Journal of Cellular Biochemistry 88:493–505 (2003)

# IRES-Dependent Translational Control of *Cbfa1/Runx2* Expression

**Zhou-Sheng Xiao, Leigh G. Simpson, and L. Darryl Quarles***

Department of Medicine, Center for Bone and Mineral Disorders, Duke University Medical Center, Durham, North Carolina 27710

**Abstract** The P1 and P2 promoters of the *Cbfa1/Runx2* gene produce Type I and II mRNAs with distinct complex 5′-untranslated regions, respectively designated UTR1 and UTR2. To evaluate whether the 5′-UTRs impart different translational efficiencies to the two isoforms, we created SV40 promoter-UTR–luciferase reporter (luc) constructs in which the translational potential of the 5′-UTR regions was assessed indirectly by measurement of luciferase activity in transfected cell lines in vitro. In MC3T3-E1 pre-osteoblasts, UTR2 was translated approximately twice as efficiently as the splice variants of UTR1, whereas translation of unspliced UTR1 was repressed. To determine if the UTRs conferred internal ribosome entry site (IRES)-dependent translation, we tested bicistronic SV40 promoter-Rluc-UTR-Fluc constructs in which Fluc is expressed only if the intercistronic UTR permits IRES-mediated translation. Transfection of bicistronic constructs into MC3T3-E1 osteoblasts demonstrated that both UTR2 and the spliced forms of UTR1 possess IRES activity. Similar to other cellular IRESs, activity increased with genotoxic stress induced by mitomycin C. In addition, we observed an osteoblastic maturation-dependent increase in IRES-mediated translation of both UTR2 and the spliced forms of UTR1. These findings suggest that *Cbfa1* UTRs have IRES-dependent translational activities that may permit continued *Cbfa1* expression under conditions that are not optimal for cap-dependent translation. J. Cell. Biochem. 88: 493–505, 2003.


**Key words:** osteoblasts; OSF2; differentiation; expression

The *Runx* transcription factor family consists of three members that share a common gene structure in which two promoters drive expression of isoforms that differ in their 5′-UTRs and N-termini but are identical with respect to their DNA-binding domains and C-termini, which are encoded by exons 2–8 [Ogawa et al., 1993; Satake et al., 1995; Mundlos et al., 1997; Stewart et al., 1997; Geoffroy et al., 1998; Thirunavukkarasu et al., 1998; Xiao et al., 1998; Drissi et al., 2000]. The second family member, *Cbfa1/Runx2* (also known as *Cbfa1*, *Osf2*, PEBP2αA, and AML3), controls skeletalgenesis prenatally and osteoblast-mediated bone formation postnatally [Ducy et al., 1999; Banerjee et al., 1997; Zeng et al., 1997; Ryoo et al., 1998; Ducy et al., 1999; Jimenez et al., 1999]. The Type II isoform (also called major til-1, *Cbfa1*.iso or *Cbfa1*/p57) is transcribed from the distal P1 promoter and encodes a 529-aa protein that begins with the 19 amino acids MASNSLFSAVTPCQQSFFW derived from exon 1 [Stewart et al., 1997; Xiao et al., 1998; Fujiwara et al., 1999; Drissi et al., 2000] (Fig. 1A,B). The Type I isoform (also called PEBP2alphaA, *Cbfa1*/org or *Cbfa1*/p56) is transcribed from the proximal P2 promoter and encodes a 513 amino acid protein that begins with the 5 amino acids MRIPV located in exon 2 [Ogawa et al., 1993; Satake et al., 1995; Mundlos et al., 1997] (Fig. 1A). In humans, haploinsufficiency due to loss of function mutations within the common region of CBFA1/RUNX2 causes cleidocranial dysplasia (CCD), an autosomal dominant disorder characterized by short stature, delayed closure of the fontanel, hypoplastic clavicles, and dental abnormalities

Grant sponsor: National Institutes of Arthritis and Musculoskeletal and Skin Disorders; Grant number: RO1-AR-37308; Grant sponsor: National Institues of Health; Grant number: RO1 AR-45955.

*Correspondence to: L. Darryl Quarles, MD, Box 3036, DUMC, Durham, NC 27710.
E-mail: quarl001@mc.duke.edu

