the pAS2-1 vector (junACT). The pAP1-Luc and pRL-TK plasmids were obtained from CLONTECH.

Ccfa1 and c-Fos-Jun Cooperatively Bind a Collagenase-3 Promoter Fragment—Previously, we and others identified two yeast two-hybrid vectors using a small scale LiAe yeast transformation procedure (CLONTECH) and plated on tryptophan/leucine drop-out plates. Transformed clones were picked out of both a GAL4 activation domain (AD) plasmid and a GAL4 binding domain (BD) plasmid. Five separate transformant colonies were taken for each sample and assayed for β-galactosidase activity in duplicate using o-nitrophenyl β-D-galactopyranoside. β-Galactosidase activity was quantified by spectrophotometric readings and converted to β-galactosidase units by normalization to cell number.

RESULTS

Ccfa1 and c-Fos-Jun Cooperatively Bind a Collagenase-3 Promoter Fragment—Previously, we and others identified two

Physical Interaction of the AP-1 Factors and Ccfa1

The Y187 Saccharomyces cerevisiae host yeast strain was transformed with various combinations of the yeast two-hybrid vectors using a small scale LiAe yeast transformation procedure (CLONTECH) and plated on tryptophan/leucine drop-out plates. Transformed clones were picked out of both a GAL4 activation domain (AD) plasmid and a GAL4 binding domain (BD) plasmid. Five separate transformant colonies were taken for each sample and assayed for β-galactosidase activity in duplicate using o-nitrophenyl β-D-galactopyranoside. β-Galactosidase activity was quantified by spectrophotometric readings and converted to β-galactosidase units by normalization to cell number.

Protein-Protein Interactions in vitro—Plasmids containing the coding regions of Ccfa1, Ccfa2, Ccfaβ, c-Fos, c-Jun, the RD of Ccfa1, and lucerase were transcribed by SP6 or T7 RNA polymerase and translated in rabbit reticulocyte lysates using 50 μCi of [35S]methionine in vitro. Equal volumes of cold and labeled translation products were mixed and incubated at 30 °C for 30 min and then cleared. The cleared supernatants were incubated with 3 μl of either anti-c-Fos or anti-c-Jun antisera and 100 μl of Nonidet P-40 buffer. The immunocomplexes were then incubated for 2 h at 4 °C with 25 μl of Staphylococcus aureus cells, precipitated, and washed three times with Nonidet P-40 buffer. The samples were resuspended in SDS sample buffer, boiled for 5 min, and resolved by 12% SDS-PAGE.

Transfection Experiments—For the helical phasing experiments, the wild type –148, the –148 + 3, and the –148 + 10 plasmids were transiently transfected into UMR 106-01 cells using LipofectAMINE transfection reagent and treated with 10 μM PTH for 24 h where indicated. The cells were then lysed, analyzed for CAT activity, and normalized to protein levels. For the AP-1 binding site lucerase reporter study, combinations of pCMV expression vectors (10 ng total in each case) were co-transfected with 100 ng of the pAP1-Luc firefly lucerase reporter plasmid and 1 ng of the control, Renilla lucerase plasmid (pRL-TK) using Superfect transfection reagent (Qiagen). The cells were serum-starved for 24 h to minimize endogenous promoter activation and lysed, and firefly lucerase activity was assayed using a luminometer and a Promega lucerase assay system and normalized to Renilla lucerase activity. For in vivo co-immunoprecipitation studies, 100-nm dishes with 70% confluent COS-7 cells were transfected with 4 μg each of c-Fos, c-Jun, and Ccfa1-Myc or just c-Fos and c-jun expression vectors using Superfect transfection reagent. The cells were grown for 2 days and lysed.

