in vivo *18*: 517-524 (2004)

Ets2 Transcription Factor Inhibits Mineralization and Affects Target Gene Expression During Osteoblast Maturation

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Abstract. *Our goal is to understand how Ets family transcription factors affect the genetic programs that control bone development. Modest overexpression of Ets2 in transgenic mice leads to Down's syndrome-like bone abnormalities. We observed that in the MC3T3-E1 in vitro model of osteoblast development, mature osteoblasts have very high levels of Ets2 relative to the immature preosteoblasts. We hypothesized that overexpression of Ets2 could have noticeable effects on gene expression, and found that exogenous Ets2 expression results in a complete lack of mineralized matrix in stable Ets2 transfected cells. Our cDNA microarray-based expression profiling of preosteoblasts vs. differentiated osteoblasts revealed several genes previously unrecognized as having roles in osteoblast maturation and up-regulated only in the mature osteoblasts. The promoters of these genes and known osteoblast marker genes were examined for Ets transcription factor binding sites (EBSs). Interactions of these sites with Ets2 protein were tested by EMSA. In vitro expressed Ets2 protein was able to form a protein:DNA complex with both known (Bsp, Opn, ON) and novel (Btg2, CysC, Lum) bone-related genes. In addition, Cbfa1 was found to interact with Ets2, forming a complex on the Opn promoter.*

Abbreviations: Ets1, v-ets erythroblastosis virus E26 oncogene homolog 1; Ets2, v-ets erythroblastosis virus E26 oncogene homolog 2; p42-Ets1, a naturally occurring variant of Ets1; Cbfa1, core binding factor alpha1; Btg2, B-cell translocation gene 2; CysC, cystatin C; Lum, lumican; Bsp, bone sialoprotein; Opn, osteopontin; ON, osteonectin; TCRa, T-cell receptor alpha; Fli1, Friend leukemia virus integration 1; Col1a, collagen, type I, alpha 1; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; EMSA, electrophoretic mobility shift assay; EBSs, Ets binding sites.

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Key Words: Microrrays, osteoblasts, MC3T3-E1, Ets2, Cbfa1.

The MC3T3-E1 cell line was derived from mouse calvaria and undergoes an ordered and time-dependent developmental sequence including cell proliferation, differentiation and matrix formation similar to the *in vivo* process of bone formation. Within 30 to 35 days in culture, cells express many osteoblast marker genes and ultimately produce multilayered nodules and a mineralized extracellular matrix (1-5). During this process, Ets1 and Ets2 gene products are reciprocally expressed (6-8). Proliferating MC3T3-E1 cells express high levels of Ets1 transcripts, whereas differentiated osteoblasts express low levels of Ets1. However, Ets2 is expressed at higher levels than Ets1 during the differentiation and mineralization phases (6-8). A number of genes regulated by Ets1 and Ets2 are also active in osteoblast proliferation, differentiation, matrix mineralization and bone nodule formation *in vitro* (6, 9). Due to the serial nature of classic methods such as Northern blots, it was not possible to examine the expression of all such genes in developing osteoblasts by conventional methods. Rather, we used cDNA microarray technology to rapidly compare the expression of thousands of genes between early and late stages of MC3T3-E1 osteoblast maturation, finding that the most highly expressed genes during the proliferation phase were cytoskeleton-associated protein 2 (Ckap2), transforming acidic coiled-coil containing protein 3 (Tacc3) and stathmin 1 (Stmn1) (8). RT-PCR and Northern analysis confirmed that CysC, Btg2 and Lum are previously unrecognized genes expressed during the MC3T3-E1 mineralization phase (8, 10). This genomic approach has allowed us to focus upon specific subsets of genes that are Ets1 and/or Ets2 targets and which are likely to be essential to the specific function(s) of osteoblasts. In addition, we identified potential Ets binding DNA sequences in the promoters of several of these genes and tested their ability to bind Ets proteins. We also examined stably transfected MC3T3-E1 osteoblasts, that express Ets2 at all times, for altered morphology and Ets target gene expression.

Materials and Methods

Cell culture. MC3T3-E1 cells were maintained in minimal essential medium (α-MEM) containing 10% fetal bovine serum and 1% penicillin/streptomycin antibiotic mix. Cells were seeded at a density of 2.5 x 10^4 cells per cm^2 and sub-cultured twice weekly (1:8 ratio). For the induction of osteoblast phenotype, cells at 80% confluence were grown in the above medium supplemented with 10 mM β-glycerol phosphate and 50 mM ascorbic acid.