Received 14 August 2002; Accepted 14 August 2002
DOI 10.1002/jcb.10375

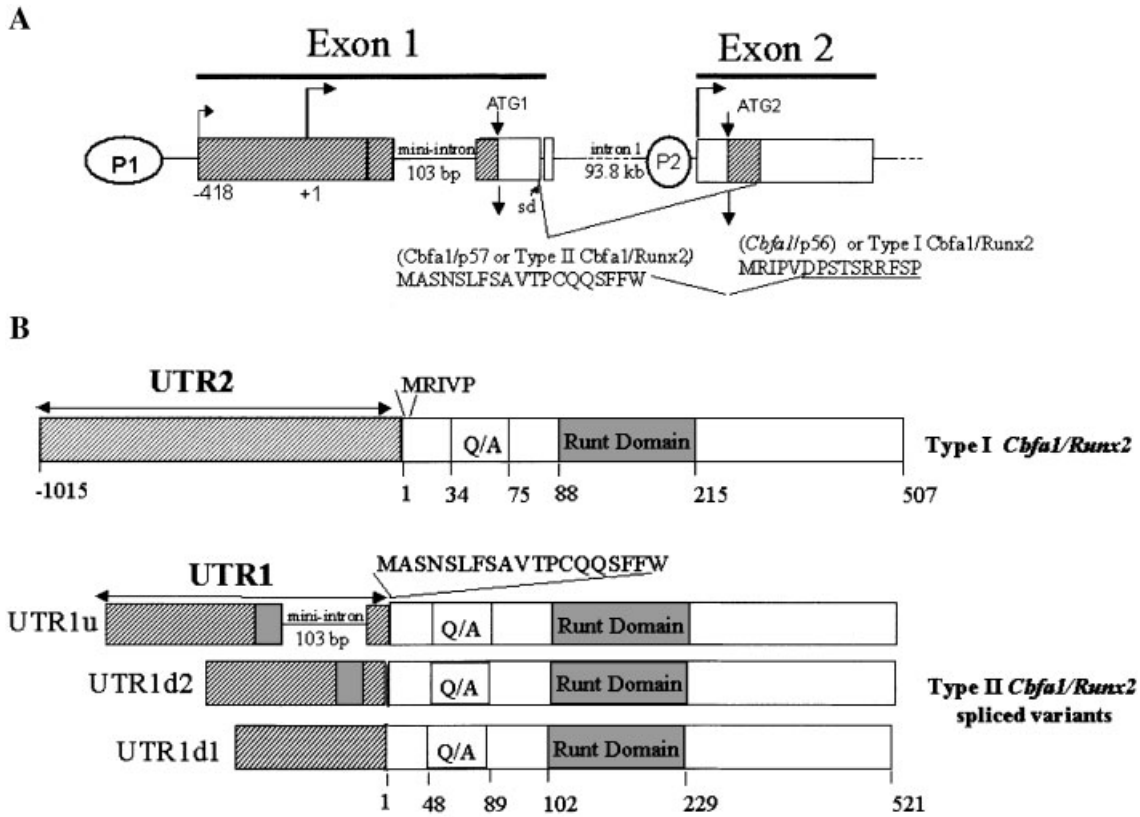


**Fig. 1.** *Cbfa1/Runx2* gene structure and N-terminal isoforms. **A**: Schematic of *Cbfa1/Runx2* gene showing location of P1 and P2 promoters, organization of Exons 1 and 2, and the respective origins of the 5′-UTRs and coding sequences for Type II and I *Cbfa1/Runx2* isoforms. **B**: Major transcripts derived from the P1 and P2 promoters with different 5′-UTRs (UTR1 and UTR2) and additional variants of UTR1 derived from alternative splicing. The *Cbfa1/Runx2* gene produces Type II and I gene products of similar function, suggesting that the purpose of the complex gene structure, consisting of two promoters and distinct 5′-UTRs, is to impart dual transcriptional and translational control of isoform expression.

[Mundlos et al., 1997]. A similar phenotype is observed in haploin sufficient mice with targeted disruption of the Runt domain. Null mice exhibit a cartilaginous skeleton [Komori et al., 1997; Otto et al., 1997] as well as impaired chondrocyte differentiation in certain parts of the skeleton [Kim et al., 1999; Takeda et al., 2001; Ueta et al., 2001] and abnormalities in tooth development [D'Souza et al., 1999; Jiang et al., 1999; Bronckers et al., 2001].

Although alternate promoter usage is thought to be the primary mechanism for regulation of *Cbfa1/Runx2* isoform expression, indirect evidence suggests that translational control may also play a role. First, discrepancies between *Cbfa1* protein and mRNA expression have been noted both in vitro and in transgenic mice [Xiao et al., 2001; Lengner et al., 2002]. Second, recent studies [Sudhakar et al., 2001] have shown that increased expression of *Cbfa1/Runx2* protein during osteoblast maturation is mediated by increased translational efficiency. Third, *Cbfa1/Runx2* UTR1 and UTR2 have complex UTRs with respect to their length and secondary structure potential, which could potentially impede translation by classic cap-dependent mechanisms [Morris and Geballe, 2000; Pestova et al., 2001; Dever, 2002]. Translation initiation of most RNAs occurs by a ribosomal scanning mechanism in which the 40S ribosomal subunit binds to the m(7)G-cap and then moves along the mRNA until an initiation codon is encountered. However, some cellular mRNAs can be translated via an alternative cap-independent mechanism mediated by internal ribosome entry site (IRES) elements in the 5′ untranslated region [Vagner et al., 2001]. Example of other cellular mRNAs include *c-myc* and *Fgf2*, in which IRES activity allows translation in situations where the

cap-dependent translation machinery is attenuated [Creancier et al., 2000, 2001; Stoneley et al., 2000]. Finally, there is a precedent for translational control by 5′-UTR elements in related Runx family members. In this regard, IRES-dependent translation was demonstrated for the UTR2 of the related *Cbfa2/Runx1*, in association with megakaryocyte, but not erythroid differentiation of multipotent precursor cells [Pozner et al., 2000]. Whether *Cbfa1/Runx2* 5′-UTRs possess similar IRES-dependent translational capabilities have not been evaluated.

In the current studies, we examined whether UTR1 and UTR2 exert translational control of *Cbfa1/Runx2* isoform expression in vitro. To assess the effects of different 5′-UTRs on overall translational efficiency, we tested whether UTR1 or UTR2 could alter translation of a heterologous message using osteoblastic and fibroblastic cell lines transfected with monocistronic constructs, in which the 5′-UTR of luciferase was replaced with the splice variants of UTR1 or UTR2. To assess the contribution of IRES to translational efficiency, we created bicistronic constructs in which either UTR2 or the splice variants of UTR1 were placed prior to the second cistron. Our findings suggest that elements in both UTR1 and UTR2 can impede cap-dependent translation and that UTR1 and UTR2 both possess IRES-dependent translational activity, which likely permits fine-tuning of *Cbfa1/Runx2* expression across a wide range of cellular conditions that do not favor cap-dependent translation.

## MATERIALS AND METHODS

### Monocitronic and Bicistronic Constructs

To create the monocistronic constructs, UTR1u (containing the mini-intron), UTR1d1, and UTR1d2 were amplified by polymerase chain reaction (PCR) using the primer sets 5′-CAGTACAAGCTTGTGTGAATGCTTCATTCGCC-3′ and 5′-GAGTCCATGGAGTCCCTCCTTTTTTTTCCA-3′ from previously reported cDNA [Xiao et al., 1998]. The fragment of UTR2 was amplified using the primer sets 5′-GCATCGAAGCTTCTGAAGTTAACAACGAAAAATTAAC-3′ and 5′-CAGTATCCATGGCACAACAGCCACAAGTTAGC-3′ from exon 2 of mouse genomic DNA. The PCR products were inserted into PGL3-Promoter (Promega) between the *Hin*dIII and *Nco*I sites to generate PGUTR1uF, PGUTR1d1F and PGUTR1d2F, and PGUTR2F, respectively (Fig. 2A).