Yeast Two-hybrid Experiments—The Y187 Saccharomyces cerevisiae host yeast strain was transformed with various combinations of the yeast two-hybrid vectors using a small scale LiAe yeast transformation procedure (CLONTECH) and plated on tryptophan/leucine drop-out plates. Transformed clones were picked out of both a GAL4 activation domain (AD) plasmid and a GAL4 binding domain (BD) plasmid. Five separate transformant colonies were taken for each sample and assayed for β-galactosidase activity in duplicate using o-nitrophenyl β-D-galactopyranoside. β-Galactosidase activity was quantified by spectrophotometric readings and converted to β-galactosidase units by normalization to cell number.
elements in the collagenase-3 promoter, the AP-1-binding site, and the RD-binding site that are required for PTH-stimulated activity in the rat osteoblastic osteosarcoma cell line, UMR 106-01 (11, 12). Further, we demonstrated an increase in DNA binding of the AP-1 subunits c-Fos and c-Jun to the AP-1-binding site in response to PTH treatment (11). To determine whether c-Fos-c-Jun and Cbfa1 can bind to the same rat collagenase-3 promoter molecule (containing the AP-1-binding site and RD-binding sites), the hexahistidine-tagged fusion proteins of c-Fos and c-Jun and the glutathione S-transferase-tagged fusion protein of Cbfa1 were purified. Either c-Fos-c-Jun, Cbfa1, or combinations of Cbfa1 and c-Fos-c-Jun were incubated with an end-filled labeled rat collagenase-3 promoter probe and analyzed by electrophoretic mobility shift assays. Incubation of labeled probe with c-Fos-c-Jun alone produced two shifted complexes, identified as c-Fos-c-Jun heterodimer and c-Jun-c-Jun homodimer by preincubating with specific antibodies (Fig. 1A). Antibodies to c-Fos, c-Jun, or equal amounts of IgG were incubated with their respective proteins before adding probe. Competition was further performed with 50-fold molar excess of unlabeled rat collagenase-3 promoter probe. Incubation of labeled probe with Cbfa1 produced another shifted complex. This complex was characterized as Cbfa1 bound to the promoter fragment by incubating protein samples with anti-Cbfa1 antibody, equal amounts of IgG, or 50-fold excess of IgG were incubated with their respective proteins before adding probe. Competition was further performed with 50-fold molar excess of unlabeled rat collagenase-3 promoter probe prior to adding labeled probe (Fig. 1B). To determine whether c-Fos-c-Jun and Cbfa1 preferentially bind to the same collagenase-3 promoter molecule, binding reactions were performed with a fixed amount of Cbfa1 and with increasing amounts of c-Fos-c-Jun (Fig. 1C). The addition of c-Fos and c-Jun to the binding reaction resulted in the formation of a ternary complex that demonstrated slower mobility than the shifts generated with either c-Fos-c-Jun or Cbfa1 alone. The specificity of proteins in the ternary complex was assessed by cold competition using either AP-1-binding site or RD-binding site oligonucleotides. A 500-fold excess of unlabeled rat collagenase-3 AP-1-binding site probe competed for the observed c-Fos-c-Jun binding to labeled collagenase-3 probe and resulted in the observation of the Cbfa1-DNA shift. Likewise, a 500-fold excess of unlabeled rat collagenase-3 RD-binding site probe inhibited the formation of the observed Cbfa1-DNA shift. Moreover, the cold RD-binding site probe partially prevented the formation of the ternary complex and resulted in formation of c-Fos-c-Jun-DNA and c-Jun-c-Jun-DNA complexes that exhibited faster mobility than the c-Fos-c-Jun-Cbfa1-DNA ternary complex. When increasing amounts of c-Fos-c-Jun, in the absence of Cbfa1, were incubated with labeled probe, increased intensity of the specific c-Fos-c-Jun complexes was observed, but no ternary complex formation was noted (Fig. 1D). This result signifies that higher levels of c-Fos-c-Jun alone were not responsible for the formation of the slower moving shift. Similar slow mobility, ternary complex formation was also noted by adding increasing Cbfa1 to fixed amounts of c-Fos-c-Jun (data not shown). The specificity of proteins in the ternary complex was assessed by cold competition with either AP-1-binding site or the RD-binding site oligonucleotides (Fig. 1E). A 500-fold excess of unlabeled rat collagenase-3 AP-1-binding site probe competed for the observed c-Fos-c-Jun binding to labeled collagenase-3 probe, whereas a 500-fold excess of unlabeled rat collagenase-3 RD-binding site probe competed for the observed Cbfa1 binding to labeled collagenase-3 probe. As before with c-Fos-c-Jun, incubation of increasing amounts of Cbfa1 with labeled probe increased only the intensity of the Cbfa1-DNA complex and, alone, did not result in the formation of the slow moving ternary complex shift (data not shown).

