FAST TRACK
Journal of Cellular Biochemistry 105:965–970 (2008)

Journal of Cellular Biochemistry

Progressive Recruitment of Runx2 to Genomic Targets Despite Decreasing Expression During Osteoblast Differentiation

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ABSTRACT

The mRNAs encoding Runx2, a master osteoblast transcription factor, and its target gene Osteocalcin (OC), are commonly used as markers of osteoblast differentiation. We found that while OC mRNA levels do indeed increase during development of the osteoblast phenotype in MC3T3-E1 cultures, Runx2 mRNA levels surprisingly decrease. Neither translational control of Runx2 (based on Western analysis) nor regulation of its DNA-binding ability (assessed by electrophoretic mobility shift assay) could explain the unexpected opposite patterns of Runx2 and OC expression. Instead, a series of chromatin immunoprecipitation (ChIP) assays during osteoblast differentiation revealed that early on, when Runx2 protein amount and DNA-binding activity are maximal, it is practically absent from the OC promoter. At later stages, Runx2 is recruited to the OC promoter while Runx2 mRNA, protein, and in vitro DNA binding progressively decrease. We also followed Runx2 occupancy at a novel genomic target discovered by ChIP-Chip analysis of cells in which the OC promoter is maximally occupied. The results revealed that Runx2 is recruited to this locus and to the OC promoter with a remarkably similar temporal pattern. These observations highlight a mechanism that restrains Runx2-mediated transcriptional control by confining its access to genomic targets to a narrow window of time. The need for such stringent control is consistent with the severe consequences of Runx2 over-expression in vivo. J. Cell. Biochem. 105: 965–970, 2008.

KEY WORDS: Runx2; OSTEOCALCIN; OSTEOBLAST; DIFFERENTIATION; MC3T3-E1

Osteocalcin (OC) is a γ-carboxylated protein produced primarily by osteoblasts during bone formation. It is both incorporated into the bone extracellular matrix (ECM) and secreted into the circulation, where for decades it has been used clinically as a marker of bone turnover [Lian and Gundberg, 1988]. Additionally, circulating non-carboxylated OC may play a central role in energy metabolism [Lee et al., 2007]. In mice, OC expression is first detectable on embryonic day 15.5, concomitant with mineralization [Desbois et al., 1994]. Likewise, in several osteoblast culture models, OC mRNA levels and promoter activity have been shown to peak late during development of the bone phenotype [Owen et al., 1990; Frenkel et al., 1996, 1997; Quarles et al., 1997; Xiao et al., 1997]. Despite these striking associations, OC is not necessary for bone formation or development [Ducy et al., 1996]. Still, tissue-restricted developmentally controlled transcription from the OC promoter has rendered it a popular research tool in the pursuit of transcriptional regulatory mechanisms of osteoblast differentiation and bone formation [Lian et al., 1998]. Most notably, the master osteoblast transcription factor, Runx2, was discovered by way of its interaction with the so-called osteoblast-specific element 2 (OSE2) of the OC promoter [Ducy and Karsenty, 1995; Merriman et al., 1995; Banerjee et al., 1997; Ducy et al., 1997]. We report here that Runx2 is recruited to the OC promoter during maturation of MC3T3-E1 osteoblast cultures in parallel with increasing OC mRNA levels; however, this phenomenon occurs in spite of decreasing Runx2 expression. Additionally, we report that the developmentally regulated

Grant sponsor: NIH; Grant numbers: DK071122, CA109147, HL38578, T32 GM067587; Grant sponsor: NIH; Grant numbers: RR10600-01, CA62528-01, RR14514-01.

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Received 21 May 2008; Accepted 30 July 2008 • DOI 10.1002/jcb.21900 • 2008 Wiley-Liss, Inc.
Published online 26 September 2008 in Wiley InterScience (www.interscience.wiley.com).

occupancy profile of Runx2 at the OC promoter is shared by at least one other genomic locus.

