Expression of the human oestrogen receptor-α gene is regulated by promoter F in MG-63 osteoblastic cells

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(O)estrogen receptor-α (ERα), a hormone-dependent transcription factor belonging to the steroid/thyroid-hormone-receptor superfamily, plays an essential role in the development and maintenance of the skeleton. Here we report the analysis of an unexplored sequence inside the bone-specific distal promoter F (PF) with respect to the regulation of ERα gene expression in bone. This sequence, 785 bp in size, is localized upstream of the assigned transcription start site of exon F, at $-117\,140$ bp from the originally described transcription start site $+1$. It contains a TA reach box, a conventional CAAT box and potential regulatory elements for many transcription factors, including Cbfa1 [OSE2 (osteoblast-specific element) core binding factor], GATA-1 [(A/T)GATA(A/G) binding protein], Sox5 [sex-determining region Y (SRY)-type HMG bOX protein, belonging to a subfamily of DNA-binding proteins with an HMG domain], Sry, AP1 (activator protein 1) and CP2 (activator of γ-globin). It is able to strongly activate the luciferase reporter gene in MG-63 osteoblastic-like cells, but not in MCF7 breast-cancer cells. This is in agreement with different transcripts that we found in the two cell types. The footprinting and electrophoretic mobility-shift assays (EMSAs) showed that, inside the region analysed, there were some sequences that specifically reacted to nuclear proteins isolated from MG-63 cells. In particular, we identified two regions, named PF*a* and PF*b*, that do not present binding sites for known transcription factors and that are involved in a strong DNA–protein interaction in MG-63, but not in MCF7, cells. The analysis of three transcription factors (GATA-1, Sry and Sox) that might bind the identified footprinted areas suggested a possible indirect role of these proteins in the regulation of ERα gene expression in bone. These data provide evidence for different promoter usage of the ERα gene through the recruitment of tissue-specific transcription activators and co-regulators.

Key words: control of gene expression, oestrogen receptor α, osteoblasts, transcription factors.

INTRODUCTION

The (o)estrogen receptors (ERs) belong to the nuclear-receptor superfamily and are transcription factors (TFs) that regulate a vast array of genes and physiological responses [1–3] in the female reproductive system, brain, cardiovascular system, adipose tissue and bone.

The two forms of ER, ERα and ERβ, are present in osteoblasts [4,5], indicating that oestrogen plays a direct role in the regulation of osteoblast functions, but limited information is available about the regulation of ER expression during bone development. Current information suggests that the effect of oestrogen in regulating cell differentiation, bone turnover and skeletal development is mostly mediated by ERα [6]. Consequently, the recruitment of regulatory *trans*-acting factors by osteoblasts or by bone-marrow stromal cells, and the stringent control on bone-specific regulatory regions of ERα gene promoters may determine the levels of ERα and the oestrogen responsiveness of the osteoblasts.

At the genomic level, distinct promoters, separated by nearly 150 kb of DNA, control the expression of the ERα gene, which appears to be strictly regulated by both transcriptional and post-transcriptional mechanisms [7–10]. The complex transcription unit of this gene and the generation of transcripts differing in their 5′-untranslated regions have recently been well described by Kos et al. [7]. Several results suggest that multiple promoters are utilized in a tissue-specific manner, strongly contributing to the regulation of ERα expression [7,11–16]. As far as bone is concerned, it was recently demonstrated that the upstream F mRNA (upstream RNA containing also exon F) isoform plays the major role in the generation of ERα in osteoblast cells [6], and at least two different ERα proteins were detected [6]. Nevertheless, protein factors and molecular mechanisms that direct its expression are not defined at present. Therefore, whereas some factors which positively or negatively modulate nuclear-receptor activity and which may help in mediating oestrogen-receptor transcriptional activity have been identified as nuclear proteins able to interact with proximal promoters of ERα gene [17–19], the so-called bone-specific distal promoter F (PF) is still under investigation.

It has been reported that osteoblastic cell differentiation is controlled by multiple TFs, including Cbfa1/Runx2, Osx, Hox, Osf1, Sry and Sox [20–22], which play a critical role in various stages of skeletal development. In order to investigate whether some of these TFs are involved in the regulation of physiological levels of ERα in osteoblastic cells, we analysed a 785 bp sequence localized inside the PF of the ERα gene, in front of exon F, for the presence of specific *cis*-acting elements, and for its functional role in mediating the transcription of ERα gene in MG-63 osteoblast-like cells. In particular, the results obtained provide a detailed map of important different classes of proteins that may contribute to the control of ERα gene transcription in bone. By footprinting and electrophoretic mobility-shift assay (EMSA) analysis we have identified in the PF sequence-specific DNA–protein interactions that are prevalent in MG-63 cells, but not in MCF7 breast-cancer cells, defining the DNA–protein complexes that might be involved in the regulation of specific ERα transcripts in bone.

Abbreviations used: DTT, dithiothreitol; EMSA, electrophoretic mobility-shift assay; ER, (o)estrogen receptor; ERE, (o)estrogen response element; FBS, fetal-bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HRP, horseradish peroxidase; α-MEM, α-minimal essential medium; PF, bone-specific distal promoter F; RT, reverse transcription; TF, transcription factor.

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These observations represent a preliminary step towards the identification of factors whose deficiency may predispose to osteopenic disorders, and the development of new therapeutic strategies designed to enhance bone differentiation through use of, for example, nucleic-acid-based drugs, such as TF decoys, against specific nuclear proteins [23] (synthetic decoys compete for binding of the TF to the consensus sequences in target genes).

EXPERIMENTAL

Cell culture, transfections and reporter assay

MG-63 and MCF7 cells were maintained at 37 °C in 5 % CO_2, in α-minimal essential medium (α-MEM) (Sigma Aldrich) supplemented with 10 % (v/v) fetal-bovine serum (FBS) (Roche). The transient transfections were performed for 24 h on cells grown to 60 % confluency, in six-well plates with Phenol Red-free MEM (Gibco BRL) plus 10 % charcoal-stripped FBS.

Transfections were performed by using FuGENE™ 6 transfection Reagent (from Fugent L.L.C., Madison, WI, U.S.A., via Roche). Cells were co-transfected using 5 μg of specific promoter construct plus 1 μg of β-galactosidase coding pBKCMV β-galactosidase reporter plasmid (Stratagene) as previously described [24]. The promoter construct pGL3B/F was generated directionally by cloning into pGL3B the *Bgl*II/*Mlu*I F fragment amplified by PCR using the following primers:

Forward primer:
5′-CGACGCGTACTTACTGCATAAACCACACC-3′

Reverse primer:
5′-GAAGATCTTTGAAGAGAAGATTATCACTC-3′.

