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CBFa(AML/PEBP2)-Related Elements in the TGF-β Type I Receptor Promoter and Expression With Osteoblast Differentiation

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Abstract Organization of the transforming growth factor-β (TGF-β) type I receptor (TRI) promoter predicts constitutive transcription, although its activity increases with differentiation status in cultured osteoblasts. Several sequences in the rat TRI promoter comprise *cis*-acting elements for CBFa (AML/PEBP2α) transcription factors. By gel mobility shift and immunological analyses, a principal osteoblast-derived nuclear factor that binds to these sites is CBFa1(AML-3/PEBP2αA). Rat CBFa1 levels parallel expression of the osteoblast phenotype and increase under conditions that promote mineralized bone nodule formation in vitro. Fusion of CBFa binding sequence from the TRI promoter to enhancer-free transfection vector increases reporter gene expression in cells that possess abundant CBFa1, and overexpression of CBFa increase the activity of transfected native TRI promoter/reporter plasmid. Consequently, phenotype-restricted use of *cis*-acting elements for CBFa transcription factors can contribute to the high levels of TRI that parallel osteoblast differentiation and to the potent effects of TGF-β on osteoblast function. J. Cell. Biochem. 69:353-363. © 1998 Wiley-Liss, Inc.

Key words: AML/CBF/PEBP2; CBFa1; differentiation; osteoblasts; regulatory elements; transforming growth factor-β; receptor

Conventional transforming growth factor-β (TGF-β) type I, II, and III receptors (TRI, TRII, and TRIII) occur on many cells. Changes in the ratios of individual TRs correlate with cell phenotype, with physiological or pathological states [Massague et al., 1994; Kingsley, 1994; Centrella et al., 1995a], and in some instances with differences in TGF-β-dependent biological effects [Centrella et al., 1991, 1995b; Chen et al., 1993; Sankar et al., 1995, 1996]. Activation of TRI appears essential for TGF-β activity [Wrana et al., 1994; Wieser et al., 1995], and specific increases in the proportion of TRI mRNA and protein occur in parallel with variations in TGF-β activity on differentiating osteoblasts [Centrella et al., 1995b]. Nonetheless, little is known about control of TRI expression in any tissue.

The rat TRI promoter lacks TATA and functional CCAAT boxes, and TRI transcription initiates at several sites compatible with the presence of a CpG island and multiple *cis*-acting elements for transcription factor Sp1. Reporter constructs with TRI promoter fragments deleted internally or from the 5′ or 3′ ends reveal cooperation among several dispersed elements for maximal expression. In addition, TRI promoter activity is significantly higher in differen

Abbreviations: TGF-β, transforming growth factor-β; TGF-β type I, type II, and type III receptors, TRI, TRII, TRIII; AML, acute myelogenous leukemia; CBF, core binding factor; PEBP, polyomavirus enhancer binding protein.

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tiated osteoblast-like cells [Ji et al., 1996, 1997]. Together, these results suggest regulation by multiple elements for basal, phenotype-restricted, and growth factor- or hormone-regulated TRI expression.

To identify constitutive and conditional *cis*-acting elements in the TRI promoter, oligonucleotide segments surrounding the region of transcription initiation were characterized by binding of *trans*-acting nuclear factors from cells committed to osteogenesis [Ji et al., 1996, 1997]. Several sequences analogous to regulatory elements in the osteocalcin promoter were observed. Osteocalcin is one of the best recognized markers of osteoblast differentiation. Certain well-studied regions in the rat and mouse osteocalcin promoters include response elements for dihydroxyvitamin D_3 and glucocorticoid, and two conserved domains termed OC boxes that bind phenotype-restricted *trans*-acting factors [Merriman et al., 1995; Geoffrey et al., 1995; Ducy and Karsenty, 1995; Banerjee et al., 1996]. OC Box II contains a sequence (5′-AACCACA-3′) defined as a consensus motif (5′-[T/G/A]ACC[G/A]C[A/G]-3′) for the prototype of a small group of transcription factors whose expression is relatively tissue restricted. First isolated from calf thymus and embryonal F9 carcinoma cells, these factors associate with certain viral enhancer cores and are termed core-binding factor (CBF) or polyoma enhancer binding protein (PEBP). Other studies show that they are homologues of proteins initially recognized by frequent gene translocations associated with acute myelogenous leukemias and termed AMLs [Hiebert et al., 1996; Ito and Bae, 1997]. To date, three independently regulated genes are known to encode "a" subunits of these factors. Functional "a" subunits contain DNA binding and heterodimerization regions homologous to the runt domain *(rd)* of the segmentation gene *runt* involved in pair rule gene expression, somatic sex determination, and neural development in *Drosophila* [Golling et al., 1996]. In general, binding of "a" subunits to DNA is enhanced by dimerizing with ubiquitously expressed "b" subunits [Hiebert et al., 1996; Ito and Bae, 1997]. Recently, mice with targeted disruption of the gene encoding one "a" subunit, termed CBFa1, were produced. Mineralized skeletal elements consistent with osteoblast-dependent bone formation were not evident in these animals, and few if any cells with osteoblast morphology were apparent [Komori et al., 1997; Otto et al., 1997]. These mice are considered models for the skeletal disease cleidocranial dysplasia [Mundlos et al., 1997; Otto et al., 1997]. Variations in CBFa2 and CBFa3 appear to have important effects on lymphoid cells, and perhaps on cells in other connective tissues [Hiebert et al., 1996; Ito and Bae, 1997].