EXPERIMENTAL PROCEDURES

CELL CULTURE

A subclone derived from the MC3T3-E1 osteoblastic cell line was used in this study [Smith et al., 2000]. Cells were maintained in α-MEM supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA). Starting at confluence, 10 mM sodium β-glycerophosphate and 50 μg/ml ascorbic acid (Sigma, St. Louis, MO) were added to support differentiation.

RNA ANALYSIS

RNA was collected from cells using the Aurum Total RNA Mini Kit (Bio-Rad, Hercules, CA) and quantitated by NanoDrop (Thermo Scientific, Waltham, MA). One microgram was then used to generate cDNA with the SuperScript III cDNA synthesis kit (Invitrogen). Two microliters from a 1:15 dilution of the resulting cDNA was used for real-time PCR with iQ^{TM} SYBR Green Supermix (Bio-Rad). Real-time PCR was performed on an iCycler with MyiQ single-color detection system (Bio-Rad). Primer sequences are given in Table I.

PROTEIN ANALYSIS

Cell extracts were prepared using the Nuclear Extraction Kit (Active Motif, Carlsbad, CA) following the *Preparation of Whole-Cell Extract* protocol. Protein concentration was determined with the Bio-Rad Protein Assay Kit (Bio-Rad) and 10 μg were used for Western blot analysis. Gel shift and super-shift assays were performed essentially as described with 20 μg protein [Luppen et al., 2003]. Anti-Runx2 antibodies (sc-10758, Santa Cruz, CA) were used for both Western and super-shift analyses.

ChIP ASSAYS

ChIP was performed essentially as described [Jia et al., 2003]; however, for the MC3T3-E1 cells, we found that an extended period of sonication (approximately 6 min) was required to achieve the optimal average chromatin fragment size of 500-bp. Primers used to assess Runx2 occupancy at specific genomic sites are listed in Table I. For chromosome-wide location analysis, the ChIP samples were amplified using the Whole Genome Amplification Kit (Sigma), labeled, and hybridized to NimbleGen array #7 of the MM8 tiling set (NimbleGen, Madison, WI). Peak calling was performed as described [Jia et al., 2008]. Despite excellent technical reproducibility, only a few peaks were validated by qPCR or shared by an independent biological replicate, demonstrating possible limitations of Runx2 ChIP-Chip analysis.

RESULTS

PARADOXICAL INVERSE RELATIONSHIP BETWEEN Runx2 AND OSTEOCALCIN EXPRESSION DURING OSTEOBLAST DIFFERENTIATION

OC mRNA levels steadily increase as the osteoblast phenotype progresses in various models, including MC3T3-E1 cultures (Fig. 1A). Because OC is a well-established target of the osteoblast master transcription factor Runx2, we monitored Runx2 mRNA throughout this process. Surprisingly, there was a steady decline between days 1 and 14 (Fig. 1B). This demonstrates that Runx2 is negatively regulated at the mRNA level during osteoblast development in the MC3T3-E1 model, in stark contrast to the positive regulation of OC (Fig. 1A).

Translational control of Runx2 has been reported [Sudhakar et al., 2001], and so we measured total Runx2 protein levels during MC3T3-E1 osteoblast differentiation. Despite evidence for translational up-regulation or protein stabilization immediately after confluence, (compare days 1 and 4 in Fig. 1B vs. 1C), Runx2 protein levels decreased thereafter (Fig. 1C), in agreement with the decreasing mRNA levels (Fig. 1B). Thus, the developmental up-regulation of OC occurs in spite of decreasing Runx2 protein levels, suggesting that Runx2 is subject to post-translational regulation.

DEVELOPMENTAL UP-REGULATION OF OSTEOCALCIN COINCIDES WITH ENHANCED Runx2 OCCUPANCY

Post-translational regulation of Runx2 may affect its ability to interact with DNA [Qiao et al., 2004]. To address this possibility, we monitored binding activity by EMSA with a Runx2-binding OSE2 probe. A specific complex that could be completely super-shifted by Runx2 antibodies first appeared on day 4 and diminished gradually until day 14 (Fig. 1D), which was consistent with Runx2 protein levels (Fig. 1C). This indicates that the intrinsic DNA-binding activity of Runx2 does not change substantially during development. Still, post-translational regulation could result in augmentation of Runx2 activity despite the decrease in its total protein levels and DNA-binding activity.