After 24 h incubation, cells were lysed in a lysis buffer and luciferase activity was measured using the Luciferase Assay System (Promega). Protein concentration was determined using the Bradford Reagent for Protein Determination (Sigma-Aldrich). All the measured luciferase activities were normalized against the protein concentration and corrected for transfection efficiency with the β-galactosidase assay. Normalized luciferase activities were shown as relative luciferase activities, where that of the pGL3 promoter vector was taken as 100 %. The results were the averages for three independent transfection experiments for each plasmid.

In vitro DNase I footprinting analysis

Nuclear extracts were prepared from cultured cells essentially as described in [25], and protein concentration was determined using Bradford reagent. DNA–protein binding reactions were carried out in a total volume of 50 μl; 40 μg of nuclear extracts were incubated with 2 μg of poly(dI-dC) non-specific DNA (Pharmacia Biotech), 5 μl of 10 × binding buffer [1 M Tris/HCl (pH 7.5)/2 M KCl/1 M $MgCl_2$/1 M dithiothreitol (DTT)/0.5 M EDTA/0.1 % Triton X-100/50 % (v/v) glycerol] and 5–10 ng [(1–2) × 10^4 c.p.m.] of 5′-end-labelled probe for 30 min at room temperature, followed by DNase I digestion for 1 min using 0.2 unit of DNase and incubation at the same temperature. The reactions were stopped by adding 95 μl of stop solution (0.2 M NaCl/30 mM EDTA/1 % SDS/80 μg/ml yeast RNA), followed by one phenol/chloroform (1 : 1, v/v) extraction. Each sample was then precipitated by addition of 3 vol. of 95 % ethanol, incubated at − 20 °C overnight and DNA was recovered by centrifugation at 4 °C for 30 min. The DNA was dried, resuspended in 6 μl of loading solution and heated to 95 °C for 3 min before loading on a denaturing 6 % polyacrylamide sequencing gel. The double-strand DNA probes were prepared by PCR using the following primers:

F1: 5′-CAAGGCTTGTAGAGAAGAAA-3′
F2: 5′-TTACAGCCCCTTTGCTACTT-3′
F3: 5′-GTGAGAAGCTAGACCTCTG-3′
F4: 5′-CCTGCTTTTAGTTTAGCTGT-3′
F5: 5′-CATGCCTGTCTAGACTTCAA-3′
F6: 5′-TGAGATTTTCCAATCCTAGT-3′
R1: 5′-TCCTGCAGCAATTTTTAAGT-3′
R2: 5′-GCACTTCTTGTTACCGATTA-3′
R3: 5′-AAACATCATTCAGCACATAA-3′
R4: 5′-ATCATGTGTTAATCTGCCTG-3′
R5: 5′-GGAAATGCAGTAACTCAAAA-3′
R6: 5′-ACTGTCTTCTTATGCTATAGAAT-3′

Gel mobility-shift assay (EMSA)

Nuclear extracts were incubated with 0.1 ng (6000 c.p.m.) of ^{32}P-labelled probes in 1 × binding buffer [10 mM Tris/HCl (pH 7.5)/20 mM KCl/10 mM $MgCl_2$/10 mM DTT/5 mM EDTA/0.01 % Triton X-100/0.5 % glycerol] containing 1.2 μg of poly(dI-dC) · poly(dI-dC) (Sigma) for 30 min at room temperature. Specific competitors including unlabelled probes were added at 100-fold excess [24]. The DNA–protein complexes were separated from the uncomplexed DNA on 6 % polyacrylamide gels in 0.25 × Tris/borate/EDTA (TBE; 1 × TBE is 45 mM Tris/borate/1 mM EDTA) by electrophoresis at 150 V. Gels were dried and then exposed to X-ray film.

In supershift studies, 2 μg of the appropriate monoclonal antibody were incubated with the binding mix for 30 min at 22 °C before addition of the probe.

Northern-blot analysis

Northern analysis was performed using total RNA prepared by TRIzol extraction (Life Technologies) electrophoresed on 1 % Mops-containing gels. Prehybridization and hybridization with ^{32}P-labelled specific probes was performed as previously described [26].

Real-time quantitative reverse-transcription (RT)-PCR

Reactions were performed and monitored using an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Rotkreuz, Switzerland). The PCR 2X master mix was based on AmpliTaq Gold DNA polymerase (Applied Biosystems). In the same reaction, cDNA samples were analysed both for canonical ERα or isoform F and the reference gene [glyceraldehyde-3-phosphate dehydrogenase (GAPDH)] using a multiplex approach as described in the PerkinElmer User Bulletin no. 2. The probe for GAPDH was fluorescently labelled with VIC™, whereas probes for the genes of interest were labelled with FAM™. Cycle temperature and times were as previously described [23]. Expression levels were calculated by normalizing the mRNA amount to the GAPDH RNA using the procedure with the $2^{\Delta\Delta Ct}$ formula as previously described [22].

Immunocytochemistry

Immunocytochemistry analysis for ERα, GATA-1 and Sry/Sox was performed employing the streptavidin–biotin method using Ultraystain Polyvalent-HRP Immunostaining Kit (Ylem, Rome, Italy; HRP is horseradish peroxidase). Cells grown in chamber slides were fixed in cold 100 % methanol and permeabilized with 0.2 % (v/v) Triton X-100 (Sigma) in TBS (Tris-buffered saline; 136 mM NaCl/2.6 mM KCl/25 mM Tris, pH 7.4). Cells were