Stable transfection of the human Ets2 cDNA into MC3T3-E1 cells. The human Ets2 cDNA fragment was released by BamHI digestion of the construct pSG5Ets2 described in Seth *et al.* (11). The 1.5 kb purified fragment (GeneClean) was ligated into a linearized CMV promoter expression plasmid (pcDNA3, Invitrogen, California, USA) at the BamHI site creating pcDNAEts2. Separate cultures of MC3T3-E1 cells (80% confluent) were transfected with the pcDNA3 vector or the pcDNAEts2 construct using the "Lipofectamine" reagent kit (Invitrogen). After two divisions (~ two days), G418 was added to the medium for seven days. Colonies were isolated and trypsinized, transferred to individual plates and expanded for further use. RNA extracted from representative cultures for each clone was examined for expression of the transfected human Ets2 gene and the clonal cell line with the highest expression level was used for all subsequent analyses.

Von Kossa staining. Detection of mineralized deposits characteristic of osteoblastic matrix formation was done by Von Kossa staining to monitor the development of matrix mineralization, as we have described (12).

RNA extraction and Northern blot analysis. Total RNA was extracted using the guanidium thiocyanate method (13). Northern blot analysis was performed as described (13, 14). Probes were derived by restriction enzyme digestion as follows: 1 kb rat Bsp by EcoR1 digestion of pBS-Bsp (from Dr. J. Sodek); 327 bp rat OC by EcoR1 digestion of pT7T3 (from Dr. J. Aubin); 1.4 kb rat Opn by Not1 digestion of pBS-SK-Opn (from Dr. B. Mukherjee), 1.6 kb mouse Ets2 by BamH1 digestion of the Ets5779 plasmid (15), 1.5 kb human Ets2 by BamH1 digestion of the Ets7459 plasmid (16), 270 bp Cbfa1 by EcoR1/Xba1 digest of a Cbfa1 cDNA plasmid (Dr. Y. Ito (17)), 1.6 kb rat Col1a by Pst1 digest of the full cDNA cloned into pBS-SK (D. Rowe from Dr. J. Aubin) and 1 kb mouse GAPDH by Pst1 digest of the NM522 plasmid (vector pT7T3). Probes derived from 300 bp mouse CysC, 350 bp Btg2 and 350 bp Lum fragments were released by EcoR1 digestion of PCR fragments cloned into the TOPO vector as described (8). Purified inserts were used as probes after labelling with 32P-dCTP by random hexamer priming. Hybridizations were performed overnight at 50˚C and filters were washed at 65˚C as described (14). For blots hybridized multiple times, filters were washed 3 times at 65˚C for 30 minutes in 0.2X SSC/0.1% SDS, dried and exposed to X-ray film. After imaging, filters were stripped by soaking for 10 minutes in boiling 10 mM Tris (pH 7.5)/ 0.1% SDS, and imaged again to ensure complete removal of labelled cDNA before use in subsequent hybridizations.

Identification of EBSs in promoter regions. A database of results from differential hybridization of microarrayed cDNA spots (Incyte) with cDNA probes derived from day 34 and day 3 of MC3T3-E1 culture was analyzed (8). Genbank accession numbers for cDNAs producing greater than 1.7-fold balanced differential expression in the mineralization stage (day 34) were bioinformatically linked to mouse Unigene clusters (NCBI). Corresponding human Unigene clusters were linked through Homologene (NCBI). Promoter sequences for the human and mouse mRNAs linked to these Unigene clusters were identified by examination of related sequence records in Genbank for published references to transcriptional start sites and/or promoter analysis.

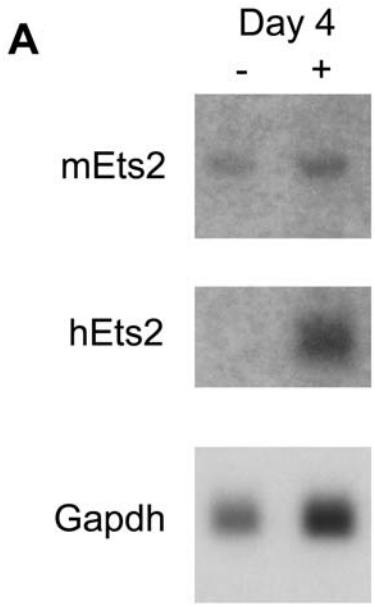

Figure 1. *Ets2 expression in transfected and untransfected MC3T3-E1 osteoblast cells. Shown are autoradiograms resulting from hybridization of radiolabelled probes as indicated with a Northern blot containing total RNA extracted from vector transfected (-) MC3T3-E1 cells and Ets2 transfected (+) MC3T3-E1 cells after four days of culture.*

Electrophoretic mobility shift assays. Protein: DNA interactions were visualized by EMSA essentially as described elsewhere (16, 18). Briefly, synthetic oligonucleotides corresponding to candidate EBSs were annealed to form double-stranded DNA, radioactively labelled, gel purified and allowed to bind with freshly *in vitro* synthesized protein produced using the TnT kit (Promega, Wisconsin, USA) from plasmids bearing coding regions for each of the Ets genes and Cbfa1 in the presence or absence of unlabelled competitor oligonucleotides. Reaction mixtures were then separated by electrophoresis through non-denaturing 4% polyacrylamide gel and visualized by autoradiography. The sequences of the oligonucleotides for CysC, Lum, Btg2, Opn, Bsp and ON are given below, with consensus EBS sites (C/A GGAA A/G/T A/C/T) in bold, and consensus Cbfa1 complex binding sites (T/G/A ACC G/A C A/G) (19) or G/A CC G/A CA (20)) underlined.