Our present results demonstrate several binding sites in the TRI promoter for CBFa(AML/PEBP2) transcription factors, differing at most by a penultimate 3′ terminal nucleotide from consensus sequences defined for the prototypical "a" subunit termed CBFa2(AML-1/PEBP2αB) found in T and B cells. We find subunit CBFa1 enriched in parallel with osteoblast differentiation and partial discrimination at certain TRI promoter sites. Consequently, phenotype-restricted events may regulate TRI promoter activity during osteoblast differentiation and augment activity by ubiquitous factors that control basal TRI expression.

MATERIALS AND METHODS

Cell Cultures

Using procedures approved by the Yale Animal Care and Use Committee, parietal bones of 22 day old Sprague-Dawley rat fetuses (Charles River Breeding Laboratories) were dissected from sutures and digested for five 20-min intervals with collagenase. The first digestion releases undifferentiated periosteal cells and a pool of the last three digestions is enriched for cells characteristic of differentiating osteoblasts. Primary cultures of each group were plated at $3–4 \times 10^3$ cells/cm^2 in Dulbecco's modified Eagle's medium with 20 mM Hepes (pH 7.2), 100 μg/ml ascorbic acid, penicillin, streptomycin, and 10% fetal bovine serum (FBS) and were confluent ($5–6 \times 10^4$ cells/cm^2) by 6–7 days. Proliferating cultures were collected at 60–70% confluence. Every 3–4 days, confluent cultures were re-fed the same medium, except that ascorbic acid and serum were reduced by one-half. Differentiated cultures were collected at 1 week after confluence. For mineralization, cultures were supplemented with 3 mM β-glycerol phosphate and collected 2 weeks after confluence. Mineralized nodules were only observed in osteoblast-enriched cultures, were evident 3–4 days after adding β-glycerol phosphate, and accumulated throughout 2 week of incubation [Centrella et al., 1996a]. Clonal rat osteoblast-like ROS 17/2.8 cultures (Dr. Gideon Rodan; Merck Sharp and Dohme, West Point,

PA) and skin fibroblasts from fetuses used to prepare primary bone cells were cultured and treated analogously [Centrella et al., 1995; Ji et al., 1996].

Nuclear Extracts

Cultures were rinsed with phosphate-buffered saline (PBS) containing phosphatase inhibitors (1 mM sodium orthovanadate, 10 mM sodium fluoride) on ice. Cells were scraped into buffer, collected by centrifugation, and nuclear extracts were prepared as in previous studies [Lee et al., 1988; Thomas et al., 1996; McCarthy et al., 1996]. Briefly, cells were lysed in hypotonic buffer (10 mM Hepes (pH 7.4), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol) with the phosphatase inhibitors, protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 1 µg/ml pepstatin A, 2 µg/ml leupeptin, 2 µg/ml aprotinin), and 1% Triton X-100. Nuclei were collected by 5-min centrifugation at 3,500*g* and resuspended in hypertonic buffer (0.42 M NaCl, 0.2 mM Na_2 EDTA, 25% glycerol, phosphatase, and protease inhibitors above). Nuclei were extracted for 30 min on ice, insoluble material was cleared by 5 min centrifugation at 12,500*g*, and soluble proteins were stored at -75°C.

Electrophoretic Gel Mobility Shift Assays

Double-strand oligonucleotide probes were annealed by heating to 95°C and cooling to 25°C in 10 mM Tris–HCl (pH 8.0), 1 mM EDTA, 5 mM $MgCl_2$. Probes were end-labeled to 1–3 × 10^5 cpm/ng DNA with [α-^{32}P]dCTP and Klenow fragment of *Escherichia coli* DNA polymerase I and gel purified. Nuclear extracts (5 µg protein) were incubated in binding buffer consisting of 25 mM Hepes (pH 7.5), 80 mM KCl, 2 mM dithiothreitol, 0.5 mg/ml bovine serum albumin (BSA), 62.5 µg/ml poly(dI/dC), 12.5% glycerol on ice with 3×10^4 cpm (0.1 ng) of ^{32}P-probe in a total of 20 µl. In competitive binding studies, unlabeled native or mutated oligonucleotide (Table I) were added just before ^{32}P-probe. To assess transcription factor immunologically, nonimmune (Santa Cruz, Santa Cruz, CA) or rabbit polyclonal IgGs [Myers et al., 1996] were preincubated with nuclear extract before adding ^{32}P- probe. Protein–DNA complexes were resolved on 5% nondenaturing polyacrylamide gels in 0.5 × TBE buffer (90 mM Tris borate (pH 8.3), 2 mM EDTA) at 20°C with 130 V for 2.5 h. Gels were dried and analyzed by autoradiography [Ji et al., 1997; McCarthy et al., 1996].