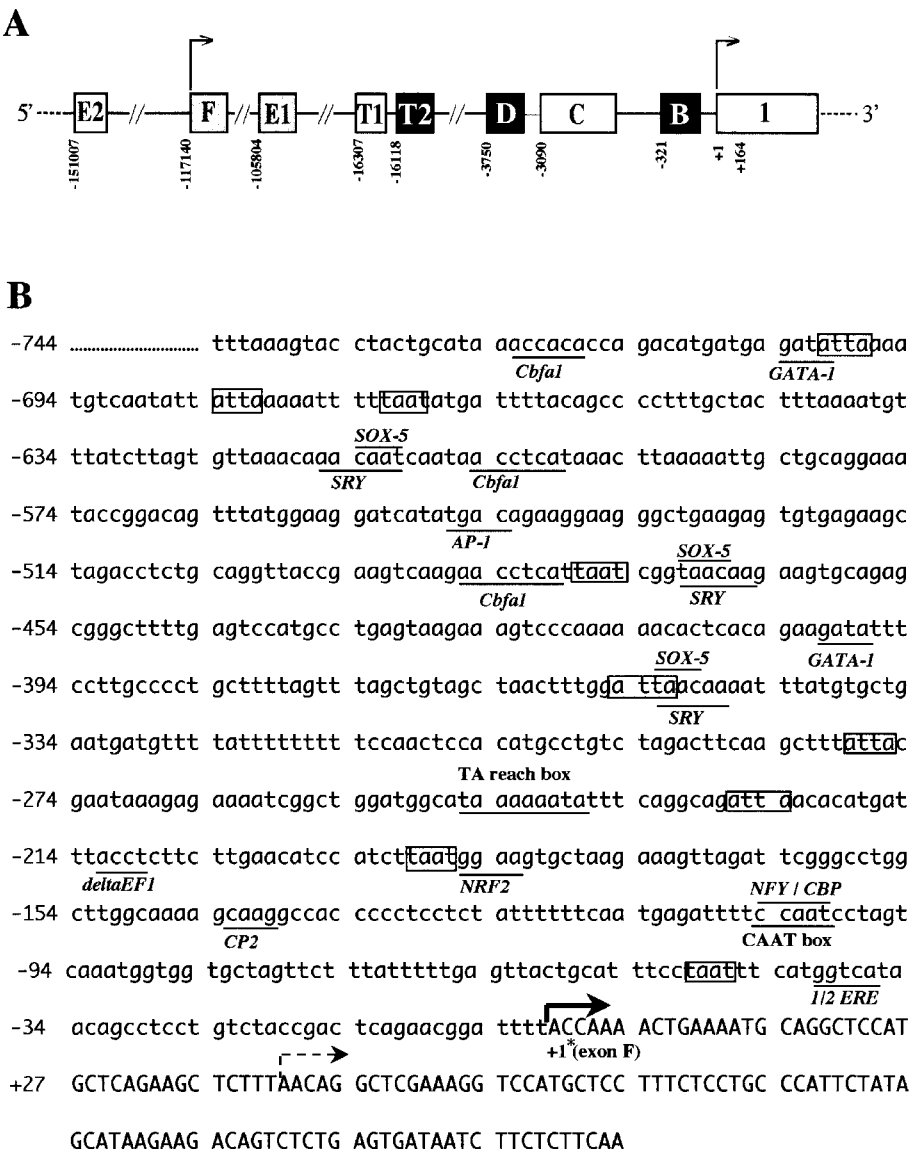

Figure 1 Nucleotide sequence of the PF region analysed in the present study

(**A**) A schematic representation of 5′ upstream region of the human ERα gene is shown. The nomenclature is in accordance with the recently described genomic organization of the human ERα gene promoter as suggested by Kos et al. [7]. The exons are indicated with boxes and the numbers below exons correspond to the distance from canonical transcription start site +1 in bp. The arrows represent two of the several promoters. The splicing acceptor site position at +164 inside exon 1 is indicated. (**B**) In the sequence of PF, here reported, the arrow corresponds to the exon F transcription start site, named here +1* and corresponding to position −117 140 with respect to the originally described transcription start site +1 of exon 1 [27]. In relation to +1* the upstream sequence was numbered. The broken arrow symbolizes the transcription initiation site of exon F observed by Thompson et al. [35]. The sites of possible *cis*-regulatory elements are underlined. The *taat* core motifs of Hox genes are boxed.

incubated in 3 % (v/v) H_2O_2, and the endogenous peroxidase was blocked with Super Block reagent (Ultraystain Polyvalent-HRP Immunostaining Kit; Ylem). Afterwards, the primary antibody, a monoclonal antibody for human ERα (rat anti-human H222, 1 : 60 dilution; generously provided by Professor Geoffrey Green, Ben May Institute for Cancer Research, Department of Biochemistry and Molecular Biology, Cancer Research Center, University of Chicago, Chicago, IL, U.S.A.) or monoclonal antibody for GATA1 (rat anti-human, 1 : 1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) or polyclonal antibodies for Sry and Sox (goat anti-human, 1 : 250 dilution; Santa Cruz Biotechnology) was applied and incubated at 4 °C overnight. Cells were then incubated at room temperature with anti-(polyvalent biotinylated antibody) (Ultraystain Polyvalent-HRP Immunostaining Kit; Ylem). After rinsing in TBS, streptavidin–HRP (Ultraystain Polyvalent-HRP Immunostaining Kit; Ylem) was applied and followed by the addition of substrate/chromogen mix (AEC Cromogeno Kit; Ylem). Following washing, cells were mounted in glycerol/PBS (9 : 1, v/v) and observed using a Leitz microscope.

RESULTS

Computer-assisted analysis of TF-binding sites

To characterize the distal PF of the ERα gene, we analysed a region of 785 bp in size, localized upstream of the assigned transcription start site of exon F (Figures 1A and 1B), at −117 140 bp from the originally described transcription start site +1 [27]. This sequence

(EMBL accession no. dJ404G5) was compared with sequences of the other ERα gene promoters without revealing any matches, and was then extensively searched for identity with previously described regulatory elements. The analysis, performed with the MatInspector V2.2 TRANSFAC 4.0 database program, revealed the presence of numerous consensus binding sites for known TFs distributed on the entire sequence. As shown in Figure 1, a search for typical promoter elements showed that PF lacked the canonical TATA box, but contained a TA reach box (TAAAAAATA) and a canonical CCAAT box, suggesting that the transcription of exon F is most probably determined both by classical and non-classical control elements. The entire sequence contained sites with a high level of identity with possible *cis*-regulatory elements, some of which resemble the consensus sequence of TFs. In particular, we found binding sites for TFs that are critical for osteoblast differentiation, establishment of the skeleton and organ development.

The sequence frequently contained the dinucleotide TA. In particular, numerous TAAT cores recognized by homoeodomain-containing proteins, such as Hox TFs [21], that are said to be indispensable for the establishment of the skeleton, were found. Three consensus sequences for Runx2/Cbfa1, which is an essential TF for osteoblast differentiation [20], were identified.