CysC 5'-AGCTCTCCTCC**TTTTCCG**GCGGCCAC
Lum PI 5'-AGCTAGTGAGTTCCTG**AGGAAATC**CACAA
Lum PII 5'-AGCTTGCA**TTTTCCT**GAGATTCTT
Lum PIII 5'-AGCTCTCTC**TCTTCCC**CCAACCCC

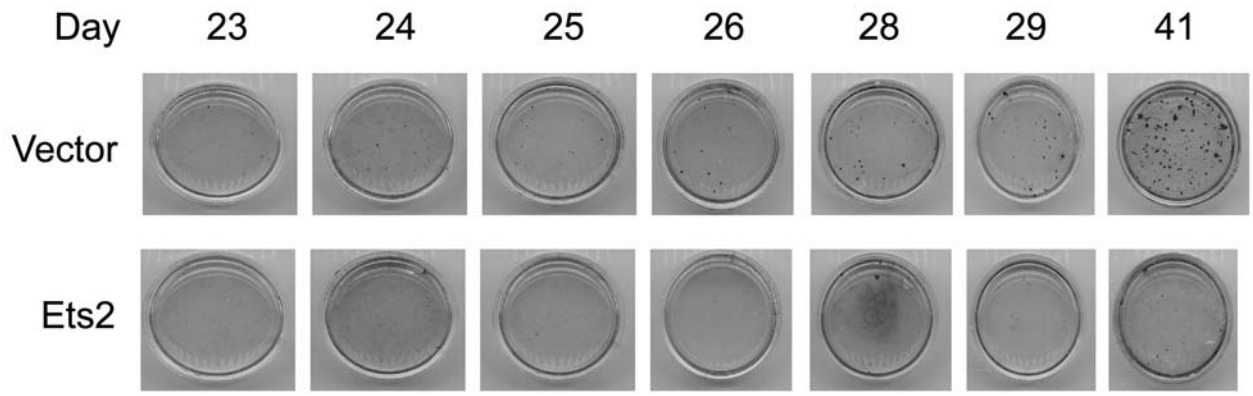

Figure 2. *Mineralized matrix in Ets2 transfected and untransfected MC3T3-E1 osteoblasts. Cultures of MC3T3-E1 cells stably transfected with the hEts2 expression vector (lower row) or the empty vector (upper row) were grown for the indicated days and then Von Kossa stained to reveal areas mineralized matrix (black).*

Btg2 PI	5'-AGCTCTTGACCT**ATTTCCC**TG**ACTTCCT**TGG TGGTTGACCTTG
Btg2 PII	5'-AGCTGGAG**GGGATGT**TTCAGAATGA CCTGAGC
Opn	5'-CGCGTTTTTAACCACAAAACCAGAGG **AGGAAGT**GTAG
Bsp	5'-AGCTAGGCA**GTTTCCTT**TGCAAAC**TTAGG AAAT**GTTCAGTT
ON	5'-CGCGCTCTGAGTG**GTTTCCT**GTTGCCTG

Results

Development of Ets2-overexpressing MC3T3-E1 cell lines. Two separate cell lines were created by stable transfection of MC3T3-E1 cells, one transfected with the empty CMV-promoter expression vector pcDNA3 alone and the other transfected with the pcDNAEts2 vector which harbors the coding region for human Ets2 within the pcDNA3 vector (12, 15). mRNA extracted on identical days from parallel cultures of the Ets2 and vector only cell lines was used for Northern analysis. Expression of human Ets2 mRNA in the pcDNAEts2 cell line was detectable from day 4 and not at all in the pcDNA3 cell line (Figure 1).

Ets2 overexpression affects bone nodule formation in MC3T3-E1 cells. The extent of mineralization and appearance of the Ets2 and vector-transfected MC3T3-E1 cells was observed by Von Kossa staining and recorded over the normal course of the mineralization phase from days 23-41 of culture (Figure 2). Comparison of matrix mineralization shows that, whereas many sites of mineralization are seen on day 41 of the control culture, mineralization of the Ets2-transfected culture is severely limited. After day 26 the control culture starts to mineralize, whereas no mineralization is detected with Ets2-transfected cells, indicating that Ets2 overexpression impairs mineralization of the osteoblastic MC3T3-E1 matrix.