Immunoblots

Nuclear protein (40 µg) was fractionated by electrophoresis through 8% denaturing polyacrylamide gels and electroblotted onto Immobilon P membranes (Millipore, Bedford, MA). Membranes were washed in TBST buffer (10 mM Tris-Cl [pH 8.0], 15 mM NaCl, 0.05% Tween-20), blocked in 5% defatted milk in TBST, incubated with 1:2,000 dilution of primary antibodies [Myers et al., 1996], washed, and incubated with a 1:3,000 dilution of goat anti-rabbit IgG conjugated to horseradish peroxidase (HPO) (BioRad, Hercules, CA), developed with ECL (Amersham, Arlington Heights, FL) reagents, and visualized by chemiluminescence [Ji et al., 1997].

Constructs

Transfection constructs pES1.0 and pSXN1C (shown diagrammatically in Fig. 1) were used

TABLE I. Oligonucleotide Sequences for Gel Electrophoretic Mobility-Shift Assays*

Name	Sequence	Position of PS in TRI promoter
PC1	5′-CGTATTAACCACAATACTCG-3′	—
PC2	5′-GGCCGGAGCGACCGCAGCGG-3′	—
PSX	5′-GGCCGGAGCGACC**T**CGGCGG-3′	—
PS1	5′-GGCCGGAGCGAGGCCGCGGCGGCGGCG-3′	−81 to −75
PS2	5′-AAGAAGTTGAACCGCGGACCGTATTGA-3′	−546 to −540
PS3	5′-TGGGCCGCTATACTCCAGCCACAGCAGGGTAGCT-3′	−307 to −313
PS4	5′-CTTCTGCCTCTAACCACGCCTCCGCAC-3′	−245 to −251
PS5	5′-GACAGCATTTTTCCGCAGAACCTTTT-3′	−1095 to −1101
PS6	5′-GTCACGGCGGCCGCATGCGTACCGA-3′	−1071 to −1077
SP1	5′-GTACATTCGATCGGGCGGGGCGAGCGATC-3′	

*Oligonucleotides PC1 and PC2 contain consensus *cis*-acting elements (underlined) for CBFa [Ito, 1996; Hiebert et al., 1996], PSX contains a consensus element for CBFa disrupted by substitution of **T** as shown (boldtype), and SP1 contains a consensus element for Sp1 [Ji et al., 1997; Hagen et al., 1994]. Oligonucleotides PS1–PS6 contain sequences of the rat TRI promoter analogous or related to CBFa binding sequences, positioned in the promoter as indicated.

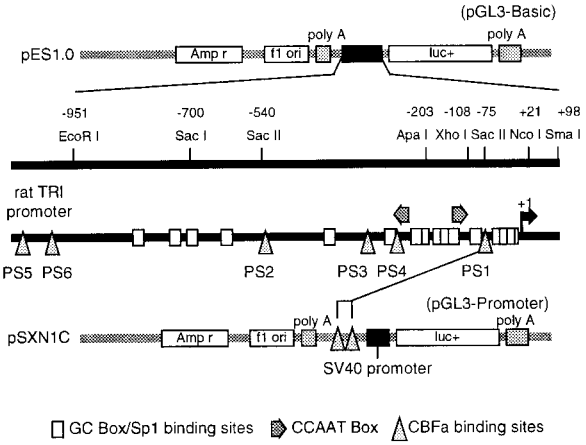

Fig. 1. Characteristics of the rat TRI promoter and transfection constructs used to assess control of TRI promoter activity by nuclear factor CBFa. The relative positions of six CBFa binding sequences (triangles), 16 Sp1 binding sequences (squares), and two CCAAT boxes (arrows) in the rat TRI promoter (these studies and Ji et al. [1997]) are indicated. +1 refers to a major site of transcription initiation [Ji et al., 1996]. The *Eco*RI and *Sma*I restriction sites used to obtain TRI promoter sequence inserted into pGL3-Basic vector to form pES1.0 is shown from above, and the two CBFa binding sequences from the TRI promoter inserted into pGL3-Promoter vector are shown from below.

to assess effects of CBFa on TRI promoter activity. pSXN1C was generated by phosphorylating double-stranded DNA oligonucleotide [5′-**CTAGA**GCCGGAGCGAGGCCGCGGCG-GCGGCGGGGAGGTG**T**-3′] [3′-**T**CGGCCTCG-CTCCGGCGCCGCCGCCGCCCCTCCAC**AG-ATC**-5′] (minimal CBFa element PS1 underlined, cohesive ends in bold type) with T4 polynucleotide kinase and ATP, and ligating into pBluescript-KSII (Stratgene, LaJolla, CA) previously linearized with *Spe*I to produce homologous cohesive ends. Insert with two copies of oligonucleotide was released with *Bam*HI and *Sal*I and cloned directionally into the upstream site of pGL3-Promoter (Promega, Madison, WI) previously digested with the same enzymes. pES1.0, containing the 3′ 1 kb portion of the rat TRI promoter, and expression constructs for each CBFa subunit were described previously [Ji et al., 1996; Banerjee et al., 1997].