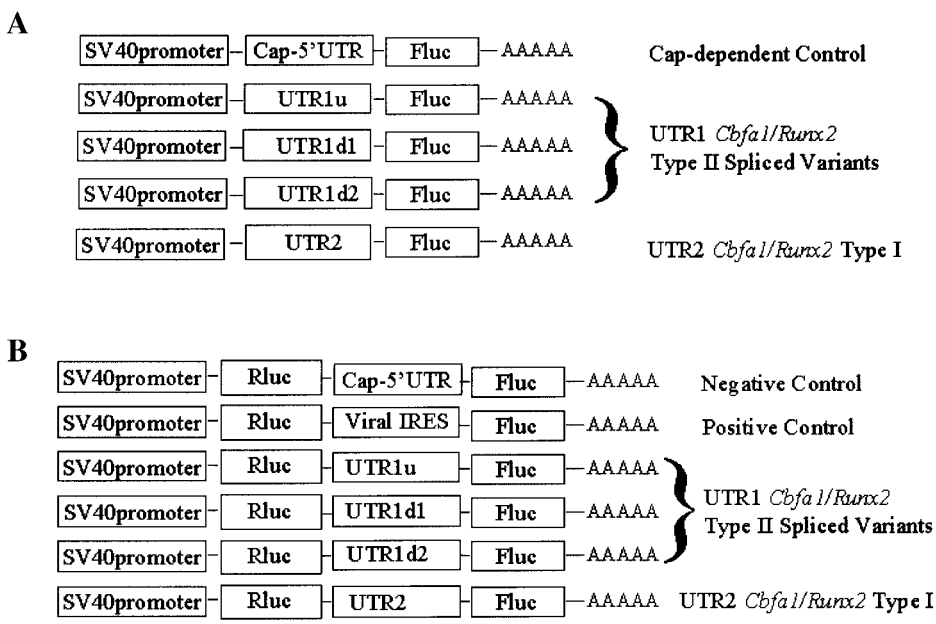


**Fig. 2.** Constructs for assessing translational activity. **A**: Monocistronic constructs. The control construct consists of SV40 driving expression the Fluc transcript containing its cap-dependent 5′-UTR. The test constructs have the luciferase 5′-UTR replaced with UTR2, or the UTR1 spliced variants. **B**: Bicistronic constructs for assessing IRES activity. SV40 drives expression of transcripts containing Rluc, an interposed UTR, and Fluc. Fluc activity is present only if there is IRES-dependent translation by the interposed UTR. The interposed UTRs consist of a viral IRES2, UTR2, and UTR1 spliced variants. Since the constructs are driven by the same promoter, differences in luciferase activity are used as a measure of translational activity.

To create the *Runx2* bicistronic constructs, the above UTR-firefly luciferase (Fluc) cassettes were excised and inserted into PRL-SV40 (Promega) at the XbaI site downstream of the *Renilla* luciferase (RLuc) coding region. We also created control bicistronic vectors in which the intercistronic UTR was obtained from a cap-dependent 5′-UTR with no IRES activity (negative control) or from (pIRES2), a modified encephalomyocarditis IRES that exhibits no cap-dependent activity (positive control (Fig. 2B). The endogenous 5′-UTR of Rluc directs cap-dependent translation of Rluc, whereas Fluc is produced only if the intercistronic UTR confers IRES-dependent translation. Expression of results as the ratio of Fluc: Rluc controls for differences in transfection efficiency and mRNA half-life.

### Cell Culture

We used mouse MC3T3E1 cells as a model of osteoblast development [Quarles et al., 1992]. These cells recapitulate the osteoblast maturational sequence when cultured in α-minimum essential medium (αMEM) in the presence of ascorbic acid and β-glycerol phosphate [Quarles et al., 1992]. The pluripotent C3H10T1/2 and C2C12 cell lines were grown in basal medium eagle (BME) and Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT). NIH3T3 cells were grown in DMEM (Gibco-BRL, Gaithersberg, MD) supplemented with 10% fetal bovine serum. All growth media contained 100 U/ml penicillin and 100 μg/ml streptomycin and the cells were incubated in a humidified incubator with 5% $CO_2$ at a temperature of 37°C.

### RNA Isolation, RT-PCR, and Real-Time PCR

To determine the tissue expression pattern of *Cbfa1*/*Runx2* isoforms, we used the MTC™ Panel I (Clontech Laboratories, Inc., Palo Alto, CA) that contains twelve normalized, first-strand cDNA preparations from poly A + RNA from mouse heart, brain, spleen, lung, liver, skeletal muscle, kidney, testis, 7-, 11-, 15-, 17-day-old embryo. *Cbfa1*/*Runx2* Type I and II specific primers used in these studies are listed in Table I. The Type I isoform primer sets are to the region 1,006–1,454 bp (GenBank accession No. D14636), and the Type II specific primer sets are to the region 1–285 bp (GenBank accession No. NM_009820). In addition, we used mouse long bone total RNA to perform One-Tube RT-PCR (Roche Diagnostics Co., Indianapolis, IN) as previously described [Xiao et al., 1998]. The reverse transcription reaction was incubated at 50°C for 30 min. PCR was performed with thermal cycling parameters of 94°C for 30 sec, 60°C for 30 sec, and 68°C for 45 sec for 35 cycles followed by a final extension at 68°C for 7 min. All predicted products were separated by agarose gel electrophoresis and stained with ethidium bromide. In addition, to increase the sensitivity for detecting *Cbfa1*/*Runx2*, gel separated products were blotted on Nytran membrane (Schleicher & Schuell), immobilized on the membrane by UV crosslinking using a Stratalinker (Stratagene, LaJolla, CA 92037), and hybridized with radiolabeled *Cbfa1*/*Runx2* probe (1–531 bp;