Bone marker gene expression is down-regulated by Ets2 in transfected MC3T3-E1 cells. In order to examine osteoblast-related gene expression in the presence and absence of exogenous Ets2, a Northern blot of total RNA extracted from MC3T3-E1 cell line cultures on days 8-41 was serially hybridized with probes for mouse Bsp, Col1a, Opn and Cbfa, as well as the mouse and human Ets2 genes (Figure 3). Btg2, Lum and CysC genes were also included in this Northern analysis as we found the promoter sequences for each bind Ets1 and Ets2 proteins. A probe for the murine "housekeeping" gene GAPDH was used to control for differences in the amount of mRNA on the blot (Figure 3). Comparison between lanes on each day reveals lower levels of Bsp, Col1a and Btg2 mRNA when hEts2 is also expressed. Opn expression is slightly higher in each lane corresponding to the expression of stably transfected hEts2. CysC expression parallels that of GAPDH, suggesting that its expression was unaffected by Ets2 overexpression. Lum expression is much lower in lanes from cultures that overexpress Ets2 (Figure 3).

Ets transcription factor binding sites. Functional promoter regions of genes identified as up-regulated during the mineralization stage of MC3T3-E1 osteoblast culture (when the endogenous Ets2 gene is also up-regulated) were examined for Ets binding sequences (8). Table I lists the fold up-regulation discovered by earlier microarray analysis, as well as positions of consensus EBSs within bone-related functional promoter sequences found in Genbank.

Ets2 binds promoters of genes up-regulated during mineralization of the MC3T3-E1 osteoblast matrix. Cystatin C (CysC): The promoter sequence for mouse CysC in Genbank U10098 was used to design oligonucleotides encompassing EBSs within nucleotides-105-126 relative to the start of transcription (21). Both Ets1 and Ets2 proteins