**Fig. 1.** Cooperative binding of Cbfa1 and c-Fos-c-Jun. A, purified c-Fos and c-Jun proteins were incubated with an end-labeled rat collagenase-3 promoter probe containing both the AP-1- and RD-binding sites for 20 min. The specific protein in the shifted complex was identified by the inclusion of a specific antibody. An antibody to c-Fos, c-Jun, or the same concentration of IgG was included in the incubation before adding labeled probe. Competition was performed with 50-fold molar excess of unlabeled rat collagenase-3 promoter probe. The protein-DNA complexes were resolved by native polyacrylamide gel (4%) electrophoresis. B, gel shift assay was carried out using purified Cbfa1 as described above. An antibody to Cbfa1 or the same concentration of IgG was included in the incubation before adding labeled probe. Competition was performed with 50-fold molar excess of unlabeled rat collagenase-3 promoter probe. C, labeled rat collagenase-3 promoter probe was incubated in the presence of c-Fos-c-Jun (lane 2), Cbfa1 (lane 3), or c-Fos-c-Jun plus Cbfa1 (lanes 4–10). In lanes 4–8, increasing amounts of c-Fos-c-Jun were used. In lanes 9 and 10, 500-fold molar excess of either cold RD-binding site or cold AP-1-binding site oligonucleotide was included. Competitors were incubated with the protein for 5 min prior to the addition of labeled probe. In lane 1, no protein was added. The specific shifted complexes are indicated. D, labeled rat collagenase-3 promoter probe was incubated with increasing amounts of c-Fos-c-Jun, and specific shifted complexes c-Fos-c-Jun and c-Jun-c-Jun are indicated. E, labeled rat collagenase-3 promoter probe was incubated with a constant amount of c-Fos-c-Jun. At a high concentration of Cbfa1 in the binding reaction, there was formation of a ternary retarded complex (c-Fos-c-Jun-Cbfa1), as indicated. Competition was performed with either cold RD-binding site or cold AP-1-binding site oligonucleotide at 500-fold molar excess concentrations.

**Cbfa1 and c-Fos-c-Jun Co-immunoprecipitation—**We next investigated the physical interaction between c-Fos, c-Jun, and Cbfa1 both *in vitro* and *in vivo*. When labeled Cbfa1 was incubated with cold c-Fos alone or cold c-Jun and c-Fos together and
co-immunoprecipitated with c-Fos antibody, labeled Cbfa1 was detected (Fig. 2A). Similarly, when labeled Cbfa1 was incubated with cold c-Jun alone or cold c-Jun and Cbfa1 together and co-immunoprecipitated with c-Jun antibody, labeled Cbfa1 was found. Two bands were detected for Cbfa1 that presumably correspond to two isoforms of Cbfa1, one beginning with methionine 1 (Type III Cbfa1) and one beginning at methionine 69 (Type II Cbfa1) (27). Similar results were observed with the runt domain of Cbfa1 (RD) (Fig. 2B). Also, Cbfa2 interacted with c-Fos (Fig. 2C) and c-Jun (Fig. 2D), and these interactions were increased with the addition of Cbfa1 (Fig. 2E). Cbfb-β alone did not interact with the AP-1 factors (Fig. 2E).

To verify the in vitro protein-protein interactions in vivo, lysates from COS-7 cells overexpressing either c-Fos, c-Jun, and c-Myc-tagged Cbfa1 or c-Fos and c-Jun alone were immunoprecipitated using anti-c-Fos, c-Jun, or normal rabbit IgG. As shown in Fig. 3A, Cbfa1 co-immunoprecipitated with both c-Fos and c-Jun, as detected with anti-c-Myc antibody. Some background was detected in samples mixed with IgG alone, but this was always less than that seen with the anti-c-Fos or c-Jun antibodies. The band was specific for Cbfa1-Myc, because it was not detected in the absence of Cbfa1-Myc transfection. Fig. 3B illustrates the presence of c-Fos and c-Jun in both types of cell lysates and the presence of Cbfa1-Myc in the lysate from c-Fos-c-Jun-Cbfa1-Myc-transfected cells, but not the lysate from cells transfected with only c-Fos-c-Jun. We attempted to co-immunoprecipitate endogenous Cbfa1 with c-Jun and c-Fos, in the osteoblastic osteosarcoma cell line, UMR 106-01, which contains endogenous Cbfa1, c-Fos, and c-Jun, but were unsuccessful in achieving this, even with PTH treatment (data not shown).