**TABLE I. Sequence of Primers Used in Quantitative PCR and RT-PCR Assessment of *Cbfa1*/*Runx2* Expression**

| | Forward primer | Reverse primer |
|---|---|---|
| Quantitative PCR | | |
| Cyclophilin A | 5′-CTGCACTGC CAAGACTGAAT-3′ | 5′-CCACAATGTTCATGCCTTCT-3′ |
| *Runx2* UTR1 total | 5′-GCCTCACAAACAACCACAGA-3′ | 5′-TTAAACGCCAGAGCCTTCTT-3′ |
| UTR1u | 5′-AAGTCTATGTACTCCAGGCATAC-3′ | 5′-AACCATACCCAGTCCCTGTT-3′ |
| UTR1d1 | 5′-GCCTCACAAACAACCACAGA-3′ | 5′-TGCTTGCAGCCTTAAATGAC-3′ |
| UTR1d2 | 5′-CACAGTCCATGCAGGAATATTTA-3′ | 5′-TAGAACTTGTGCCCTCTGTTGT-3′ |
| UTR2 | 5′-CGTCACCTCCATCCTCTTT-3′ | 5′-AGCCACAAGTTAGCGAAGT-3′ |
| RT-PCR | | |
| *Cbfa1*/*Runx2* Type I | 5′-GGCTGTTGTGATGCGTATTCCTGTA-3′ | 5′-TAACCACAGTCCCATCTGGTACCTC-3′ |
| *Cbfa1*/*Runx2* Type II | 5′-ATGCTTCATTCGCCTCACAAACAA-3′ | 5′-GAAGCGCCGGCTGGTGCTCGGATC-3′ |
| *G3PDH* | 5′-ACCACAGTCCATGCCATCAC-3′ | 5′-TCCACCACCCTGTTGCTGTA-3′ |

"*Runx2* UTR1 total" refers to primers that amplify all three alternatively spliced UTR1 transcripts (UTR1u, UTR1d1, UTR1d2) of the Type II *Cbfa1*/*Runx2* isoform. UTR2 refers to the 5′-UTR of Type I *Cbfa1*/*Runx2* isoform. Glyceraldehyde 3-phosphate dehydrogenase (G3PDH) was used as a control for mRNA integrity.

GenBank accession No. NM_009820) that recognizes both *Cbfa1/Runx2* isoforms. The membrane was washed and exposed to X-ray film.

For quantitative real-time PCR, 1.5 μg total RNA was denatured for 5 min at 65°C in the presence of 0.5 ηmols random hexamer, snap cooled in ice water, then reverse transcribed in 100 μl using the TaqMan reverse transcription kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. PCR reactions contained 50 ηg template (cDNA or RNA), 300 ηM each forward and reverse primer, and one time SybrGreen PCR Master Mix (Applied Biosystems, Foster City, CA) in 50 μl. Samples were amplified for 40 cycles in an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) with an initial melt at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. PCR product accumulation was monitored at multiple points during each cycle by measuring the increase in fluorescence caused by the binding of SybrGreen I to dsDNA. The partial cycle at which a statistically significant increase in *Cbfa1/Runx2* product was first detected (threshold cycle, Ct) was normalized to the Ct for cyclophilin A to permit inter-sample and inter-assay comparison. A passive reference dye, ROX, was used to normalize for variations in volume and/or dye concentration between sample wells. Post-amplification melting curves were performed to confirm that a single PCR product was produced in each reaction. The contribution of contaminating genomic DNA to the observed product was determined from the Ct given by the RNA template. This quantity was usually less than 0.1%. The primer sequences used are listed in Table I.

### Transfections

For transient transfections cell lines were co-transfected with the test construct (1.0 μg) plus pSV β-gal plasmid (0.5 μg) using a Lipofectin (Gibco-BRL, Gaithersberg, MD) protocol. Briefly, $2 \times 10^5$ cells were plated in a 60 mm dish, incubated for 12 h at 37°C, rinsed twice, then transfected with DNA-liposome complexes for 5 h in serum-free medium. Cells were then rinsed twice and cultured for additional time periods from 24 to 43 h in complete growth medium. Cells transfected by the same protocol with an empty expression vector were used as a negative control. Luminescent signal from Rluc and Fluc reporter enzymes was assayed using the Dual-luciferase reporter assay kit from Promega as described by the manufacturer. Rluc activity serves as a direct measure of the overall transfection–transcription efficiency, while the Fluc/Rluc ratio monitors IRES activity. β-galactosidase activity was measured by colorimetric assay (Promega, Madison, WI) and total protein content was determined by Bio-Rad assay (Bio-Rad, Hercules, CA) based on the Bradford method using bovine serum albumin as the standard [Bradford, 1976].

In addition, to investigate the effects of osteoblast development on translation, we stably transfected MC3T3-E1 with the various bicistronic constructs. Stable transfection of MC3T3-E1 was performed by a pooled protocol that maintains the differentiation potential of these osteoblasts as previously described [Quarles et al., 1997]. Briefly, MC3T3-E1 cells grown to 40–60% confluence in a 60-mm dish were co-transfected with a bicistronic construct (5 μg/dish) and 0.5 μg of pSV2neo, and selected by incubation in media containing 500 μg/ml G418 (Life Technologies, Inc.). To induce differentiation, MC3T3-E1 cells expressing the bicistronic constructs were grown in αMEM supplemented with 10% (v/v) FBS, 0.13 mM of ascorbic acid, 5 mM β-glycerol phosphate.

### Secondary Structure Modeling

Minimal free energy structures for UTR1u (407 bp) and UTR2 (1015 bp) were computed using mFold version 3.1 [Mathews et al., 1999; Zuker et al., 1999]. The calculated free energy values of the structures shown are −81 for UTR1u and −444 for UTR2.