Transfections

For studies with pSXN1C, cells at 50% confluence were transfected with optimal reporter plasmid DNA (0.2 μg/cm^2 culture) using 0.5% Lipofectin™ (Life Technologies). For transcription factor forced expression studies, cells at 50% confluence were transfected with a limiting amount of TRI promoter/reporter plasmid pES1.0 (0.04 μg/cm^2) and an optimal amount of expression construct for a single CBFa subunit (0.006 μg/cm^2 for ROS 17/2.8 and 0.02 μg/cm^2 for undifferentiated perisoteal cells). Cells were transfected for 3 h, re-fed medium containing 5% fetal bovine serum (FBS), and cultured for an additional 48 h. Cells were rinsed and extracted with cell lysis buffer (Promega), nuclei were cleared by centrifugation at 12,000*g* for 5 min, and luciferase was measured in supernatants with commercial kits (Promega). Data were corrected for relative protein and co-transfected reporter plasmid [Bradford, 1976; Ji et al., 1996; Banerjee et al., 1997].

Statistical Analysis

Biochemical data were analyzed after multiple determinations and expressed as means ± SEM. Statistical differences were assessed by analysis of variance with commercial software (SigmaStat®). Post-hoc analysis was by the Student-Newman-Keuls method and considered significant with P values of <0.05.

RESULTS

Constitutive and Restricted Elements in the TRI Promoter

The 3′ 1.0-kb segment of the rat TRI promoter, containing many *cis*-acting elements, a CpG island, and several Sp1 binding sites [Ji et al., 1996, 1997] drives high-level reporter expression in transfected fetal rat bone cells. The 3′-terminal 0.2-kb sequence defines a basal promoter with 30–50% of maximal activity that is suppressed by the loss of an essential Sp1 site. Gel mobility shift analyses with oligonucleotides corresponding to clusters of *cis*-acting elements in this region revealed multiple binding sites for Sp1 and Sp3 [Ji et al., 1997]. In contrast to results with probe ^{32}P-SP1, which contains a consensus binding sequence for Sp1 and Sp3 [Hagen et al., 1994], nuclear factor that bound to a CBFa consensus probe ^{32}P-PC1 (Table I) was enriched in osteoblast-like cells by relation to undifferentiated periosteal bone cells or dermal fibroblasts (Fig. 2). For consistency, factors reactive with CBFa(AML/PEBP2) sites will generally be referred to as CBFa in this report.

Multiple CBFa Binding Sites in TRI Promoter

Sequence analysis revealed six consensus or very closely related CBFa binding sequences

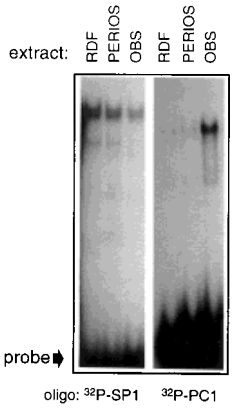

Figure 2.

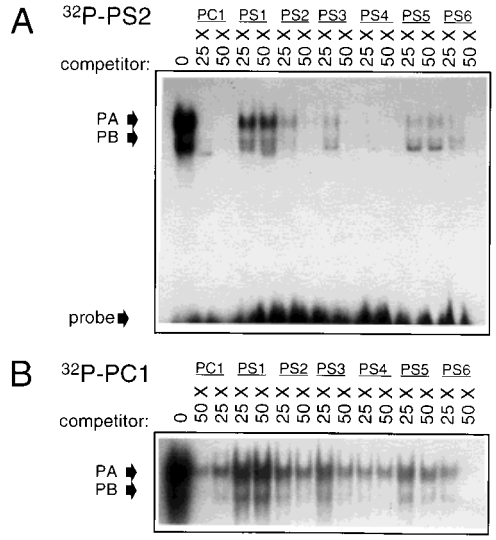

Figure 3.

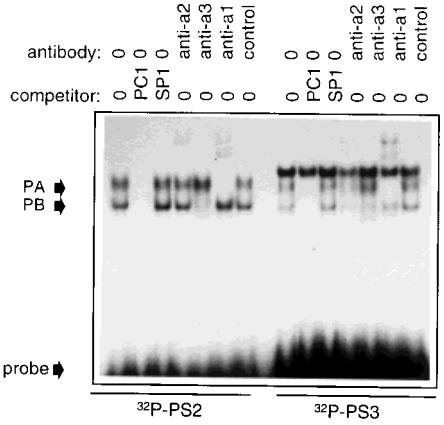

Figure 4.

Fig. 2. Differential nuclear factor binding to DNA encoding Sp1 and CBFa consensus elements in extracts from osteoblastic cells. Nuclear extract (5 μg protein) from postconfluent fetal rat dermal fibroblasts (RDF), undifferentiated periosteal bone cells (PERIOS), or osteoblast-enriched cultures (OBS) was combined with 0.1 ng of probes ^{32}P-SP1 or ^{32}P-PC1 as indicated, and resolved on nondenaturing 5% polyacrylamide gels. Analogous results occurred in four separate studies.

Fig. 3. Six CBFa binding sites in TRI promoter. Nuclear extract (5 μg protein) from postconfluent osteoblast-enriched cultures was combined with 0.1 ng of probe ^{32}P-PS2 from the TRI promoter **(A)**, or CBFa consensus probe ^{32}P-PC1 **(B)**, without or with 2.5 or 5 ng of the unlabeled oligonucleotides indicated, and resolved on nondenaturing 5% polyacrylamide gels. Analogous results occurred in four separate studies.