Yeast Two-Hybrid Interactions—The yeast two-hybrid system was utilized to verify the in vitro and in vivo co-immunoprecipitation results and to more easily assess the regions involved in the interactions of the proteins. Equal amounts of DNA were used in each sample, and for each reaction that involved only one fusion construct, equal amounts of corresponding empty pACT-2 or pAS2-1, coding for the GAL4 activation domain or the GAL4 binding domain, respectively, were included. When Cbfa1 or the RD of Cbfa1 was combined with c-Jun, 3- and 2-fold increases, respectively, in GAL4 promoter activity were observed compared with c-Jun alone, indicating protein-protein interaction (Fig. 4A). Further, this interaction was similar to the interaction found between c-Jun and c-Fos, two transcription factors whose dimerization has been well characterized. All interactions were statistically significant (p < 0.001 for Cbfa1, p = 0.03 for the runt domain, and p < 0.003 for c-Fos) compared with c-Jun alone as determined by Student’s t test. As shown in Fig. 4B, a significant (p = 0.003) increase in GAL4 activity was also noted for interaction of the runt domain of Cbfa1 with c-Fos compared with c-Fos alone. Additional deletions of c-Jun (Fig. 4E) were performed to further identify the areas of interaction with Cbfa1. A significant increase in GAL4 promoter activity was noted with the combination of the junACT deletion with RUNX2 (p = 0.03) com-
transformant colonies were taken for each sample and assayed for
and with the RD/BD construct in the
JunZIP/BD was transfected with the AD expression vector in the
fifth bar
and with the Cbfa1/BD construct in the
plasmid was transformed with the AD expression vector in the
fourth bar
was transformed with the BD expression vector. The junACT/BD fusion
vector in the
sents the transformation of the c-Fos/AD fusion plasmid with the
BD expression vector. The
RD, c-Fos, or Cbfa1 proteins fused to the AD were transformed with the
leucine zipper regions but not the activation domain, were
containing the leucine zipper region but not the activation domain, were
fused to the GAL4 binding domain in the pAS2–1 vector. The cDNAs for
Cbfa1, the RD of Cbfa1, and c-Fos were fused in frame with the GAL4
AD in the pACT-2 vector. Yeast strain Y187 was transformed with combinations of the fusion plasmids and plated on tryptophan/leucine
drop-out medium. A, in the first three bars, the expression plasmids for
RD, c-Fos, or Cbfa1 proteins fused to the AD were transformed with the
BD expression vector. The fourth to seventh bars represent the transforma-
tion of the c-Jun/BD fusion plasmid with the AD expression vector or
the Cbfa1, RD, or c-Fos AD fusion expression plasmids, respectively. B, in the first bar, the RD/AD fusion plasmid was transformed with the
BD expression vector. The c-Fos/BD fusion plasmid was transformed with the
AD expression vector in the second bar. The third bar represents the transformation of the c-Fos/AD fusion plasmid with the
AD expression vector and with the Cbfa1/AD fusion plasmid, respectively. In the first bar, the RD/AD fusion plasmid was transformed with the
BD expression vector. The junACT/BD fusion plasmid was transformed with the AD expression vector in the second bar and with the Cbfa1/BD construct in the third bar. JunZIP/BD was transfected with the AD expression vector in the fourth bar and with the Cbfa1/BD construct in the fifth bar. D, in the first bar, the RD/AD fusion plasmid was transformed with the BD expression vector. The junACT/BD fusion plasmid was transformed with the AD expression vector in the second bar and with the Cbfa1/BD construct in the third bar. JunZIP/BD was transfected with the AD expression vector in the fourth bar and with the RD/BD construct in the fifth bar. Five separate transformant colonies were taken for each sample and assayed for
β-galactosidase by spectrophotometry. The data represent the means ± S.E. of five individual yeast clones. An asterisk in A–C indicates statistical signifi-
cance (p < 0.05). D, diagram of the two c-Jun deletions, junZIP (amino acids 176–334), and junACT (amino acids 1–253), and the full-length rat c-Jun (334 amino acids). One transactivation domain (TA) is indicated for c-Jun, which is also found in junACT. The ++++ and LLLL regions represent the basic DNA-binding region and the leucine zippers found in both c-Jun and junZIP. The proline/glutamic acid-rich (PG) region and proline/glutamic acid-rich (PG) region in c-Jun and the respective deletions are also indicated.