### Western Blot Analysis

Nuclear extracts were prepared using NE-PER™ (Pierce Chemical Co., Rockford, IL). Protein concentations were determined with a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). SDS–PAGE and immunoblotting were carried out by modifications of previously described methods [Takeda et al., 2001]. Thirty microgram of nuclear protein were resolved on NuPAGE™ 4–12% Bis-Tris Gel (Invitrogen Co., Carlsbad, CA) and transferred to PVDF membranes (0.2 μm, Bio-Rad, Hercules, CA) for 90 min at 2.5 mA/cm$^2$ at room temperature using a Semi-Dry blotting system (Millipore Co., Chicago, IL). The membranes were blocked with SuperBlock™ Blocking Buffer in TBS

(Pierce Chemical Co., Rockford, IL) for 2 h at room temperature, washed once for 10 min in TBS/0.05% tween (TBST), and incubated for 60 min with RUNX2 antibody (*sc*-10758, Santa Cruz, CA) diluted 1:1,000 in TBST. The membranes were then washed twice (30 min each) with TBST, incubated with horseradish peroxidase conjugated donkey anti-rabbit antibody (*sc*-2004, Santa Cruz, CA) diluted 1:5,000 in TBST for 60 min then washed $3 \times 20$ min in TBST.Chemiluminescent signal was developed using an ECL kit (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) according to the manufacturer's instructions.

### Statistical Analyses

One-way analysis of variance (ANOVA) was performed with each isoform group. Values sharing a superscript within an isoform group are not different at $P < 0.05$. Values represent the mean $\pm$ SEM of a minimum of three separate experiments.

## RESULTS

### mRNA Expression of *Cbfa1/Runx2* Type I and II Isoforms

The expression of *Cbfa1/Runx2* Type I and II isoform transcripts was compared in mouse tissues using RT-PCR and PCR (Fig. 3). RT-PCR showed that the Type II message increases progressively from embryonic d7–d17. Additional, larger transcripts were present on day 15 and 17, likely representing UTR1 splice variants [Xiao et al., 1998]. Under the conditions studied, embryonic expression of the Type I isoform was more restricted, being most prominent on d11. Both isoforms were expressed in adult bone and lung, but Type II transcripts were also expressed in testis and skeletal muscle (weak signal). Neither isoform was detected in heart, brain, spleen, liver, or kidney by this technique. The effect of osteoblast maturation on *Cbfa1/Runx2* isoform expression was assessed in MC3T3-E1 osteoblasts cultured in the presence of ascorbate and β-glycerol phosphate [Quarles et al., 1992]. We observed a small (approximate 1.5-fold), but reproducible, increase in the amount of message for the Type II UTR splice variants and no change in the unspliced Type II or the Type I message in MC3T3-E1 cells during a 14 days of culture during which these cells undergo progressive osteoblastic maturation (Fig. 4A). Differences in the levels of mRNAs that differ only in their 5′-UTRs suggest a possible role of the 5′-UTR in translational control of protein expression.

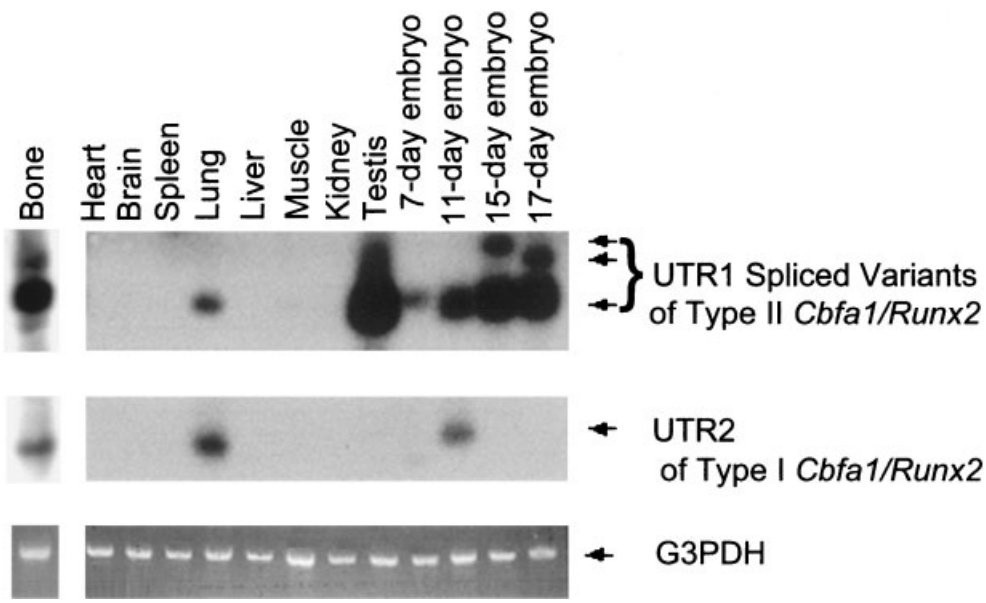


**Fig. 3.** *Cbfa1/Runx2* Type I and II isoform expression during embryogenesis and in different tissues. RT-PCR and PCR analysis were performed as described in Materials and Methods. **Upper panel**, products generated by *Cbfa1/Runx2* Type II specific primers; **middle panel**, products generated by *Cbfa1/Runx2* Type I specific primers; **lower panel**, G3PDH control.