Three consensus binding sites for Sry [28] and Sox [22] (Sry-box related) transcriptional regulators, which recognize certain AT-rich sequences, were found at −355, −471 and −616, in relation to the start site of exon F transcription. One Ap-1 site and two GATA-1 like [29] sites were positioned at −547, −401 and −704 respectively. A consensus DNA sequence for members of the CP2 family [30] of TFs was also detected. In addition, *cis*-elements for DNA-binding proteins, such as CBP (CCAAT binding protein) and Nrf2 [31], able to interact with tertiary proteins like p300, which has been shown to have potent histone acetyltransferase activity [32], were positioned. Binding sites for negative TFs were not found, except for a sequence at −212, recognized by a DNA-binding protein named delta EF1/ZEB-1, that acts as transcriptional repressor in skeletal development [33].

Functional characterization of the promoter activity

In order to determine whether the transcription of upstream exon F was controlled by these possible *cis*-elements, transient transfection experiments using a luciferase reporter construct driven by the 785 bp sequence (Figure 2A) were performed. As illustrated in Figure 2(B), the transfection of MG-63 osteoblastic-like cells with 5 μg of 785-luc resulted in a 25-fold induced luciferase activity compared with the basal level. This activation was only modestly increased in MCF7 breast-cancer cells (1.5-fold induction). The experiments were performed in triplicate and results are presented as means ± S.D.

The different expression of the construct in MG-63 cells compared with MCF7 cells may be the result of differential expression of essential TFs in the two cell lines or underlying differences in transcriptional control. This suggests that the sequence analysed may be crucial for ERα transcription in MG-63 osteoblast-like cells.

While both reporter gene assays measured PF-regulated luciferase activity, cell-dependent expression of co-activators and co-repressors could alter the expression of luciferase activity. Therefore the variation in the results may be due to the type of cell transfected.

Analysis of ERα gene expression

Expression of appropriate ERα isoforms in cell lines selected for these studies was evaluated by RT-PCR quantitative analysis with a specific probe designed between the exon F and the exon E1

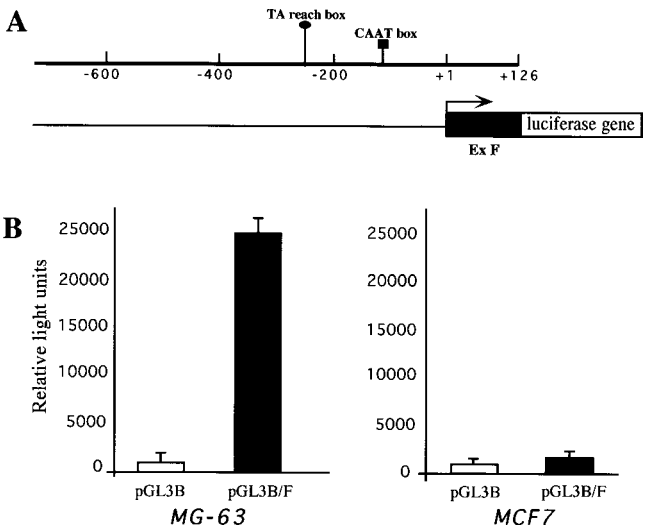

Figure 2 PF-induced transcription from a basal promoter

(**A**) Schematic representation of the construct used in this experiment. (**B**) MG-63 and MCF7 cells were transiently transfected with 5 μg of pGL3B 785-luc (pGL3B/F) or pGL3 basic vector (pGL3B) (promoter-less vector). Cells were harvested 24 h after transfection, and cell extracts were assayed for luciferase activity. The luciferase activity represents the average value for three independent experiments normalized by protein concentration of the transfected cells.

for the analysis of upstream transcripts, and a probe designed between the exon 5 and exon 6 to estimate the total ERα RNA. As shown in Figure 3(A), the mRNA levels for ERα in MG-63 and MCF7 cells differed substantially: both canonical ERα mRNA and upstream transcripts were expressed to a significantly greater extent in highly ERα-positive MCF7 than in MG-63 cells. Whereas in the MCF7 cells the levels of total ERα RNA were higher than the levels of upstream transcripts, all ERα transcripts detected in MG-63 cells were derived only from upstream transcriptional events, because total ERα expression coincided with F isoform expression. This confirmed the activity of distal promoters, including the PF, in bone, and cannot exclude the activity of promoters that are more upstream than PF, both in MCF7 and in MG-63 cells.

In order to validate this observation and to estimate the correct size of the transcripts, Northern-blot analysis was performed using exon 1 probe from the published human ERα sequence [29]. As shown in Figure 3(B), the presence of ERα mRNA was detected both in MCF7 and MG-63 cells, but with a different pattern. The probe hybridized mostly to 6.3 kb canonical mRNA and faintly to 7.5 kb F isoform in breast-cancer MCF7 cells, whereas, in MG-63 osteoblastic-like cells, only a prominent signal at the 7.5 kb region was found. This is the transcript size expected when the distal promoters such as promoter F are used and exon F splices to +163 [6,15]. The same Northern blot was reprobed with β-actin as positive control.

In agreement with mRNA expression data, the immunocytochemical experiments, reported in Figure 3(C), revealed the presence of the nuclear ERα protein in both cell lines, but not in the ERα-negative MDA-MB-231 breast-cancer cells used as control.

Promoter sequences and nuclear protein interactions

To characterize the potential binding sites for TFs within the PF promoter, *in vitro* DNase I footprinting experiments were performed and the activity of nuclear extracts from MG-63 osteoblastic-like cells and MCF7 breast-cancer cells were compared. The efficacy and the quality of both nuclear extracts