Fig. 4. Relative association of osteoblast-derived CBFa with CBFa binding sequences from the TRI promoter. Nuclear extract (5 g protein) from post-confluent osteoblast-enriched cultures was combined with 0.1 ng of probes ^{32}P-PS2 or ^{32}P-PS3 from the TRI promoter, without (0) or with 5 ng of unlabeled CBFa consensus oligonucleotide (PC1) or Sp1 consensus oligonucleotide (SP1), or 0.5 μg of antibody to CBFa2/AML-1 (anti-a2), CBFa3/AML-2 (anti-a3), CBFa1/AML-3 (anti-a1) [Myers et al., 1996], or nonimmune IgG (control) as shown, and resolved on nondenaturing 5% polyacrylamide gels. Analogous results occurred in four separate studies.

within the 3′ 1.1 kb of the TRI promoter (Table I and Fig. 1). The CBFa binding sites that we termed PS2 and PS4 each contain previously defined consensus sequences. The four other sites that we termed PS1, PS3, PS5, and PS6 differ by one nucleotide in the penultimate 5′ position (Table II). To examine whether these sequences in TRI could bin CBFa, we prepared oligonucleotides with sequences derived from the TRI promoter where they formed a central core. We first tested their abilities to compete for nuclear factor with two different ^{32}P-labeled oligonucleotides containing consensus binding sequences for CBFa's. Analogous to results in Figure 2 with ^{32}P-PC1 (with a generic CBFa consensus binding sequence), ^{32}P-PS2 (with a CBFa consensus sequence that occurs in the TRI promoter) formed nuclear factor complexes designated as PA and PB (Fig. 3A). Complex PB formed more readily with ^{32}P-PS2, but it was also apparent after a longer exposure in samples from reactions with probe ^{32}P-PC1 (Fig. 3B). Oligonucleotides with each of the six CBFa sites from the TRI promoter successfully competed for nuclear factor with either ^{32}P-labeled consensus probe, although complex PA that formed with ^{32}P-PS2 appeared to be somewhat more easily displaced by several TRI-derived sequences. We also ^{32}P-labeled each PS oligonucleotide and examined nuclear factor binding. Consistent with the presence of other nuclear factor binding sequences that occur in these regions (see, e.g., earlier studies with ^{32}P-PS1 in Ji et al. [1997] and current studies with ^{32}P-PS3 in Fig. 4, below), other nuclear factor complexes that form with ^{32}P-PS4, -PS5, and -PS6 partially suppress the appearance of complexes that contain CBFa by autoradiographic analysis (data not shown). While not associating directly with CBFa binding sequences, other nuclear factors can compete with CBFa's by binding other sequences on radiola-

TABLE II. Relative Homology of CBFa Binding Sites From the Rat TRI Promoter*

Element	Sequence	Homology	Relative potency
Consensus	A A A 5′-GACCGCG-3′ T		
PC1	5′-AACCACA-3′	7/7	++++
PSX	5′-GACC**T**CG-3′	6/7	0
PS1	5′-G**G**CCGCG-3′	6/7	+
PS2	5′-AACCGCG-3′	7/7	+++
PS3	5′-A**G**CCACA-3′	6/7	++
PS4	5′-AACCACG-3′	7/7	++++
PS5	5′-T**T**CCGCA-3′	6/7	++
PS6	5′-G**G**CCGCA-3′	6/7	++++

*Consensus binding sequences for CBFa transcription factors were defined previously [Hiebert et al., 1996; Ito and Bae, 1997]. Letters above or below the consensus sequence indicate the variability previously reported at these positions. Differences from previously defined consensus sequences are indicated by boldface type.

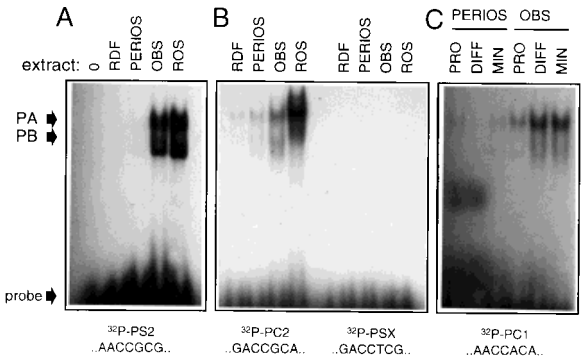

Fig. 5. Nuclear factor association with CBFa binding sites increases with differentiated osteoblast status. Nuclear extract (5 μg protein) from RDF, PERIOS, and OBS as in Figure 1, or highly differentiated ROS 17/2.8 osteosarcoma-derived (ROS) cell cultures was combined with 0.1 ng of probes ^{32}P-PS2 from the TRI promoter (**A**), CBFa consensus probes ^{32}P-PC2 (**B**),or ^{32}P-PC1 (**C**), or mutated probe ^{32}P-PSX in (B), and resolved on nondenaturing 5% polyacrylamide gels. A,B: Nuclear extracts were derived from post-confluent cultures. C: Nuclear extracts were derived from subconfluent proliferating cultures (PRO), 1 week postconfluent cultures (DIFF), or cells cultured for 2 weeks with β-glycerolphosphate to promote mineralized nodule formation (MIN). Analogous results occurred in three separate studies.

beled probes, whereas the proximity of other nuclear factor complexes in some instances can obscure CBFa binding. Thus, the potency of the CBFa sequences in these regions is more readily assessed in competitive binding studies with probes associating with essentially CBFa alone.