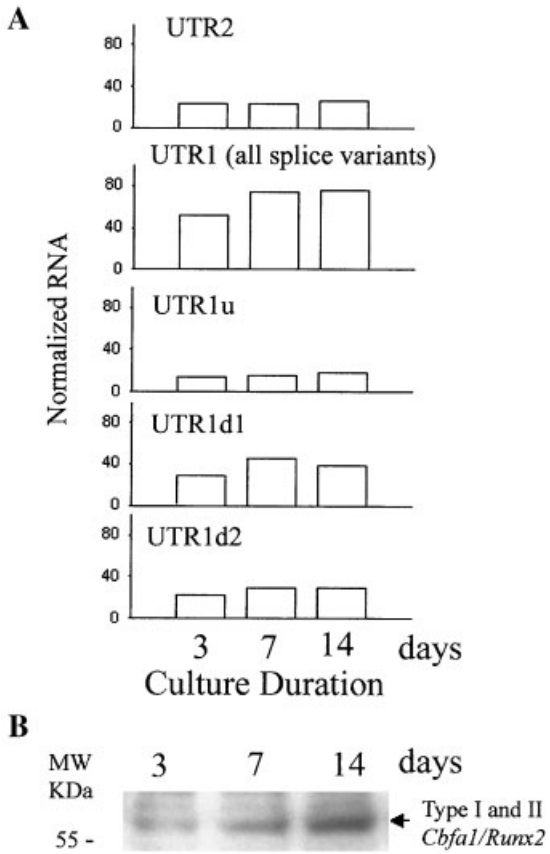


**Fig. 4.** Osteoblast maturational stage-dependent expression of *Cbfa1/Runx2*. **A**: Expression of *Cbfa1/Runx2* Type I and II isoforms transcripts in MC3T3-E1 cells during 14 days of culture under conditions that promote differentiation. Real-time PCR was performed as described in Materials and Methods. **B**: Western blot analysis of *Cbfa1/Runx2* expression in MC3T3-E1 osteoblasts at various stages of maturation. Western blot analysis was performed as described in Materials and Methods using an antibody that recognizes both Type I and II *Cbfa1/Runx2*.

### Discordance Between *Cbfa1/Runx2* Message and Protein Expression

Expression of Type I and II isoforms was assessed at the protein level by Western blot analysis in MC3T3-E1 osteoblasts as a function of maturation. Similar to others [Banerjee et al., 2001], we were unable to separate the two isoforms by size using an antibody that recognizes an epitope in common region. We found, however, that *Cbfa1/Runx2* protein expression increased roughly fivefold in MC3T3-E1 osteoblasts cultured in the presence of ascorbate and β-glycerol phosphate for 14 days (Fig. 4B). The increase in total *Cbfa1* protein detected by Western analysis is greater than the change in message levels (Fig. 4A) and is consistent with increased translational efficiency during osteoblast differentiation, a finding supported by subsequent studies (see below).

### 5′-UTR of Type I and II *Cbfa1/Runx2* Have Complex Secondary Structures

The unspliced form of UTR1 (UTR1u) contains six AUGs, four of which are associated with uORFs that may hinder cap-dependent translation. A 103 bp "mini-intron" that is removed in both splice variants UTR1d1 and UTR1d2 deletes three AUGs, two of which are associated with uORFs. UTR2 contains no uORFs. Computer modeling suggests that all of the UTRS have the potential to form complex secondary structures. Figure 5 shows a prediction of the optimal secondary structures of UTR1u, UTR1d1, UTRd2, and UTR2, which are sufficiently complex as to impede ribosomal scanning and translation.

### Translational Activity of Moncistronic Constructs Containing the 5′-UTRs of Type I and II *Cbfa1/Runx2* isoforms

We first evaluated the translation efficiency of UTR1 and UTR2 using monocistronic constructs in which the luciferase 5′-UTR was replaced by UTR2 (SV40-UTR2-Luc) or the splice variants of UTR1 (SV40-UTR1-Luc) (Fig. 2A). After transient transfection into MC3T3-E1 pre-osteoblasts and NIH3T3 fibroblasts, luciferase activity was assayed as a surrogate for translational activity (Fig. 6A). The rank order of activity in osteoblasts was UTR2 > UTR1d1 = UTR1d2 ≥ luciferase 5′-UTR > UTR1u. The reduced luciferase activity of UTR1u compared to the spliced variants could be due to impaired translation imparted by the retained mini-intron.

### IRES-Dependent Translation Activity of the 5′-UTRs of *Cbfa1/Runx2* Type I and II Isoforms

Since monocistronic constructs do not distinguish between cap- and IRES-dependent translation, we used the bicistronic constructs shown in Figure 2B test for the presence of IRES-dependent mechanisms. In these bicistronic vectors, the SV40 promoter drives RLuc in the first cistron followed by constructs with potential IRES activity in the second cistron linked to Fluc. Fluc is produced only if the intercistronic UTR confers IRES-dependent translation.

IRES activity was assessed in MC3T3-E1 preosteoblasts under basal conditions (Fig. 6B),