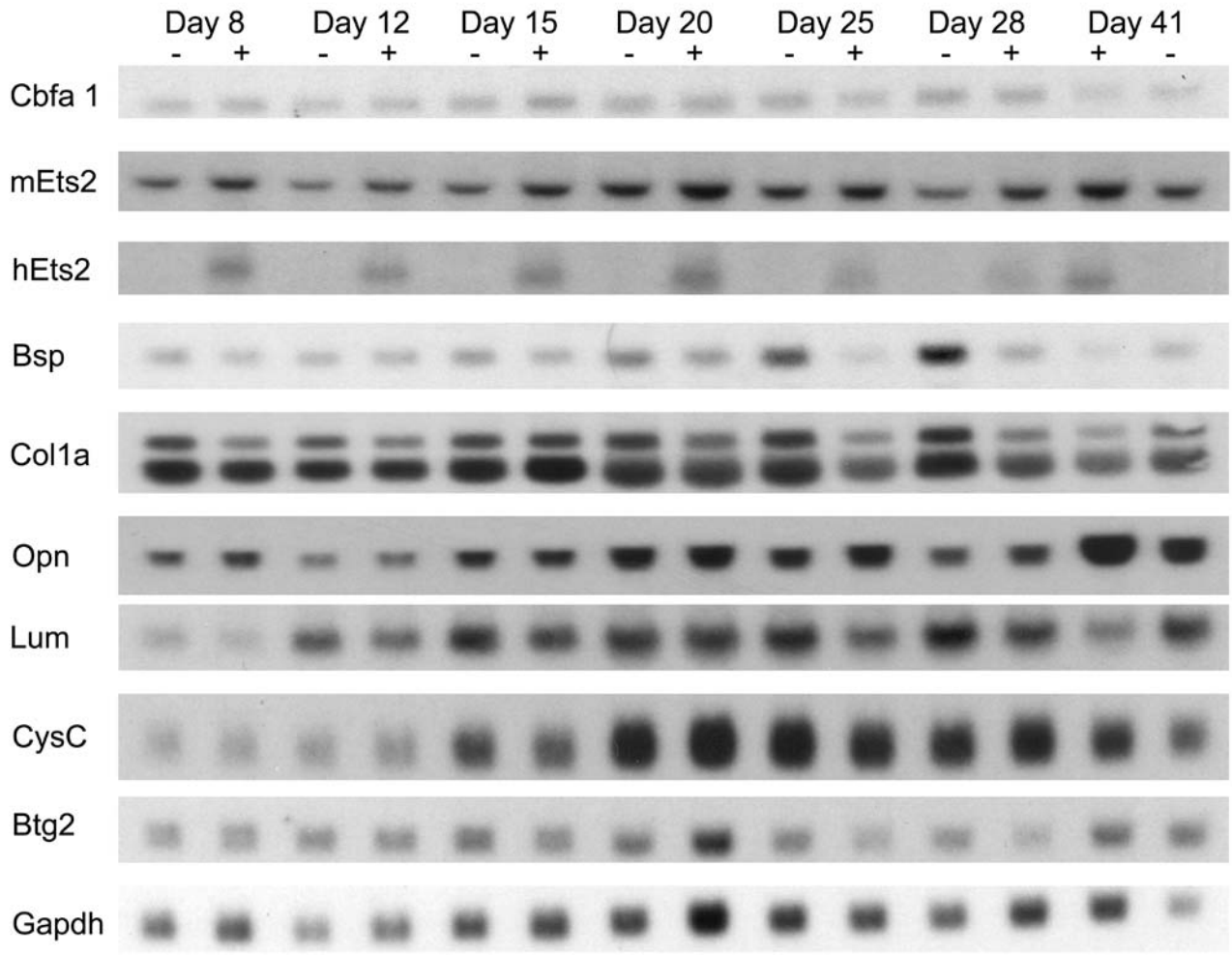

Figure 3. *Ets transcription factor and bone marker gene expression in Ets2 transfected and untransfected MC3T3-E1 osteoblast cells. Shown are autoradiograms resulting from hybridization of radiolabelled probes as indicated with a Northern blot containing total RNA extracted from stable clones of vector transfected (-) MC3T3-E1 cells and Ets2 transfected (+) MC3T3-E1 cells after the indicated days of culture.*

bound this oligonucleotide, resulting in gel-shift bands that were greatly reduced by inclusion of unlabelled competitor oligonucleotides (Figure 4A).

B-cell translocation gene 2 (Btg2): The mouse promoter sequence AF071079 was used to design oligonucleotides encompassing EBSs between nucleotides-1701-1728 (Btg2 PI) and-2525-2564 (Btg2 PII) relative to the start of translation. We performed EMSA on the Btg2 PI sequences with wild-type Ets1, Ets2 and Cbfa1 (Figure 4B). Ets2 binds the Btg2 PI oligonucleotide strongly, however wild-type Ets1 and Cbfa1 do not bind this sequence (Figure 4B). Neither Ets1, Ets2, nor Cbfa1 proteins bound to the Btg2 PII sequence (data not shown).

Lumican (Lum): Oligonucleotides encompassing EBSs within positions 194-213, 403-422 and 622-71 of the mouse Lum promoter sequence (Genbank AF186467) were synthesized. EMSA with these oligonucleotides indicated that Ets2 binds the Lum PII but not to the Lum PI or Lum PIII oligonucleotides, that Ets1 binds to Lum PIII but not to Lum PI and Lum PII, and that Cbfa1 binds to Lum PII (Figure 4C and data not shown).

Osteopontin (Opn) promoter: Oligonucleotides for EMSA with Ets1 and Ets2 were designed from the Opn promoter sequence (17). As expected, Ets1 protein and a complex of Cbfa1 with Ets1 were seen (Figure 4D). Interestingly, bands representing similar complexes were seen with Ets2 and Ets2/Cbfa1 (Figure 4D). This novel interaction between Cbfa1 and the Ets2 transcription factor on the Opn promoter indicates that regulation of Opn expression may be affected by additional proteins that could form ternary or higher order complexes.

Table I. *Consensus EBSs in functional promoter sequences of Ets target genes and genes up-regulated during MC3T3-E1 mineralization.*

Unigene Names	cDNA microarray ratio	Promoter accession	Size (nts)	Ets sites	Positions of EBS relative to transcriptional start site	Ets protein(s) binding	Ets2 affects mRNA
Lumican (Lum)	18.6	AF186467	871	3	712, 418, 208	Ets1;Ets2;Elk1;Fli1;PEA3	yes
Cystatin C (CysC)	5.0	U10098	930	1	115	Ets1;Ets2	no
Osteopontin (Opn); secreted phosphoprotein 1 (SPP1)	3.5	D14816	2980	9	2250, 2133, 2113, 2083, 1831, 1381, 1235, 913, 121	Ets1;Ets2;Fli1	yes
B-cell translocation gene 2 (Btg2); TIS21	2.2	AF361937	2202	5	1800, 1791, 1691, 969, 760	Ets1;Ets2;Elk1	yes
v-ets erythroblastosis virus E26 oncogene homolog 2 (Ets2)	1.7	M30137	1620	4	88, 647, 1087, 1260	unknown	yes
Collagen, type I, alpha 1 (Col1a1)	-1.3	X54876	1680	9	328, 354, 430, 705, 842,860, 876, 1378, 1391	unknown	yes
Bone sialoprotein (Bsp)	not done	AF071079	2472	6	386, 789, 806, 1014, 1583, 2250	Ets1	yes
Osteonectin (ON); secreted protein acidic and rich in cysteine (SPARC)	not done	J04951	638	4	27, 177, 267, 278	Ets2	not done