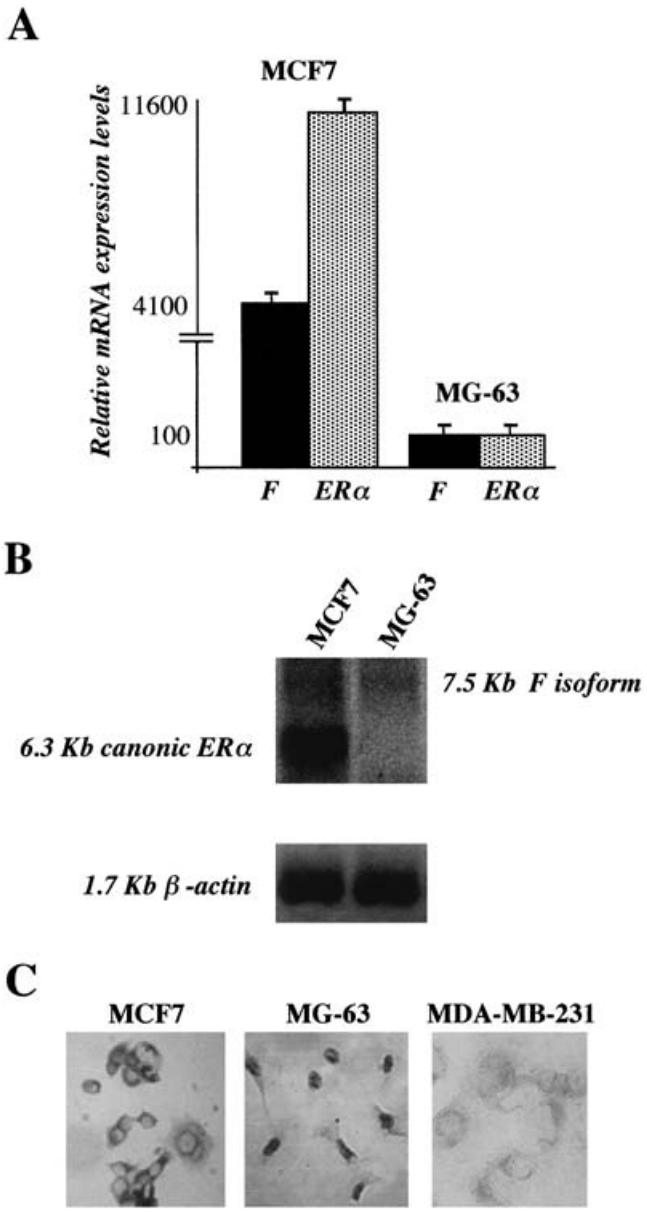

Figure 3 Analysis of ERα gene expression by quantitative RT-PCR, Northern blot and immunocytochemistry

(**A**) The cDNA obtained from MCF7 and MG-63 total RNA was subjected to quantitative TaqMan RT-PCR for upstream (*F*) and total (*ER*α) transcript analysis. The expression levels were normalized on the basis of GAPDH expression, and results of the experiments are reported as relative mRNA expression levels. Results are representative of three independent experiments carried out in triplicate; the $2^{\Delta\Delta Ct}$ method was used to compare gene-expression data; the S.E.M. was calculated. (**B**) Total RNA from MCF7 and MG-63 cells was extracted and specific transcripts for canonical ERα (6.3 kb), F isoform (7.5 kb) and β-actin were detected by Northern blot. (**C**) Immunocytochemistry for ERα nuclear protein in MCF7 and in MG-63 cells. As a negative control ERα-negative MDA-MB-231 breast-cancer cells was used.

was comparable, as demonstrated by the equivalence of binding of the extracts to the Sp1 and nuclear factor κB sequences (see Figure 4).

Different double-stranded DNA probes were produced using the pairs of primers reported in Figure 5(A) covering the entire 785 bp sequence.

The regions mainly protected from nuclease digestion by nuclear proteins were those represented in Figure 5(B). A

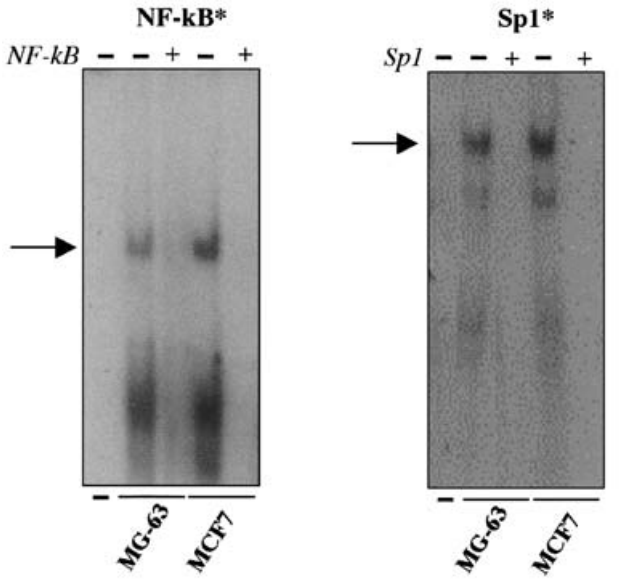

Figure 4 Binding efficiency of the extracts

Binding efficiency was determined by an EMSA. A 5 μg portion of nuclear protein extracts from MG-63 and MCF7 cells was mixed with labelled Sp1 and nuclear factor κB (NF-κB) oligonucleotides (10 000 c.p.m.), demonstrating the equivalence of binding of extracts to the sequences. The experiments were carried out in presence (+) or in absence (–) of the corresponding unlabelled oligonucleotide. The first lane (–) contains the probe alone without nuclear extracts and the arrows indicate the specific DNA–protein complexes.

generally higher level of protection was observed in MG-63 rather than in MCF7 cells. In the region from -720 to -550 protected sequences with a TAAT element and a potential binding site for Cbfa1 were detected. In addition, the region from -523 to -51 contained more faintly protected sequences whose analysis revealed the presence of two potential binding sites for previously described TFs such as Sry/Sox and GATA-1. In addition, two distinct sites with significant protection were identified at -379 and at -187 and named PF*a* and PF*b* respectively. These regions did not contain binding sites for known TFs. The major appreciable interactions were detected in the forward DNA strand for the region from -523 to -51, whereas both DNA strands of the region from -720 to -550 were involved in the protein interactions.

We next performed EMSA studies to investigate the formation of the DNA–protein(s) complex(es) by designing several oligonucleotides spanning on footprinting protected areas. As shown in Figure 6, for Cbfa1, Sry/Sox, GATA-1, PF*a* and PF*b* radiolabelled oligonucleotides a single prominent shifted band was detected. The formation of the complexes was competed for with a 100-fold molar excess of unlabelled self oligonucleotide. Nuclear extracts from MG-63 cells bound to the radiolabelled oligonucleotides with higher affinity than those from MCF7 cells. Interestingly, when the PF*b* radiolabelled oligonucleotide was used as a probe, the complex was not detectable in MCF7 cells. This result indicates that nuclear factors which are important for ERα gene transcription, in particular MG-63-specific TFs, may interact with the sequence inside the promoter F here analysed in a cell-specific manner. Therefore, the relatively weak DNA interaction of nuclear protein from MCF7 cells may explain the different promoter usage in breast and in bone.