pared with junACT alone (Fig. 4C), but the increase observed was not significantly greater than the addition of the effects observed with junACT and RUNX2 alone. An increase in GAL4

promoter activity was also noted with the combination of the
junACT deletion with RD, but it was not statistically signifi-
cant (Fig. 4D). Nevertheless, no interaction was found for the
junZIP construct, which contained the DNA-binding and
leucine zipper domains, with either Cbfa1 or RD.

Functional Interaction of Cbfa1 with c-Jun—To study the
functional interaction of Cbfa1 with c-Jun, a construct contain-
ing six AP-1-binding sites upstream of a luciferase reporter
gene (pAP-1 Luc) was co-transfected with c-Jun and Cbfa1 in
COS-7 cells. Equal amounts of DNA were included in each
sample reaction. Unlike c-Fos, c-Jun can form homodimers and
bind to the AP-1-binding site, leading to transcriptional activa-
tion and synthesis of the luciferase reporter protein. As shown in Fig. 5, transfection with low amounts (5 ng) of c-Jun
or Cbfa1 led to only slight, insignificant increases in reporter
activity above those elicited with transfection with empty
pCMV vector. When c-Jun and Cbfa1 were co-transfected, a
significant increase in promoter activity was observed com-
pared with transfection with c-Jun, Cbfa1, or pCMV alone (p
= 0.002, p = 0.001, and p < 0.001, respectively). This pattern was
observed only when small (<100 ng) amounts of protein expres-
sion vectors were co-transfected with 100 ng of reporter
construct. When the cells were transfected with larger amounts of
c-Jun expression vector, the AP-1-binding site promoter was
highly activated, but co-transfection with Cbfa1 inhibited this
increase (data not shown).

Helical Phasing between the AP-1 and RD Sites—The fact
that the RD-binding site and AP-1-binding sites are nonadja-
cent (the RD-binding site is −132 to −126, and the AP-1-
binding site is −48 to −42) in the rat collagenase-3 promoter
and are likely located in a single nucleosome particle (p
= 0.002) led us to hypothesize that the helical phasing between these
two sites is important for the assembly of a stable nucleopro-
tein complex. As shown in Fig. 6, the insertion of 3 nucleotides,
which disrupts helical phasing between the AP-1- and
RD-binding sites, decreased both the basal and PTH-induced
collagenase-3 promoter activity. In contrast, the insertion of 10
nucleotides, which maintained the helical phasing relationship,
did not alter the activity of the promoter.

FIG. 4. Yeast two-hybrid interactions of c-Jun or c-Fos with
Cbfa1 or the runt domain of Cbfa1. The cDNAs coding for c-Fos,
full-length c-Jun, JunACT, a c-Jun deletion containing the activation
domain, but not the leucine zipper region, and JunZIP, a c-Jun deletion
containing the leucine zipper region but not the activation domain, were
fused to the GAL4 binding domain in the pAS2–1 vector. The cDNAs for
Cbfa1, the RD of Cbfa1, and c-Fos were fused in frame with the GAL4
AD in the pACT-2 vector. Yeast strain Y187 was transformed with combinations of the fusion plasmids and plated on tryptophan/leucine
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BD expression vector. The junACT/BD fusion plasmid was transformed with the AD expression vector in the second bar and with the Cbfa1/BD construct in the third bar. JunZIP/BD was transfected with the AD expression vector in the fourth bar and with the Cbfa1/BD construct in the fifth bar. Five separate transformant colonies were taken for each sample and assayed for
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FIG. 5. Interaction of Cbfa1 with c-Jun on a repeated AP-1-
binding site reporter construct. A construct containing six AP-1-
binding sites upstream of a luciferase reporter gene (pAP-1 Luc) was transiently transfected (100 ng) along with 1 ng of Renilla luciferase
expression plasmid (PRL-TK) and 10 ng of pCMV, 5 ng of pCMV-Cbfa1
with 5 ng pCMV, 5 ng pCMV-c-Jun vector with 5 ng pCMV, or 5 ng
pCMV-c-Jun with 5 ng pCMV-Cbfa1 as indicated into COS-7 cells. The
cells were serum-starved for 24 h to minimize endogenous promoter
activation, assayed for firefly luciferase activity, and normalized to
Renilla luciferase expression. The data represent the means ± S.E. of
12 samples. The asterisk indicates statistical significance (p < 0.002)
comparing c-Jun with Cbfa1 to each of the other three individual
samples.