Runx2 may be subject to post-translational regulation that only affects DNA binding in vivo. To test this hypothesis, we monitored

TABLE I. Oligonucleotides Used in this Study

A. EMSA probes		
OSE2	5′-AGCTGCAATCACCAACCACAGCA-3′	5′-TGCTGTGGTTGGTGATTGCAGCT-3′
B. RT-qPCR primers		
OC	5′-TGCCAGAGTTTGGCTTTAGG-3′	5′-CATGAGGACCCTCTCTCTGC-3′
Runx2	5′-GCCTTCAAGGTTGTAGCCC-3′	5′-CCCGGCCATGACGGTA-3′
Twist2	5′-AGAGCGACGAGATGGACAAT-3′	5′-GTCATGAGGAGCCACAAGGT-3′
rpL10A	5′-CGCCGCAAGTTTCTGGAGAC-3′	5′-CTTGCCAGCCTTGTTTAGGC-3′
C. ChIP primers		
OC	5′-CTAATTGGGGGTCATGTGCT-3′	5′-CCAGCTGAGGCTGAGAGAGA-3′
Insulin	5′-TAGCACCAGGCAAGTGTTTG-3′	5′-CTGCTTGCTGATGGTCTCTG-3′
Glt28d2	5′-ATGCTGTGGTGGAAAACCTC-3′	5′-AGCTCTCCCCTTCAGTCTCC-3′
Runx2	5′-TCCTGCATGGACTGTGGTTA-3′	5′-CTTCATTCGCCTCACAAACA-3′