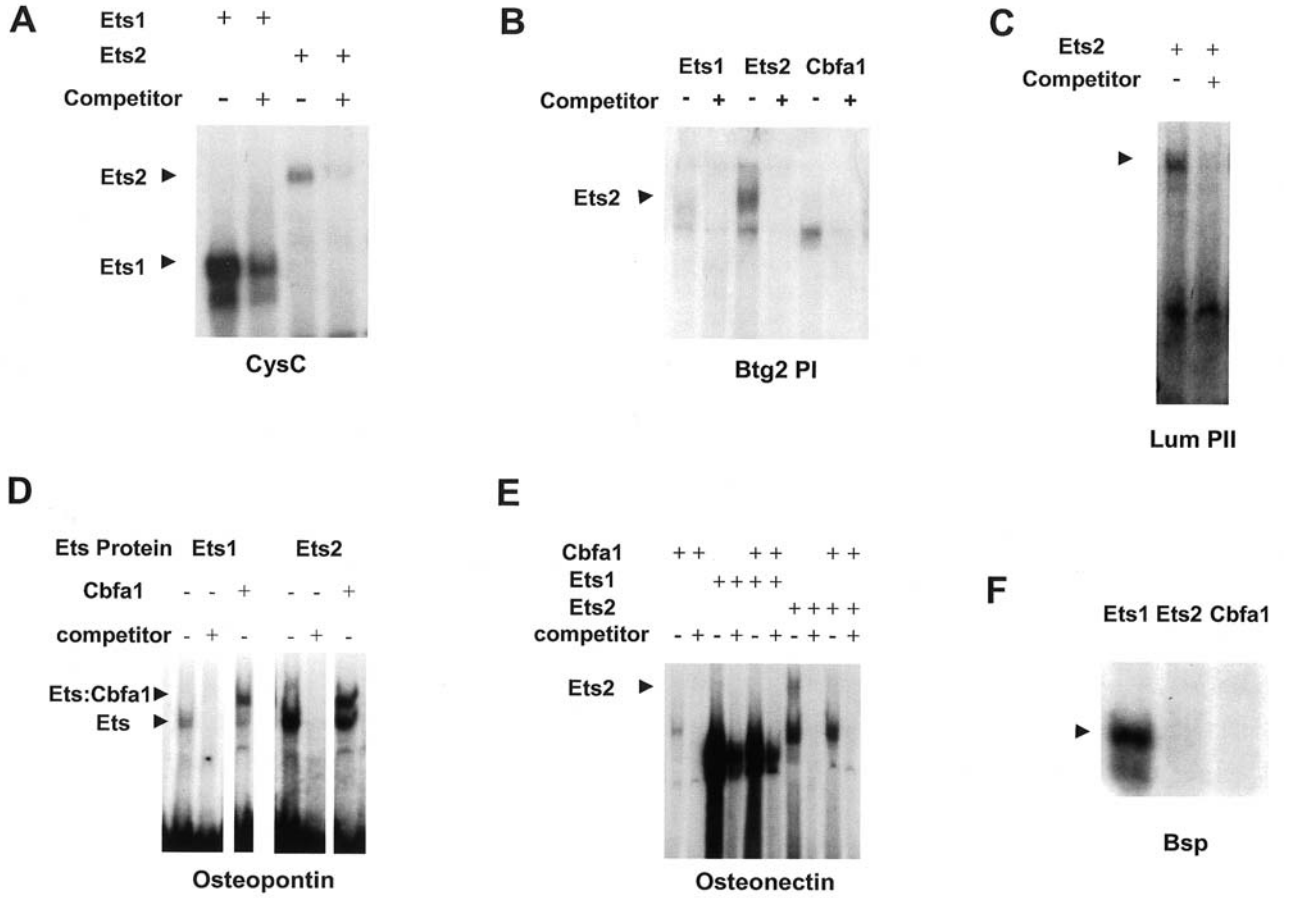

Figure 4. *Ets protein interactions on the bone-related gene promoter sequences. Shown are the autoradiograms resulting from electrophoresis of complexes formed by incubation of in vitro expressed Ets proteins as indicated (A-F) with radiolabelled oligonucleotides (-) or with both labelled and unlabelled oligonucleotides (+).*

Osteonectin (ON): The oligonucleotide used here encompasses the consensus EBS at nucleotides 1246-1270 of the ON promoter sequence U65081. Figure 4E indicates that, although Cbfa1, Ets1 and Ets2 can bind the ON promoter, the Ets proteins do not interact with Cbfa1 on the ON promoter. *Bone sialoprotein (Bsp):* The Genbank mouse Bsp promoter sequence AF071079 was used to design oligonucleotides encompassing two EBSs at positions -815 to -779 relative to the start of transcription. Only Ets1 protein was seen to bind this oligonucleotide (Figure 4F).

Discussion

Ets2 is a member of the Ets gene family of highly related transcription factors with the ability to bind to consensus *cis*-acting DNA elements in the promoters of numerous genes (reviewed in (9)). Transgenic mice with less than 2-fold Ets2 overexpression develop skeletal anomalies similar to those of trisomy-16 mice and humans with Down's syndrome, both of which have increased Ets2 gene dosage. In cultured osteoblastic cells we had observed that Ets2 is increased in mature cells, suggesting that Ets2 could have a more significant effect on Ets family target genes in mature osteoblasts relative to pre-osteoblasts. A further indication of this was derived from our cDNA microarray analysis of global gene expression between early and late stage MC3T3-E1 culture where Opn and Ets2 were found to be up-regulated (8).