Within the rat collagenase-3 promoter, two sites, the AP-1-
binding site and the RD-binding site, along with their binding
proteins form a functional complex that constitutes the major determinant of both basal activity and PTH inducibility (11, 12). The gel shift experiments presented here demonstrate that the interaction of c-Fos–c-Jun and Cbfa1 together with DNA facilitates formation of a complex that migrates slower than the c-Fos–c-Jun-DNA complex and considerably slower than the Cbfa1-DNA complex. The preferential formation of this complex in the presence of excess probe DNA constitutes evidence that the binding of c-Fos–c-Jun/Cbfa1 binding may be cooperative. If the binding were not cooperative, it would be reasonable to assume that, in the presence of excess probe, the proteins would bind separate promoter molecules, leading to separate shifts of Cbfa1-DNA and c-Fos–c-Jun-DNA complexes. This was not the case in these studies, because the addition of increasing amounts of c-Fos–c-Jun led to formation of a third, slower moving band and no band corresponding to a Cbfa1-DNA shift (Fig. 1C). Thus, it appears that Cbfa1 preferentially bound c-Fos–c-Jun/Cbfa1 binding rather than free probe. Likewise, c-Fos–c-Jun also preferentially bound Cbfa1-DNA complexes instead of free probe. Even large excesses (500-fold) of competitor AP-1-binding site and RD-binding site oligonucleotides could not entirely abolish the formation of the ternary complex. This result suggests that the binding of both sets of the proteins to their respective sites is more stable than each binding alone.

Cbfa1 and other Cbfa proteins have been shown to directly interact with a variety of other transcription factors. For example, Cbfa1 directly binds the androgen and glucocorticoid receptors (36). Cbfa1 also interacts with the basic helix loop helix transcription factor, HES-1, a mammalian counterpart of the Drosophila Hairy and Enhancer of Split, which is important for sex determination and segmentation (37). Cbfa2 and the CCAAT enhancer-binding protein interact in vitro and cooperate to activate the macrophage colony-stimulating factor receptor promoter (38). Both Cbfa1 and Cbfa2 interact with Groucho and TLE2 (39). Additionally, Cbfa1 and Cbfa2 bind Ets-1 and participate in the formation of the T cell receptor enhancer complex with lymphoid enhancer-binding factor 1 and the non-DNA-binding protein, ALY (40, 41). Finally, both Cbfa and AP-1 proteins cooperatively interact with Smad proteins (42–44).

In vitro studies performed here demonstrate that Cbfa1 and the runt domain of Cbfa1 alone are able to interact with c-Fos and c-Jun in a manner that does not require DNA binding or phosphorylation. The in vitro co-immunoprecipitation studies further show that the proteins do interact in mammalian cells but require overexpression. These results suggest a complex interaction, one that may involve dilution of competing transcription factors or, conversely, may require recruitment of additional transcription factors to stabilize a relatively weaker interaction. The yeast two-hybrid system, a system that may be free of potential competing or repressive effectors found in mammalian cells, also showed positive protein-protein interaction between the AP-1 factors and the runt domain of Cbfa1.

The yeast two-hybrid system was further employed to determine the interacting domains of c-Jun and Cbfa1. Clearly, the RD of Cbfa1 interacted with c-Fos and c-Jun and supported the findings from the in vitro co-immunoprecipitation experiments. When the amino-terminal activation domain of c-Jun was deleted, no interaction was seen with Cbfa1 or with the runt domain of Cbfa1, suggesting that the DNA-binding domain and the leucine zipper region alone do not bind Cbfa1. Conversely, some interaction with Cbfa1 was noted with the c-Jun deletion construct containing the activation, but not the leucine zipper region. Neither region interacted with Cbfa1 as well as the full-length c-Jun, suggesting that both the amino-terminal region and the leucine zipper region may both be required for full interaction. A similar pattern of interaction of the c-Jun deletions with the runt domain of Cbfa1 was also noted.