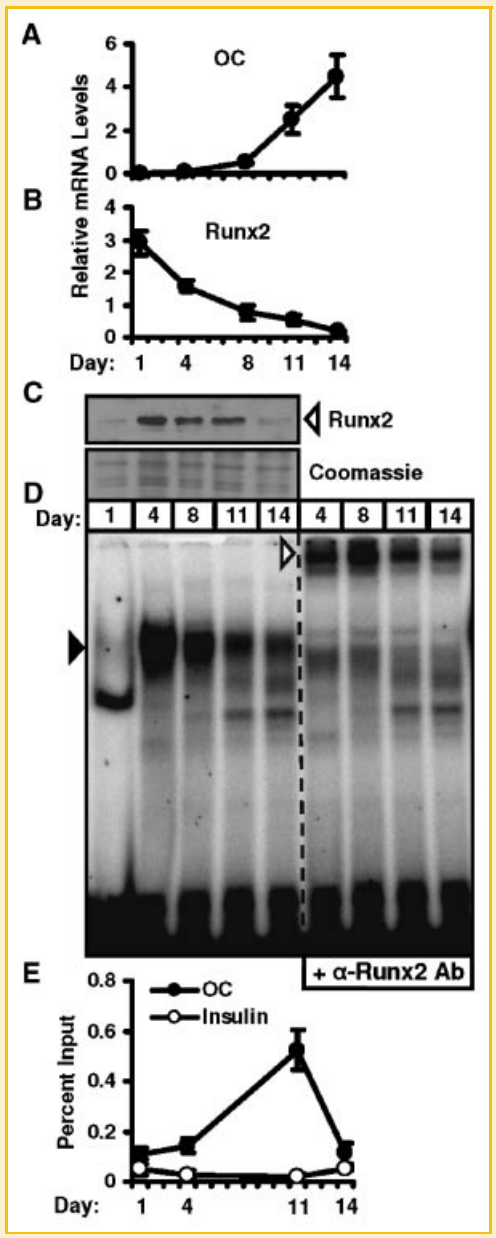

Fig. 1. Increased Runx2 occupancy in living cells, not its expression or DNA-binding activity in vitro, parallels OC up-regulation during osteoblast maturation. MC3T3-E1 cells were cultured in the presence of differentiation media for up to 14 days after confluence. Total RNA, whole-cell protein extracts, and cross-linked chromatin were collected concomitantly on days 1, 4, 8, 11, and 14. A,B: OC and Runx2 mRNA were measured by RT-qPCR and the resulting values were expressed relative to those obtained for Ribosomal protein L10A. C: Western blot analysis of Runx2. Coomassie-stained bands are shown to demonstrate equal loading. D: EMSA with an OSE2 probe was performed on the indicated days in the absence (left) or presence (right) of Runx2 antibodies. Black arrowhead—Runx2 shift; white arrowhead—super-shift. E: Runx2 occupancy in vivo was assessed by ChIP with Runx2-specific antibodies followed by qPCR with primers directed against the OC (closed circles) or Insulin (open circles) promoters. Values for each locus are expressed as a percentage of the signal obtained from input DNA. Error bars are standard deviations of triplicate measurements.

Runx2 occupancy of the OSE2 element in its native chromatin environment via ChIP assay of the OC promoter. For a negative control, we measured occupancy at the insulin promoter. Consistent with protein levels and in vitro DNA-binding activity, Runx2 was absent from the OC promoter on day 1 (Fig. 1E). By day 4, there was only a slight increase in occupancy (Fig. 1E) despite maximal protein levels and in vitro DNA-binding activity (Fig. 1C,D). Remarkably, occupancy increased between days 4 and 11 (Fig. 1E), as Runx2 mRNA, protein, and in vitro DNA-binding activity all decreased (Fig. 1B–D). These results indicate that Runx2 is blocked from binding to the OC promoter prior to the onset of OC expression, and that relief of this block coincides with a dramatic increase in OC expression. Thus, our data supports the notion that developmental regulation of OC by Runx2 is at the level of promoter occupancy, which is regulated post-translationally.

DEVELOPMENTAL REGULATION OF Runx2 OCCUPANCY AT THE Glt28d2 AND Runx2 LOCI SUGGESTS A GENOME-WIDE MECHANISM THAT RESTRICTS Runx2 ACTIVITY TO A NARROW WINDOW OF TIME

Regulation of Runx2 occupancy at the OC promoter during osteoblast development may occur via local changes in chromatin structure [Shen et al., 2003]. Alternatively, the mechanism that affects Runx2 occupancy at the OC promoter could be operative on a genome-wide scale. To address this possibility, we initially searched for additional Runx2-bound sites in the genome of MC3T3-E1 cells, in which the OC promoter was maximally occupied, by subjecting late-stage cultures to Runx2 ChIP-Chip analysis using the NimbleGen platform. Despite a high false discovery rate (see Materials and Methods Section), we identified a novel locus highly occupied by Runx2, which mapped 1.2-kb upstream from the transcription start site of Glycosyltransferase 28 domain-containing 2 (Glt28d2), a gene with no known function (Fig. 2A). Using qPCR with site-specific primers, we found that Runx2 occupancy at the Glt28d2 locus was as robust as the interaction with the OC promoter (Fig. 2B). We then performed an independent series of ChIP assays to measure recruitment of Runx2 to the Glt28d2 locus as a function of time during development of the osteoblast phenotype in MC3T3-E1 cultures. Remarkably, the dynamic profiles of Runx2 occupancy at the Glt28d2 and OC loci were nearly identical (Fig. 2C). Taken together, our results are consistent with the 4-phase model illustrated in Figure 3A. During phase I, when Runx2 mRNA is highest, its translation is blocked (Fig. 3A). In phase II, the block on translation is relieved; however, Runx2 protein is sequestered from the osteoblast genome (Fig. 3A). This sequestration, and the subsequent accessibility during phase III (Fig. 3A) may be partly attributed to a drastic decrease in the expression of Twist2 (Fig. 3B), which interacts with Runx2 and represses its function in immature osteoblasts [Bialek et al., 2004]. During phase III (Fig. 3A), as Runx2 occupies its targets, its own expression declines, possibly reflecting a negative feedback loop via OSE2-like sites at the Runx2 promoter [Drissi et al., 2000]. Indeed, ChIP analysis of the Runx2 promoter revealed no occupancy on day 4 and strong occupancy on day 11 (Fig. 3C). Finally, in phase IV (Fig. 3A), Runx2 expression and occupancy diminish, thereby closing a short window of opportunity (phase III) during which Runx2 can regulate its target genes.