The biological significance of Ets2 overexpression in osteoblast development specifically was tested by selecting clonal MC3T3-E1 cells transfected with a human Ets2 expression vector (Figure 1). The human cDNA was chosen in order to allow for rapid distinction between transcripts arising from the endogenous and transfected Ets2 genes. Overexpression of transfected hEts2 relative to endogenous Ets2 in proliferating preosteoblastic MC3T3-E1 cells was confirmed by Northern blot analysis (Figure 1). Later days of MC3T3-E1 development contained hEts2 along with increasing amounts of mEts2 message (Figure 3).

The phenotypic effect of exogenous Ets2 expression in these cells was examined by Von Kossa staining of mineralized matrix in hEts2 and vector-transfected MC3T3-E1 cultures (Figure 2). In the absence of hEts2, the cultures were heavily mineralized by day 41, as expected in this *in vitro* model of bone formation. In contrast, hEts2 expression resulted in a complete lack of mineralized matrix in the stably transfected culture. It appears that overexpression of Ets2 during the proliferation phase of MC3T3-1 development prevents the normal course of osteoblastic maturation that would lead to mineralization.

In order to observe transcriptional effects of the genetic program initiated and maintained by Ets2 expression, we used Northern blot analysis to monitor the expression of known osteoblast marker genes and three novel osteoblast genes (Figure 3). In general, these genes were down-regulated by exogenous Ets2 expression. Endogenous Ets2 appears to be up-regulated, an indication of possible Ets2 autoregulation. Cbfa1 and CysC expression appeared to be unchanged by additional Ets2. Opn expression was up-regulated by early Ets2 expression. Thus, early overexpression of Ets2 resulted in down-regulation of most bone marker gene expression, which is consistent with phenotypic changes such as lack of mineralization (Figures 2 and 3).

The possibility of direct Ets2 regulation of these genes was investigated by EMSA in order to identify those promoters that can or cannot bind Ets2 protein, the essential first step preceding transactivation of gene promoters. Bioinformatic analysis of promoter regions of the bone marker genes Col1a, ON, Bsp and Opn revealed consensus Ets binding sequences within 1000 bp of the start site for each (Table I). Potential Ets protein binding sites were also found within the promoters of Lum, Btg2 and CysC, three genes we found to be up-regulated in mature MC3T3-E1 cells (Table I) (8). Ets2 and Ets1 bind identical consensus sequences of many promoters and we included Ets1 protein for comparison with Ets2 on these sites. *In vitro* expressed Ets1 protein was able to form a protein: DNA complex on most of the promoter sequences tested (Figure 4). Ets2 protein bound every sequence except for Bsp, indicating that the down-regulation of Bsp1 by Ets2 overexpression discovered by Northern analysis would be indirect (Figures 3 and 4).

As with most transcription factors, strong activation of promoters by Ets proteins often requires interaction with additional transcription factors. One such interacting factor with known importance in osteoblast development is Cbfa1, the original osteoblast-specific transcription factor. No Cbfa1 binding was seen on the Btg2 (Figure 4B) and Lum oligonucleotides (Figure 4C), however, both the Opn (Figure 4D) and ON (Figure 4E) promoter elements resulted in shifted bands indicative of Cbfa1 binding. As expected for the Opn promoter element, an additional band was seen in EMSAs where Ets1 and Cbfa1 protein were included, confirming the interaction of these two transcription factors reported earlier (Figure 4D) (17, 22). We also found that Cbfa1 and Ets 2 interact, forming a similar complex on the Opn promoter (Figure 4D). Separately, Cbfa1, Ets1 and Ets2 each bound the ON promoter oligonucleotide, however, no cooperative Cbfa1 complexes were seen with either of the Ets proteins (Figure 4E). Both the ON and Opn sequences contain consensus Cbfa binding and EBSs, however these sites overlap in the ON promoter and are spatially separate in the Opn sequence, indicating that, as seen with other promoters, the distance between these potentially cooperative binding sites is important for the interaction of transcription factor proteins bound to them (16, 23).