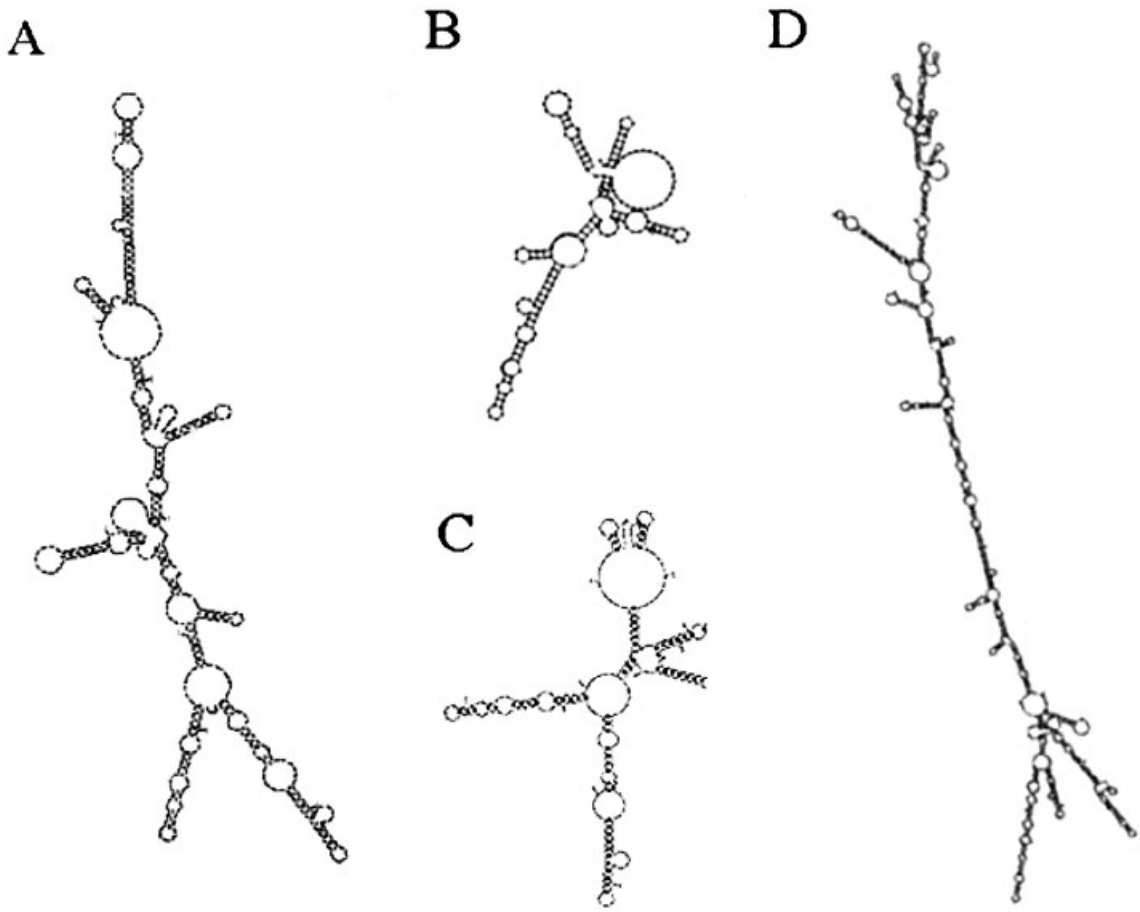


**Fig. 5.** Secondary Structure of the *Cbfa1/Runx2* 5′-UTRs. Representative optimal secondary structures for UTR1u (**A**), UTR1d1 (**B**), UTR1d2 (**C**) of Type II *Cbfa1/Runx2,* and UTR2 (**D**) of Type I *Cbfa1/Runx2.* Structures were obtained using mFold version 3.1.

during maturation (Fig. 6C) and under cell stress (Fig. 7A,B). In undifferentiated 3-day pre-osteoblasts (Fig. 6B), we found that the endogenous luciferase 5′-UTR had no appreciable IRES activity, whereas the encephalomyocarditis 5′-UTR was able to initiate translation via internal ribosomal entry. Both the UTR2 and the spliced forms of UTR1 exhibited IRES activity (Fig. 6B,C) but the level was approximately one-third of that observed with the viral IRES under these conditions. MC3T3-E1 osteoblasts undergo a temporal sequence of differentiation when grown in the presence of ascorbic acid and β-glycerol phosphate [Quarles et al., 1992]. During this maturation, the IRES activity of UTR2 and the splice variants of UTR1 (UTR1d1 and UTR1d2) increased almost fourfold (Fig. 6C), consistent with a role for IRES activity during osteoblast development. In contrast, the mini-intron in UTR1u impaired IRES-dependent translation.

Since IRES activity is also utilized during cell stress, we compared IRES activity during growth arrest induced by serum deprivation (Fig. 7A) with IRES activity during genotoxic stress induced by mitomycin C (Fig. 7B). The IRES activity of UTR2 increased in response to both serum deprivation (Fig. 8A) and genotoxic stress (Fig. 7B). In contrast, the IRES activity of UTR1d1 and d2 was similarly responsive to DNA damage induced by mitomycin C but less responsive to growth arrest.

We observed cell type-dependence differences in IRES activity (Fig. 8). All cell lines supported activity of the viral IRES and UTR1 spliced variants. In contrast, UTR2 activity was greater in MC3T3-E1 pre-osteoblasts compared to NIH3T3 fibroblasts, suggesting the presence of

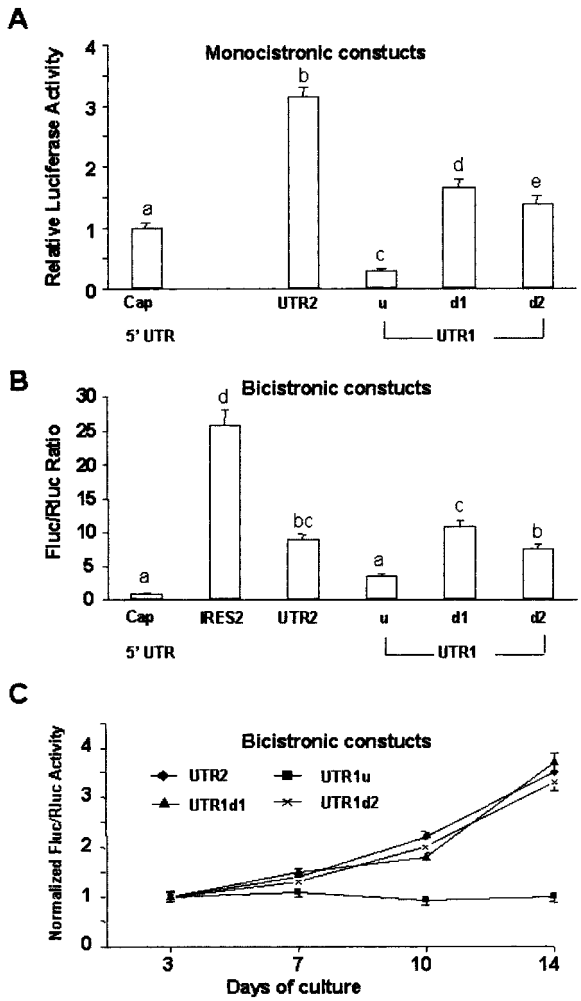


**Fig. 6.** Translational regulation of *Cbfa1/Runx2* expression. **A**: Relative luciferase activity of monocistronic constructs shown in Figure 2A in MC3T3-E1 pre-osteoblasts. **B**: Assessment of IRES activity using bicistronic Rluc and Fluc constructs containing different UTRs transiently transfected into MC3T3-E1 pre-osteoblasts. The SV40-Rluc-UTR-Fluc constructs Rluc is generated by cap-dependent translation, whereas the Fluc is produced only if the intercistronic UTR permits IRES-mediated translation. Results are expressed as a ratio of Rluc/Fluc. **C**: Effects of osteoblast differentiation on IRES-dependent translation in MC3T3-E1 cells stably transfected with bicistronic constructs (Fig. 2B) and induced to differentiate by growth for 14 days in media containing ascorbic acid and β-glycerol phosphate as described in Material and Methods. Results are expressed as Rluc/Fluc normalized to day 3. Values represent the mean ± SEM of at least three separate determinations.