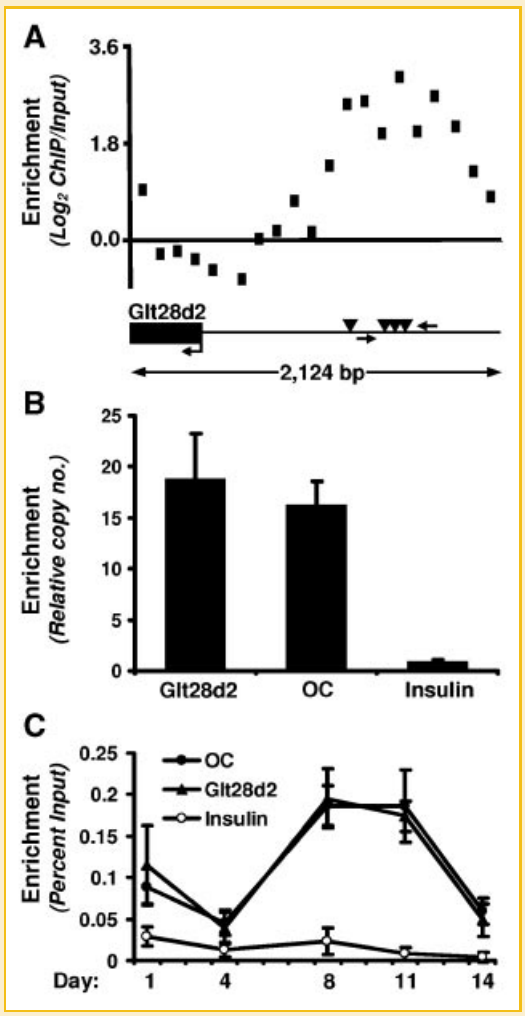

Fig. 2. Identification of a novel Runx2-occupied genomic target and characterization of its occupancy during osteoblast differentiation. A: Runx2 ChIPs were prepared from two independent late-stage MC3T3-E1 osteoblast cultures and were each subjected to ChIP-Chip analysis using NimbleGen chip #7 of the MM8 tiling set. Results from one ChIP-Chip experiment are shown for a reproducibly enriched locus. Enrichment values, based on hybridization intensity of ChIP DNA versus input DNA, are presented for twenty 50-bp probes (black squares) tiling a 2.1-kb fragment that encompasses the transcription start site (bent arrow) of the Glt28d2 gene (black rectangle). Consensus Runx2 elements are shown as black triangles and the positions of primers used to measure occupancy by qPCR are shown as horizontal arrows. B: Runx2 recruitment to the Glt28d2, as well as the OC (positive control) and the insulin (negative control) loci, was assessed by qPCR analysis of the DNA used for ChIP-Chip. C: Presence of Runx2 at the Glt28d2, OC, and Insulin loci was measured by an independent series of ChIP assays performed on samples collected from MC3T3-E1 cells on the indicated days after confluence. Values represent the percentage of signal obtained from the respective input DNA samples. Error bars are standard deviations of triplicate measurements.

DISCUSSION

Expression of Runx2, a master transcription factor required for osteoblast differentiation, is often considered an indicator of lineage progression. We observed that Runx2 mRNA levels actually decrease in MC3T3-E1 pre-osteoblast cultures during the 2-week period after confluence, in which they secrete and subsequently mineralize a bone-like ECM. Thus, while Runx2 mRNA is undoubtedly a good marker for early commitment of mesenchymal stem cells to the osteoblast lineage [Ducy et al., 1997], the present work suggests that it cannot be used to measure progression of the osteoblast phenotype in committed cells. In contrast, our results confirm the usefulness of OC mRNA as a marker for monitoring progression of the osteoblast phenotype in committed mesenchymal cells. Measurement of both Runx2 and OC mRNA is therefore an effective way to follow development of the osteoblast phenotype, with the appearance of Runx2 transcripts marking early commitment, and OC mRNA levels indicating the degree of differentiation in committed cells.