Multiple CBFa Subunits in Osteoblasts

Like ^{32}P-PS2 (with a consensus CBFa sequence), ^{32}P-PS3 (differing by one nucleotide from previously defined consensus sequences) also forms discrete gel mobility shift complexes PA and PB with nuclear protein from osteoblast-enriched cultures. For validation, both TRI-derived oligonucleotides were used to characterize osteoblast-derived nuclear factors that are reactive with consensus or related CBFa binding sequences. As in Figure 3, complexes PA and PB were both competed by CBFa consensus oligonucleotide PC1, but not by nonspecific Sp1/Sp3 oligonucleotide SP1. The identities of the nuclear proteins in complexes PA and PB were then examined by supershift and/or immunodepletion with antibody specific for the three known CBFa subunits. Essentially all of complex PA was reactive with anti-CBFa1 antibody, the major portion of complex PB was reactive with anti-CBFa3 antibody, and only a small amount of protein supershifted with anti-CBFa2 (Fig. 4). Consistent with the competitive binding studies in Figure 3, complexes PA and PB formed slightly less efficiently with ^{32}P-PS3. This was due, perhaps in part, to the presence of nuclear factor that forms a third slower migrating complex, itself insensitive to competition or immunoreaction with CBFa-or Sp1-specific reagents, and not yet further defined.

Phenotype-Dependent Levels of CBFa1 in Osteoblasts

Because the only gel-shift complexes formed by ^{32}P- PS2 are accounted for by osteoblast-derived CBFa-like proteins (Fig. 4), we next used this probe to assess changes in CBFa levels with relation to a CBFa binding sequence in the TRI promoter. Analogous to the result with consensus CBFa probe ^{32}P-PC1 (shown in Fig. 2), minimal complex formed between ^{32}P-PS2 and nuclear extract from dermal fibroblasts or undifferentiated periosteal bone cells, while efficient binding occurred with nuclear extract from osteoblast-enriched cultures, as well as from highly differentiated rat osteoblast-like ROS 17/2.8 cells (Fig. 5A). We verified this observation with a third consensus CBFa oligonucleotide probe termed ^{32}P- PC2, and further found negligible complex formation with ^{32}P-PSX, in which the consensus sequence in ^{32}P-PC2 was disrupted near the 3′ end (Fig. 5B). Higher levels of complex formation occurred with nuclear extract from primary osteoblast-enriched cultures as they progressed through the stages of proliferation, differentia-

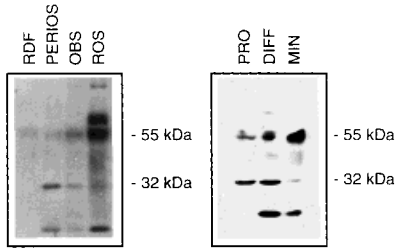

Fig. 6. Western blot analysis of CBFa1 in fetal rat cell cultures. Nuclear extract (40 μg protein) from RDF, PERIOS, OBS, and ROS cultures as in Figure 4A was resolved on denaturing 8% polyacrylamide gels, blotted, and probed with antibody to CBFa1. Left, nuclear extracts were derived from postconfluent cultures. Right, nuclear extracts were derived from PRO, DIFF, MIN cultures as in Figure 4B. Analogous results occurred in two separate studies.

tion, and mineralization, but not from similarly cultured periosteal cells (Fig. 5C).

Although antisera to CBFa1 and CBFa3 reacted with nuclear factor in gel mobility shift studies, only anti-CBFa1 antibody was efficient by Western blot analysis. Whereas CBFa1 was initially cloned from Ha-*ras*-transformed mouse NIH 3T3 fibroblasts [Ogawa et al., 1993], relatively little of this factor occurs in normal rat dermal fibroblasts (Fig. 6A). By contrast, immunoreactive 55-kDa CBFa1 and smaller bands of ≤32 kDa invariably occurred in bone cell cultures. The proportion of the 55-kDa band increased relative to the 32-kDa band in osteoblast-enriched and ROS 17/2.8 cultures, and was further enhanced when primary osteoblast-enriched cultures progressed to the mineralization state (Fig. 6B).