When the EMSA experiment was performed with the oligonucleotide containing the TAAT element, several shifted

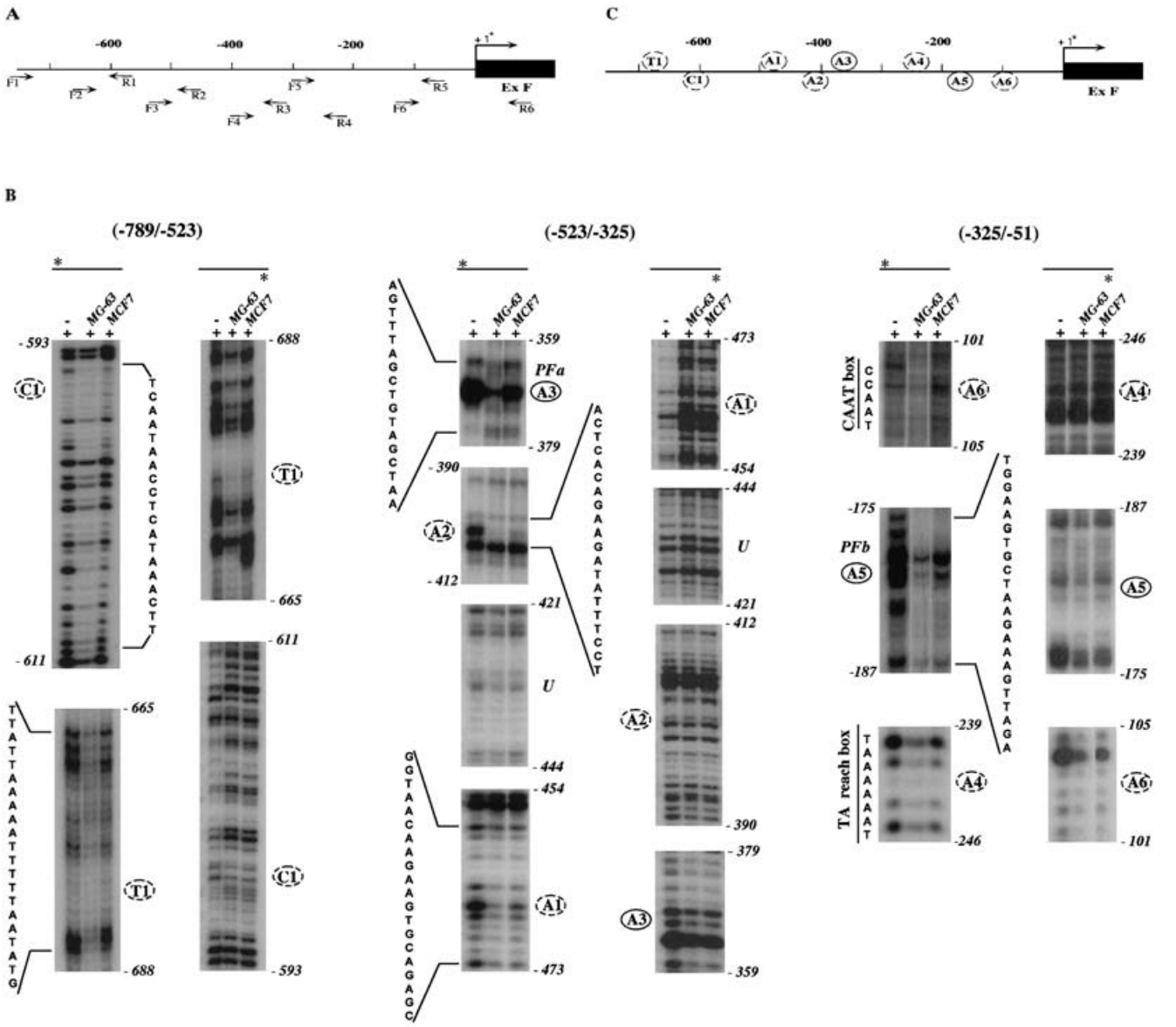

Figure 5 Identification of DNA–protein contact sites

(**A**) Location of the primers used in the footprinting experiments. (**B**) Nuclear extract proteins from MG-63 and MCF7 cells were incubated with the indicated double-stranded DNA probes (the regions − 789/ − 523, − 523/ − 325 and − 325/ − 51) labelled with [γ-^{32}P]ATP. Footprinting was carried out in the sense (left) and antisense (right) directions. Control lanes (− +) contain probe alone and digested with 0.2 unit of DNase I. The sequences in the protected regions (T1, C1 and A1–A6) are reported. 'U' represents a region without footprint that has been used in the EMSA experiments reported in Figure 6. (**C**) Schematic representation of the location of the footprinted areas. The outlined symbols represent the hypothetical binding sites for the TFs described in the text, and the PF*a* and PF*b* regions are represented by a continuous line.

bands were detected, suggesting the presence of many proteins interacting with the DNA in this region. Although footprinting data demonstrated that this region was more protected in MG-63 than in MCF7 cells, nuclear extracts from these cells showed a comparable binding capacity even if with a slightly different pattern. These findings led us to hypothesize that the proteins recognized by this sequence may be capable of altering the DNA structure of PF and acting in a cell-dependent manner through the same sequence.

Determination of TF expression

In order to investigate whether the stronger DNA–protein complexes detected at potential TF binding sites contained the specific nuclear proteins, the presence of GATA-1, Sry and Sox proteins was examined immunocytochemically using specific antibodies. As shown in the Figure 7(A), GATA-1 was diffusely distributed throughout the nucleus both in MG-63 and in MCF7. Conversely, slight Sry expression at the cytoplasmic level was observed only in MCF7, and no Sox expression was revealed in either cell lines (results not shown). These immunocytochemical results were confirmed by Western blotting (results not shown).

Data obtained suggest that Sox is either underexpressed in both the cell lines or absent.