The incongruent patterns of Runx2 and OC expression can be at least partly explained by dynamic regulation of Runx2 at multiple levels. In very early MC3T3-E1 cultures (day 1), when Runx2 mRNA is at its peak, the absence of OC expression is attributable simply to absence of Runx2 protein. This disconnect between Runx2 mRNA and protein levels likely reflects a translational block [Sudhakar et al., 2001]. On day 4, the low OC mRNA steady-state level cannot be explained by such a block, because Runx2 protein (Western blot) and DNA-binding potential (EMSA) are in fact maximal at this point. Instead, OC transcriptional silence is attributable to a post-translational block in recruitment of Runx2 to the OC promoter (ChIP). Around day 8, this block is relieved and Runx2 gains access to its genomic targets despite down-regulation of its own expression. Increased occupancy at the OC promoter presumably contributes to the parallel rise in OC mRNA levels.

The ongoing increase in OC expression despite decreasing Runx2 expression and occupancy on day 14 (compare Fig. 1A,E) indicates that Runx2 is not the sole regulator of OC transcription. Indeed, other factors are likely involved [Hassan et al., 2004]. One practical implication of this observation is that changes in Runx2 promoter occupancy are not reliable predictors of changes in target gene expression. Accordingly, we found that Glt28d2 expression does not change substantially during osteoblast development (data not shown), despite a dramatic increase in Runx2 occupancy at its putative promoter region. This is consistent with the notion that transcription of Runx2 target genes can be stimulated, repressed, or unchanged depending on the specific promoter sequence and cellular milieu [Javed et al., 2001; Pregizer et al., 2007].

During early stages of culture progression, Runx2 was excluded from its genomic targets, despite peak protein levels and DNA-binding activity. This may be due to several mechanisms: (i) Runx2 could be sequestered in the cytoplasm [Kim et al., 2003]; (ii) Runx2 and its genomic targets could be partitioned to different sub-nuclear compartments [Zaidi et al., 2001]; (iii) Runx2 could interact with a repressor protein [Bialek et al., 2004; Hassan et al., 2004]; and (iv) Runx2 might require a collaborator that is absent during these early stages [Hassan et al., 2004]. Future work is warranted to elucidate both the exclusion mechanism and the signals that overcome it, thereby allowing recruitment of Runx2 to target loci. Such signals may originate from the ECM, which appears around the time that Runx2 occupancy increases (data not shown). They may result in post-translational modifications of Runx2 itself [Franceschi and Xiao, 2003] or they may alter the expression of proteins that