Unlike c-Fos, c-Jun can homodimerize, bind the AP-1-binding site, and activate transcription. We found that transfection of COS-7 cells with small amounts of c-Jun expression vector (5 ng) resulted in only slight activation of a series of AP-1-binding site promoter sites linked to a luciferase reporter gene. When Cbfa1 was co-transfected with c-Jun, a significant increase in luciferase activity, compared with either construct alone, was noted. This result suggests a functional mechanism by which Cbfa1 binds the AP-1-binding site promoter site and then Cbfa1 interacts with c-Jun to cooperatively activate the transcription of the luciferase reporter gene. Some slight increases in reporter activity were observed with transfection of Cbfa1 with the reporter construct alone, but this could be attributed to Cbfa1 binding to endogenous AP-1 factors found in COS-7 cells, leading to a slight increase in basal reporter gene activation. Interestingly, when COS-7 cells were transfected with greater amounts of c-Jun and Cbfa1 expression vector (>100 ng), the promoter was significantly activated, but co-transfection with Cbfa1 inhibited this increase. This finding may represent squelching of c-Jun activity. Evidence of inhibition of AP-1 activity by competition for limiting cofactors has been demonstrated previously. For example, several steroid nuclear receptors inhibit AP-1 activity by competition for limiting amounts of cAMP response element-binding protein (45). Alternatively, at higher concentrations, Cbfa1 could compete with other factors for binding to c-Jun. Regardless, at lower concentrations, Cbfa1 reproducibly augments c-Jun activation of the AP-1-binding site promoter.

The final piece of evidence presented here demonstrating AP-1 and Cbfa1 interaction, further suggesting that this interaction is required for rat collagenase-3 promoter activation, is derived from shifting the helical phasing between the AP-1- and RD-binding sites. Shifting the helical phasing (3-base pair insertion) between the AP-1- and RD-binding sites reduced promoter activity, whereas the insertion of 10 nucleotides, which preserved the helical phasing, restored promoter activity. Thus, it appears that a specific helical phasing is required to properly position the AP-1- and RD-binding sites and to enable the transcription factors that bind the sites, namely c-Fos–c-Jun and Cbfa1, to interact and initiate transcription. In addition to supporting Cbfa1/AP-1 interactions, proper helical phasing may further contribute to the assembly of a higher order nucleoprotein structure for PTH-induced collagenase-3.
promoter activity. A similar requirement for a specific helical phase between factor-binding sites has been observed in the osteopontin promoter with the Cbfa- and Ets-1-binding sites, the c-fos promoter with the cAMP response element-binding protein and YY1 binding sites, and the minimal T cell receptor α gene enhancer with the activating transcription factor/cAMP response element-binding protein family and lymphoid enhancer-binding factor 1-binding sites (41, 46, 47).

Overall, these studies provide strong evidence demonstrating cooperative DNA binding and direct interaction of the AP-1 factors with Cbfa1 and, more specifically, with the runt domain factors with Cbfa1 and, more specifically, with the runt domain

interaction of Cbfa1 with c-Jun in the yeast two-hybrid experiments performed both in vitro and with crude cell lysates. The differences in these two studies may be explained by the potential for post-translational modification of the different proteins in the separate environments or that the yeast two-hybrid system may represent a situation that is more representative of in vivo interaction. In addition to exploring the physical interaction of Cbfa with AP-1, we demonstrate co-operative binding of the transcription factors to collagenase-3 promoter and illustrate the requirement for proper helical phasing between the AP-1- and RD-binding sites for full promoter activity. Taken together, these results suggest a transcriptional model where, when properly aligned, adjacent AP-1- and RD-binding site elements cooperatively recruit AP-1 and Cbfa1 proteins and facilitate protein-protein interaction between the transcription factors. This interaction could then recruit other transcriptional factors and lay the foundation for assembly of higher order transcriptional initiators. In addition to the collagenase-3 promoter, this process may also be important for activation of other osteoblastic genes, including osteocalcin and COL1A1 and COL1A2, which also contain AP-1- and RD-binding sites in their respective promoters (48, 49).
Physical Interaction of the Activator Protein-1 Factors c-Fos and c-Jun with Cbfa1 for Collagenase-3 Promoter Activation
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