To test the binding capability of GATA-1 to the identified regulatory region present in the promoter F, we next performed electrophoretic-mobility-supershift assays using the same antibody specifically targeting GATA-1, but we failed to detect a

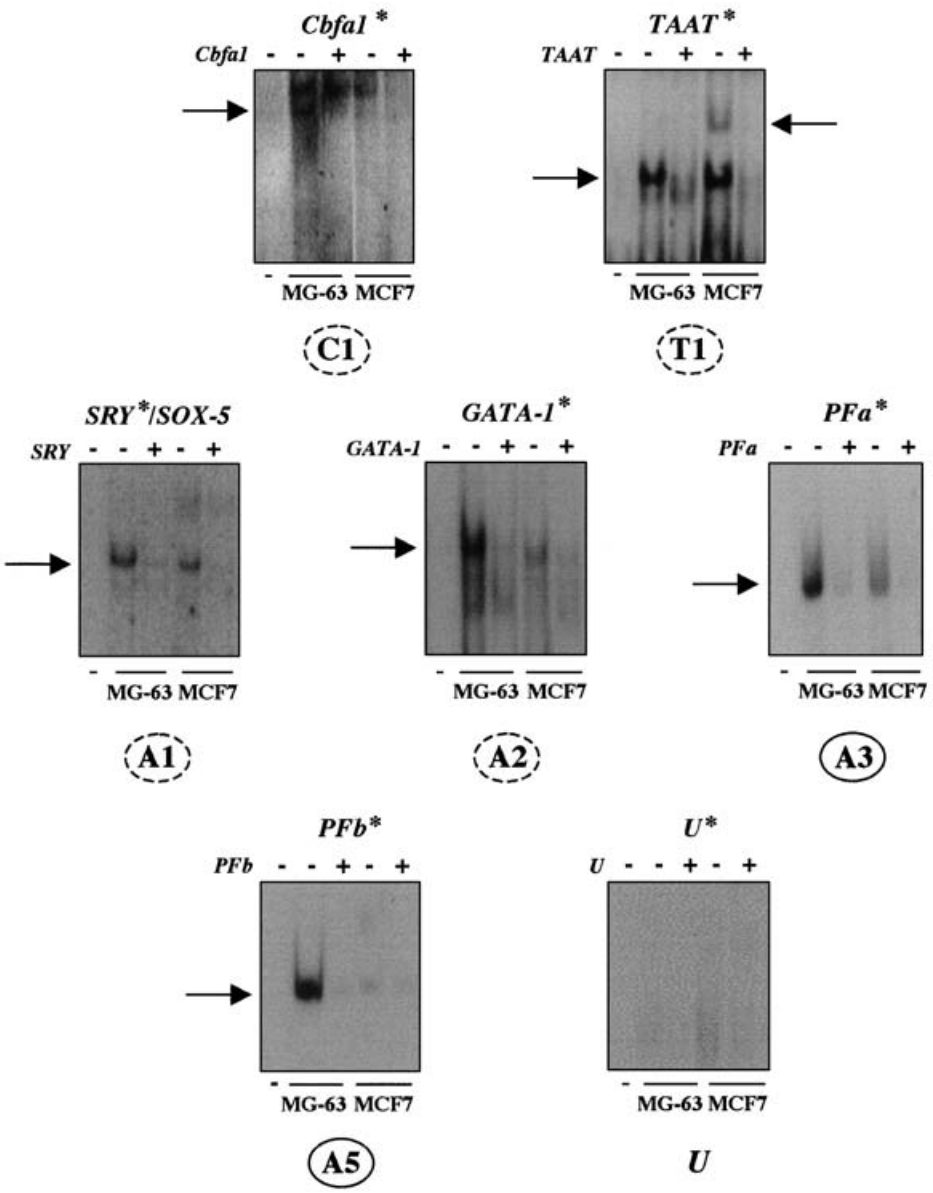

Figure 6 Gel-shift analysis with specific probes

On the basis of the footprinting experiments, six oligonucleotides were designed and used in the reported EMSA. The oligonucleotides Cbfa1*, TAAT*, SRY*/SOX-5, GATA-1*, PF*a** and PF*b** were labelled and allowed to react with the same amount of nuclear extracts from MG-63 and MCF7 cells, in the presence (+) or in the absence (–) of the corresponding unlabelled oligonucleotide. The first lane (–) contains the probe alone without nuclear extracts and the arrows indicate the specific DNA–protein complex(es). The U* probe belonging to the region − 444/ − 421 without footprint was used as a control.

supershifted complex as shown in Figure 7(B). Consistent with the previous results, we found no supershifted complex also using Sry and Sox antibodies. These results suggest that a direct binding of the TFs analysed may be excluded, even if their recruitment in a transcriptional co-operation mechanism may be postulated.

DISCUSSION

The results presented here demonstrate that PF of the ERα gene binds nuclear factors selectively present in MG-63 osteoblastic cells, and stimulates, at high levels, the transcription of the luciferase reporter gene in these cells. Consistently, we showed that the PF, and not the canonical promoter, is active in MG-63 cells because the only ERα mRNAs detectable were the upstream transcripts. Therefore these cells may be considered a good model with which to analyse the presence of regulatory proteins at the promoter F sequence. Even if recent studies indicate that the distal promoter F [6,15] is the prevalent promoter used in bone, its characterization in terms of factor-binding activity, up to now, has not been reported. In fact, the increasing complexity of the large 5′ region of the ERα gene has emerged from several studies, but the number of TFs or molecular mechanisms that have so far been described are limited, and only few specific elements in ERα promoters that are important in receptor expression have been identified.

The sequence here analysed, spanning − 117 925 to − 117 140 showed no identity with the other described promoters of the ERα gene. Unlike the PF analysed here, both promoter C and