CBFa Activity

Native TRI promoter/reporter constructs with one or more CBFa binding sequences are more active in differentiated bone cells, and appear to amplify TRI expression driven by essential Sp1 binding sequences [Ji et al., 1996, 1997]. Therefore, to focus functional studies on *cis*-acting CBFa elements independently of the Sp1 sites in the TRI promoter, we created a minimal CBFa-dependent promoter/reporter construct. Because a strong CBFa binding sequence would not distinguish subtle differences in the amounts of functional CBFa protein in bone cells, we used a weak CBFa binding sequence from the TRI promoter. Thus, we inserted two copies of oligonucleotide PS1 into vector pGL3-Promoter to produce plasmid pSXN1C. The pGL3-Promoter vector lacks enhancer sequence, but contains a viral promoter and is therefore a functional unit with enogenous activity. Consequently, the amount of reporter gene expresssion that occurs in transfected cells above that driven by the basal viral promoter will depend wholly on the presence of protein that associates with the two minimal PS1 sites. The viral promoter lacking PS1 sequences directed reporter gene expression by approximately 10 fold above pGL3-Basic, the empty vector that lacks both promoter and enhancer sequences. However, consistent with the relative levels of CBFa1 seen in each cell culture by gel mobility shift and Western blot analyses (Figs. 5, 6), the presence of the CBFa binding sequences did not further increase reporter expression in dermal fibroblasts or undifferentiated periosteal bone cells, but enhanced pGL3-Promoter activity to 20 fold in osteoblast-enriched cultures and to 45-fold in ROS 17/2.8 cells (Fig. 7A).

To focus functional studies on *trans*-acting CBFa proteins, undifferentiated periosteal cells and ROS 17/2.8 cultures were co-transfected with a maximally active native TRI promoter/reporter plasmid termed pES1.0 [Ji et al., 1996, 1997] and expression constructs encoding each CBFa subunit. We used a limiting amount of promoter/reporter construct in order to minimize its ability to sequester pre-existing CBFa from genomic transcriptional units in the ROS 17/2.8 cells, and to ensure that reporter synthesis would depend on additional CBFa expression. Even a limiting amount of TRI promoter construct directed a five- to eightfold increase in reporter gene expression in each culture model, consistent with the presence of Sp1 in these cultures and the essential Sp1 binding sites in the TRI promoter [Ji et al., 1997]. Nonetheless, forced expression of each CBFa subunit significantly increased reporter activity by 16- to 20-fold in undifferentiated bone cells where the levels of CBFa are very low. In differentiated ROS 17/2.8 osteoblasts, transfection of CBFa's caused a 16 to 30 fold increase in reporter expression. In these cells, transfection of CBFa1 or CBFa3 was approximately twofold more effective than CBFa2 (Fig. 7B).

DISCUSSION

Changes in TGF-β function parallel variations in TRI expression in cultured bone cells [Centrella et al., 1991, 1995a,b], and TRI promoter activity corresponds to its relative mRNA and protein levels in cells that express a differ-

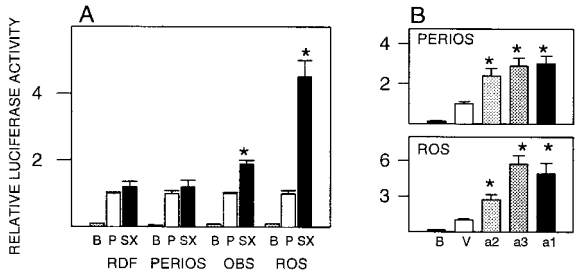

Fig. 7. CBFa-dependent effects on TRI promoter activity. **A:** Subconfluent RDF, PERIOS, OBS, and ROS cultures as in Fig. 4 were transfected with empty vector pGL3-Basic **(B)**, with pGL3-Promoter containing SV40 promoter but no enhancer (P), or pSXN1C (pGL3-Promoter with a 2-copy insert of the minimal CBFa binding sequence PS1: SX). Results are from 2–6 separate studies with 9–25 replicate cultures per condition. B: Undifferentiated PERIOS and ROS cultures were transfected with reporter construct pES1.0 [Ji et al., 1996] containing the 3′ 1-kb portion of TRI promoter and either empty expression vector pCMV5 (V) (Invitrogen), or expression constructs for CBFa2 (a2), CBFa3 (a3), or CBFa1 (a1). To assess basal activity by the minimal amount of pES1.0 used in these studies, parallel cultures were transfected with the promoter/enhancer free empty reporter construct pGL3-Basic (B). Results are from 2–3 separate studies with 6–9 replicate cultures per condition. Data are shown as relative luciferase activity. By analysis of variance, SX is significantly greater than P in OBS and ROS cultures (A) and forced expression of each CBFa subunit significantly enhanced pES1.0 activity relative to vector control (B).

entiated osteoblast phenotype [Centrella et al, 1995b; Ji et al., 1996]. Although multiple and essential transcription factor Sp1 binding sites occur in the TRI promoter, they do not explain its tissue- or phenotype-restricted expression [Ji et al., 1997]. Our current studies show that the 3′ region of the TRI promoter, highly active in bone cells, contains at least six *cis*-acting elements for CBFa transcription factors. Importantly, all six CBFa binding sequences from the TRI promoter readily associate with CBFa1 in the gel-shift complex that we termed PA, and enrichment for CBFa1 in differentiated osteoblasts coincides with higher TRI expression and promoter activity. A second CBFa binding complex, PB, was accounted for by CBFa3 by gel mobility shift analysis with specific "a" subunit antibodies, although CBFa3 appeared less abundant by Western analysis. Much less CBFa2 was evident in differentiated osteoblasts by either method. This distribution of CBFa subunits contrasts with myeloid cells where CBFa2 (T cells) or CBFa2 and CBFa3 (B cells) are more highly represented [Hiebert et al., 1996; Ito and Bae, 1997].