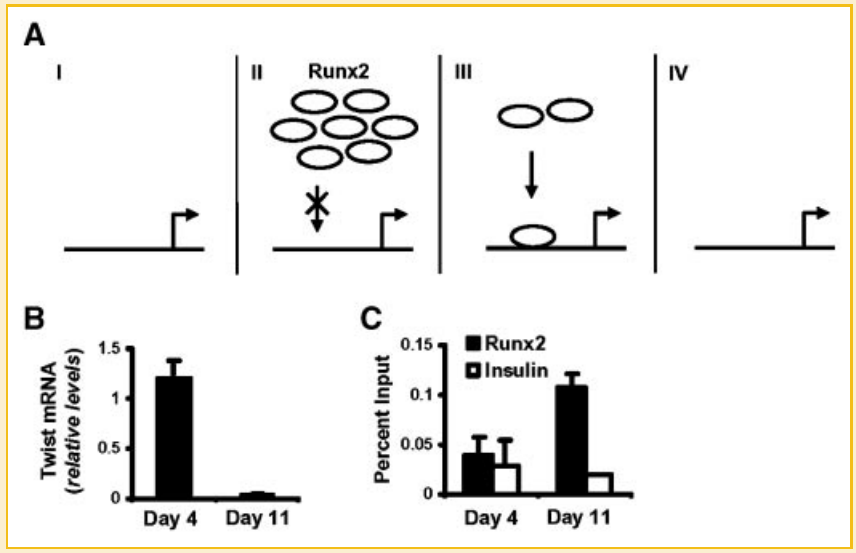

Fig. 3. Developmental regulation of Runx2 genomic occupancy: potential involvement of Twist2 and the Runx2 promoter. A: Developmental regulation of Runx2 genomic occupancy. A four-phase model is proposed for Runx2 occupancy of target loci: (I) Runx2 is absent due to translational inhibition; (II) Runx2 is highly expressed but does not occupy its targets; (III) Runx2 occupies its genomic targets but is expressed at lower levels; and (IV) Runx2 is absent due to attrition of Runx2 expression. B: Potential role for Twist2 in the transition between phases II and III. MC3T3-E1 cells were cultured in the presence of differentiation media. Twist2 mRNA levels were measured by RT-qPCR analysis on the indicated days and are expressed relative to those obtained for Ribosomal protein L10A (mean ± SD; n = 3). C: Potential role for Runx2 occupancy at its own promoter in the transition between phases II and III. Cultures parallel to those described in panel B were subjected to ChIP with Runx2-specific antibodies followed by qPCR with primers directed against the Runx2 (black bars) or Insulin (white bars) promoters. Values for each locus are expressed as a percentage of the signal obtained from input DNA (mean ± SD; n = 3).

regulate access of Runx2 to its genomic targets, such as Twist2 (Fig. 3B), Dlx3, Dlx5, or Msx2 [Hassan et al., 2004]. The incongruity between Runx2 expression and genomic occupancy is an important conclusion of the present work. It is not attributable to inter-experimental variation because all the assays—RT-qPCR, Western blotting, EMSA and ChIP—were performed on samples collected from parallel cultures. Evidence for a similar, complex regulation of Runx2 genomic occupancy can be found in a previous report employing primary rat calvarial osteoblasts [Hassan et al., 2004].

Increased Runx2 genomic occupancy is accompanied by decreased Runx2 expression. This may reflect negative auto-regulation, or it may be due to repression by other factors. In support of the former idea, Runx2 is recruited to its own promoter during the same time that its expression decreases (Fig. 3C). Regardless, osteoblasts have an effective system for quenching the expression of Runx2 around the time at which it gains full access to its genomic targets. Presumably, the resulting attrition of Runx2 protein is what eventually leads to the reduced occupancy seen on day 14 (Figs. 1E and 2C). The implication of these findings is that there is only a brief window of time during which the full transcriptional regulatory capacity of Runx2 is unleashed. The need for such stringent control is consistent with the deleterious outcome of Runx2 over-expression in vivo [Liu et al., 2001; Geoffroy et al., 2002].

In summary, we measured various aspects of Runx2 expression and function during development of the osteoblast phenotype in MC3T3-E1 cultures and found that Runx2 occupancy of genomic targets (demonstrated by ChIP assays) was the only parameter that increased in a manner consistent with promotion of differentiation. Unlike genomic occupancy, Runx2 DNA-binding potential (EMSA) and expression (RT-qPCR, Western analysis) both decreased as the cells matured. The mechanism regulating recruitment of Runx2 to its genomic targets likely involves post-translational control of Runx2 itself, as suggested by the near identical developmental patterns of occupancy at the OC and Glt28d2 loci.

ACKNOWLEDGMENTS

We thank Unnati Jariwala for technical assistance with EMSA, and Ting Chen and Fengzhu Sun for supervision of the bioinformatic analysis. This study was supported by grants DK071122, CA109147, and HL38578 from the NIH. BF holds the J. Harold and Edna L. LaBriola Chair in Genetic Orthopaedic Research and ZB holds the Ralph Edgington Chair in Medicine. SP was partially supported by NIH training grant T32 GM067587. SKB was partially supported by a California Community Foundation grant from the Arthritis Foundation Southern California Chapter. The experiments were conducted in a facility constructed with support from Research Facilities Improvement Program Grant Number C06 (RR10600-01, CA62528-01, RR14514-01) from the NIH/NCRR.

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