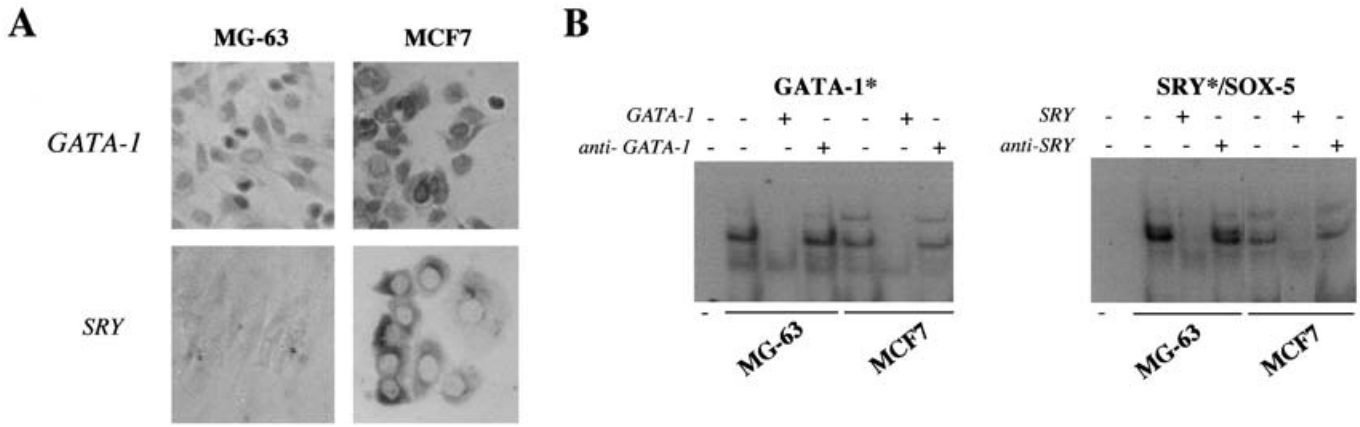

Figure 7 Analysis of the presence of GATA-1 and Sry proteins by immunocytochemistry in MG-63 cells

(**A**) The levels of GATA-1 and Sry expression were determined by testing the immunoreactivity of MG-63 and MCF7 cells with the specific antibodies. (**B**) Competition between MG-63 and MCF7 nuclear extracts and GATA-1, Sry and Sox-5 antibodies were tested in the supershift assay. The GATA-1* and Sry*/Sox-5 radiolabelled oligonucleotides, corresponding to $-412/-390$ and $-473/-454$ footprinted areas respectively, were used as probes in the reaction with the nuclear extracts from MG-63 and MCF7 cells, in the presence (+) or in the absence (–) of the corresponding unlabelled oligonucleotide, and in presence (+) or in absence (–) of 2 μg of the appropriate antibody (anti-GATA-1 and anti-Sry respectively). The first lane (–) contains the probe alone without nuclear extracts. No supershift was observed.

promoter T present several Sp1 sites [16,18,34]. Several (o)estrogen-response-element (ERE)-like elements and several half-EREs that may confer oestrogen responsiveness are scattered throughout the different promoters [34], but only one half-ERE is present in the promoter F. The sequence recognized by the ERF1 activating factor found inside promoter A [17] is absent in promoter F.

Consistent with the observations that the promoter usage varies between different tissues [7–14,35], characterization of the regulatory elements and the DNA–protein interactions that we have identified in the sequence here analysed supports the activity of the PF promoter in the bone.

Specific, even if faint, DNA–protein interactions that we have identified by footprinting and EMSA analysis overlap with binding sites for TFs putatively important for bone differentiation. These include: Cbfa1 ($-606/-600$), a TF implicated in osteoblast differentiation [20], GATA-1 ($-401/-398$), a regulator of erythroid cell differentiation [29,36], Sry and Sox ($-616/-611$ and $-471/-466$), proteins able to interact with other TFs performing a variety of important roles in vertebrate development, postulated also to act as architectural elements, promoting formation of contacts between factors bound at distant sites on DNA [22,28]. In addition, two regions, named PF*a* and PF*b*, were identified as the most strongly protected areas. DNA sequence analysis of these regions did not reveal the presence of binding sites for known TFs, suggesting that unidentified nuclear protein(s), produced only in MG-63 osteoblastic cells, may interact with elements inside PF*a* and PF*b* and play a critical role in the regulation of promoter F activity. In particular, the proximity of one *taat* element in each PF*a* and PF*b* region further supports the bone specificity.

In all cases the DNA–protein interactions were always much stronger in MG-63 osteoblastic cells than in MCF7 breast-cancer cells, as confirmed by results obtained with EMSA experiments. These differences in DNA-binding properties indicate that the PF is occupied by regulators or co-regulators that are mainly present in MG-63 cells. The faint DNA–protein interactions that are detectable in MCF7 cells may be explained by the presence of the same TFs in breast cells, but at a very low level, that is not sufficient for full promoter F activity. Alternatively, it is also possible that the same TF functions as an activator or a repressor of the specific PF depending upon the cell type, by mechanism(s) yet to be determined. Therefore, considering these interactions on the whole, a general low level activity of PF in different cell types may be postulated.

Differently from our hypothesis, only one of the three Cbfa1 consensus sites and only one of the several *taat* core motifs of Hox genes that we have identified by computer-assisted analysis appear to be involved in DNA–protein interactions in the cells analysed. It is possible that protein interactions with all binding sites are not required in the stage of differentiation of MG-63 cells. In addition, another important aspect to be considered is the influence of local chromatin structure on a co-ordinate assembly of multiple TFs. Therefore, even if MG-63 cells give a positive result when subjected to Cbfa1 immunocytochemical analysis (results not shown), it is possible that footprinting and EMSA assays, by using limited segments of DNA, failed to detect a stronger interaction within the PF sequence studied.

Immunocytochemical and Western-blot analysis detected the presence of GATA-1 protein, but failed to identify significant amounts of Sry and Sox proteins both in MG-63 and in MCF7 cells. In addition, although other bone-derived cells and experimental conditions are to be tested, data from EMSAs clearly demonstrated that, when antibodies against GATA-1, Sry and Sox were added, nuclear-factor binding to DNA was not interrupted, and neither was a supershift observed. These observations indicate that direct interactions between the TFs investigated and the *cis*-elements identified as footprinted areas are not present. Alternatively, the binding proteins are GATA-1, Sry and Sox-like factors not well recognized by the antibody used. It is also plausible that other proteins may prevent the direct recruitment of GATA-1, Sry and Sox. When the identity of these proteins is known, their potential role in the regulation of ERα gene expression and in the interplay with other TFs could be tested.

Although it is still unknown whether the region here analysed provides the unique binding sites for ERα-specific bone regulatory factors, our results are sufficient to hypothesize that the promoter F mediates the transcription of the bone-specific F isoform of ERα RNA, and that the repression of promoter F activity could induce a significant decrease in ERα gene expression in bone with a consequent possible loss of osteoblastic function.

Even if the mechanisms by which ERα acts on the process of bone modelling and remodelling [4,37–40] to maintain bone architecture have not been fully established, it is reasonable that oestrogen-dependent pathways in bone physiology and pathology are strictly correlated with the levels of ERα gene expression. Therefore the investigation of PF specific regulatory sequences and bone TFs that may be repressors and/or activators of ERα gene expression could provide new pharmacotherapeutic tools in the fight against bone disorders.

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