Having established that each CBFa binding sequence in the TRI promoter can physically associate with CBFa1, and to a lesser extent CBFa2 and CBFa3, from differentiated bone cells, we examined their ability to direct TRI promoter activity. Bone cells relatively enriched with CBFa1 and CBFa3 efficiently expressed a promoter/reporter transfection construct containing only two copies of a minimal CBFa binding element from the TRI promoter. Moreover, forced expression of CBFa, particularly CBFa1 and CBFa3, enhanced reporter expression by native TRI promoter construct pES1.0, with maximal promoter activity, even in the presence of active Sp1 sites and functional levels of Sp1 [Ji et al., 1997]. We find no other nuclear factor complexes that can account for these phenotype-related effects. Of note, pES1.0 contains four of the six CBFa binding sequences present in the TRI promoter. Unlike our previous studies with differentiated osteoblast cultures, where up to 4.1 kb of the TRI promoter directs reporter expression equivalent to that by the 3′ 1.0-kb portion found in pES1.0 (Ji et al., 1997), larger promoter fragments direct significantly less to virtually no activity in undifferentiated bone cells and in fibroblast cell cultures. Consequently, the two upstream CBFa binding sequences PS5 and PS6 may enhance TRI promoter activity further in differentiated bone cells, and perhaps overcome the presence of upstream silencer elements, although this requires more detailed analysis.

Of the CBFa complexes seen by gel mobility shift assay, PA specifically and completely reacted with antibody for CBFa1 and was relatively more sensitive to competition by oligonucleotides containing the CBFa sites PS3, PS5, and PS6 from the TRI promoter. These sites include a 3′ A and "nonconsensus" nucleotides G or T in the penultimate 5′ position. This suggests that consensus sequences previously defined for prototypical CBFa2 may be somewhat more variable. Alternately, the activity of certain CBFa subunits may be focused at sequences slightly different from sites that preferentially respond to other CBFa. In combination with tissue- or phenotype-restricted expression of specific CBFa subunits, this could help to determine some tissue-restricted biological effects.

Some of our current results are similar to recently reported studies that identify multiple CBFa binding sites in the osteocalcin promoter [Merriman et al., 1995; Ducy and Karsenty, 1995; Geoffrey et al., 1995; Banerjee et al., 1996]. Expression of osteocalcin is highly correlated with later stages of osteoblast differentiation when CBFa1 levels remain high [these studies and Banerjee et al., 1996; Ducy et al., 1997]. Although the biological role of osteocalcin is uncertain, its skeletal tissue-specific expression is tightly regulated by binding sites for CBFa and several osteotropic hormone/receptor complexes [Calvo et al., 1996; Ducy et al., 1996]. In contrast, TGF-β potently regulates bone cell replication, matrix type I collagen synthesis, alkaline phosphatase, and mineralization in vitro and in vivo, and variations in these effects appear related to the proportion of TRI on bone cells [Centrella et al., 1991, 1995a,b]. TRI, like osteocalcin, is expressed at relatively low levels by undifferentiated bone cells. However, TRI mRNA and protein levels increase relative to TRII and TRIII on cells with a more osteoblastic phenotype, and are maintained when TRII and TRIII levels specifically decline in response to bone morphogenetic protein 2-induced differentiation. In this context, the mitogenic effect of TGF-β subsides, but its ability to enhance collagen synthesis and alkaline phosphatase, two earlier markers of differentiation, increase [Centrell et al., 1995b]. Other studies reveal that later stages of osteogenesis, particularly those related to matrix mineralization, are suppressed by forced expression of TGF-β or exogenous TGF-β treatment [Breen et al., 1994; Erlebacher and Derynck, 1996]. We recently reported that TRI mRNA and protein have relatively short halflives on bone cells [Centrella et al., 1996b], indicating that net TRI levels could vary quickly in response to extracellular agents or events. Because TGF-β effects rely on active TRI expression, changes in expression of CBFa could regulate rapid transitions in sensitivity to TGF-β by bone cells or by other cells where CBFa occurs, as well as the nature of their response.

Consistent with our findings on CBFa1 expression and the importance of TGF-β and TRI for early aspects of bone cell activity, loss of CBFa1 by targeted disruption in mice completely eliminates mineralized bone formation [Komori et al., 1997; Otto et al., 1997]. Importantly, few cells with osteoblast morphology or biochemical markers are evident and mineralized skeletal matrix fails to form. Suppression of TRI expression, perhaps only to the lower levels found on fibroblastic cells where the influence of CBFa appear minimal, may not fully explain the mutant phenotype. However, even partial loss of TRI reduces the mitogenic effect of TGF-β for cells committed to the osteoblast lineage and also decreases type I collagen synthesis by differentiating osteoblasts [Centrella et al., 1986, 1991, 1995b; Hock et al., 1990], limiting both an essential cell population and the principal organic component of bone matrix. In this context, we have now noted hormone and growth factor dependent changes in CBFa1 expression that correlate with variations in TRI expression and TGF-β activity in bone-derived cell cultures (unpublished observations). Further studies to characterize TRI and other genes sensitive to CBFa in skeletal cells will clarify the important role of these factors in normal bone formation, and perhaps in metabolic bone disease.

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