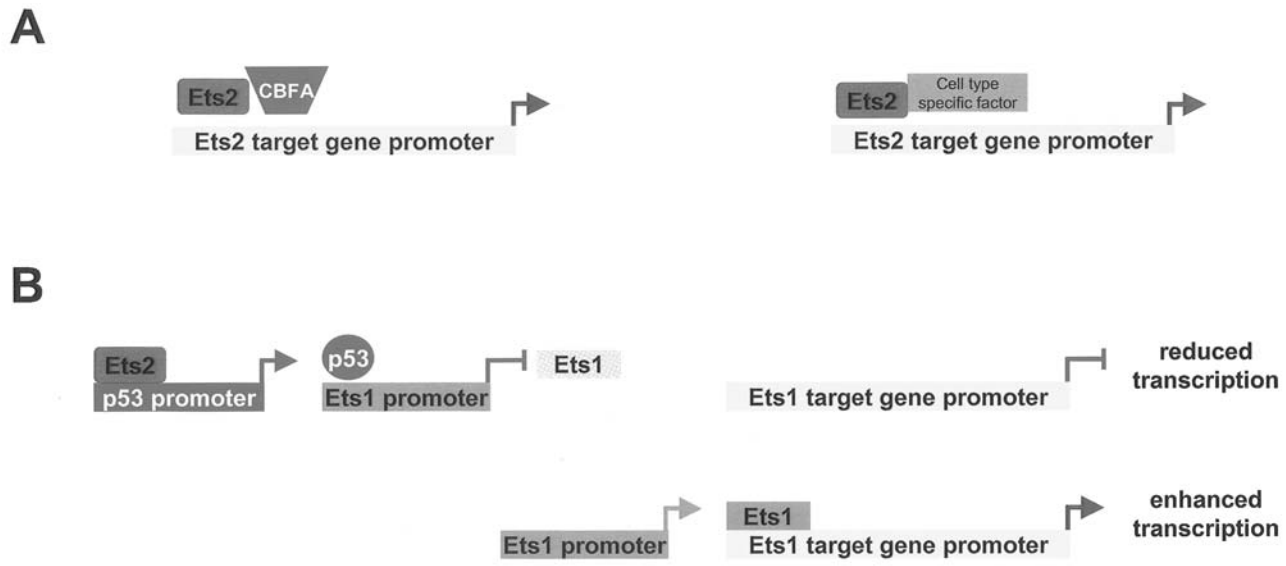

Figure 5. *A. Ets2 activation of osteoblast genes requires Cbfa binding sites in the target promoter. Other cell types may require other cell type-specific factors. B. Indirect regulation of Ets1 target genes by Ets2.*

Among the Ets target genes examined by Northern blot, only OPN was up-regulated by early expression of Ets2 (Figure 3). Cbfa1 expression was consistently high and unaffected by Ets2 overexpression, indicating that the interaction of Cbfa1 and Ets2 may be sufficient to induce the transcriptional up-regulation of OPN *in vivo*. This raises the possibility that Ets2 interaction with Cbfa1 is required for osteoblast-specific Ets2 target gene expression, as diagrammed in Figure 5A. Overall, the expression of hEts2 from day 1 of MC3T3-E1 culture had a negative effect on most bone marker gene transcription relative to vector-transfected cells (Figure 3). Expression of Ets2 during days when Ets1 is normally much higher than Ets2 could affect Ets1 and its target genes. This is possible because both Ets1 and Ets2 bind the same consensus sequence in many gene promoters, including most of those tested here. Many Ets transcription factors form ternary or higher complexes on such elements, and expression of Ets2 could directly compete for EBSs and, in the absence of appropriate ternary factors, result in relatively lower transcription activity (24).

The mechanisms for down-regulation of Ets1 and Ets1 target genes by Ets2 may also be indirect, acting through up-regulation of other transcription factors that affect Ets1 such as p53. Ets2 overexpression up-regulates expression of p53 in mouse cell culture systems (14, 16). In turn, p53 has been shown to down-regulate the expression of Ets1 target genes such as presenilin 1, while in the MC3T3-E1 system it could mediate the down-regulation of other Ets1 target genes after up-regulation by Ets2 (25). A mechanism for this is diagrammed in Figure 5B, where Ets1 target gene promoters are shown to have enhanced transcription resulting from Ets1 binding to elements of their promoters and reduced Ets1 target gene expression when the Ets1 promoter is repressed by p53.

In activated T cells Ets1 is reciprocally expressed relative to Ets2 and it was suggested that Ets1 gene products may be necessary to maintain T cells in a quiescent state (26). One might consider that the activated T cell is not merely proliferating but is actually differentiating into a cell expressing a large set of other gene products related to the new function, including lymphokines. Ets2 is also rapidly induced in cultured cells as they differentiate (27). This model may apply to the MC3T3-E1 osteoblast-like cell culture system, where preosteoblastic cells express Ets1 and mature osteoblast cells express Ets2 (6). In this context, Ets1 can be understood as part of the genetic program of a less differentiated cell type and Ets2 can be understood as promoting and maintaining a more differentially active phenotype.

Acknowledgements

This work was supported by CIHR project grant MOP37784 to A. Seth and by the CIHR Group in Matrix Dynamics (MGC48376), University of Toronto, Canada.

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Received June 30, 2004
Accepted August 6, 2004