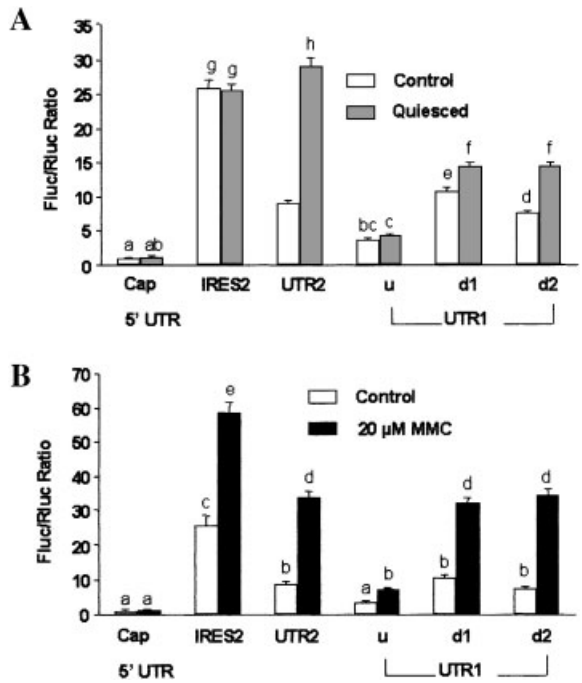


**Fig. 7.** Stress-induced IRES activity in MC3T3-E1 osteoblasts transfected with bicistronic Rluc and Fluc constructs containing different UTRs. **A**: UTR2 and UTR1 display IRES activity in quiescent cells induced by serum deprivation. **B**: Effects of genotoxic stress on IRES-dependent translation. IRES2, UTR1, and UTR2 activity were enhanced in cells treated with mitomycin C (MMC) to induce DNA damage. The 5′-UTR of Fluc did not display IRES activity. IRES activity was estimated by the Fluc/Rluc ratio. Values represent the mean ± SEM of at least three separate determinations. These studies used the SV40-Rluc-UTR-Fluc bicistronic constructs shown in Figure 2B, in which a single transcript generates Rluc by cap-dependent translation but produces Fluc only if the intercistronic UTR permits IRES-mediated translation.

cell-type regulation of *Cbfa1/Runx2* IRES activity. UTR1u demonstrated lower activity in MC3T3-E1 compared to NIH3T3, suggesting that the presence of the mini-intron also impedes translational efficiency in a cell-type specific manner. The response of pluripotent C3H10T1/2 resembled that of MC3T3-E1 osteoblasts, whereas the activities of these UTRs in C2C12 cells were similar to the responses observed in NIH3T3 fibroblasts (data not shown).

## DISCUSSION

The *Cbfa1/Runx2* gene has a complex structure consisting of two promoters that generates two major isoforms, which are identical except for their 5′-UTRs and N-termini (Fig. 1). To date, no separate function has been assigned to the protein products derived from these two promoters, suggesting that the purpose of the complex gene organization is to provide multiple mechanisms for controlling gene product expression. The importance of precise control of the time, place, and amount of *Cbfa1/Runx2* is

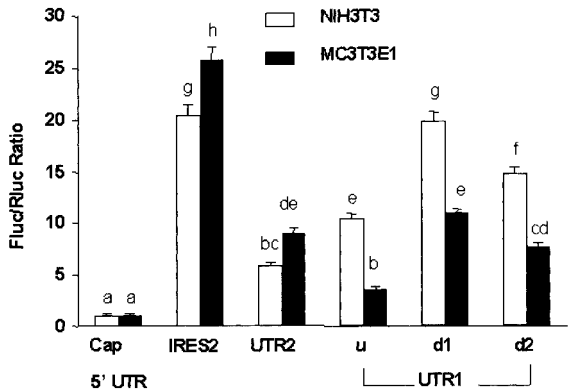


**Fig. 8.** Cell-type dependent IRES activity. NIH3T3 fibroblasts and MC3T3-E1 pre-osteoblasts were transiently transfected with bicistronic Rluc and Fluc constructs containing different UTRs as described in Materials and Methods. Translation by IRES-dependent mechanisms is measured by the Fluc/Rluc ratio. Values represent the mean ± SEM of at least three separate determinations.

implied by the paradoxical stimulation of bone resorption and bone loss, rather than expected induction of bone formation, when *Cbfa1/Runx2* is overexpressed in mice using a heterologous promoter [Geoffroy et al., 2002]. Although having a dominant role in controlling *Cbfa1/Runx2* gene expression, transcriptional mechanisms may not fully account for the observed expression of *Cbfa1/Runx2* gene products [Xiao et al., 2001; Lengner et al., 2002]. In the current studies, we provide evidence that translational mechanisms may also contribute to the regulation of the expression of *Cbfa1/Runx2* isoforms.

Several findings support a role for translational control of *Cbfa1/Runx2* isoform expression. First, *Cbfa1/Runx2* mRNA isoforms possess secondary structural features in their 5′-UTRs that are predicted to impede translation by a 5′-cap-dependent ribosome-scanning mechanism (Fig. 5). Indeed, similar to IRES regions of other mRNAs [Creancier et al., 2000, 2001; Pestova et al., 2001], we observed the existence of conserved Y-shaped elements in the 5′-UTRs of both Type I and II isoform mRNAs (Fig. 5). Second, translational control is implicated by the discordance between mRNA levels of the different isoforms and total protein expression of *Cbfa1/Runx2* during osteoblast maturation in MC3T3-E1 cells (Figs. 4A,B). Third, isoforms that differ in their respective 5′-UTRs exhibit different translational potentials. For example, we observed preferential translation of UTR2 compared to UTR1 in MC3T3-E1, using monocistronic constructs (Fig. 6A). Fourth, both UTR2 and UTR1 demonstrated IRES activity (Fig. 6B,C) that increased with serum deprivation and/or genotoxic stress induced by mitomycin C (Fig. 7), as well as osteoblastic maturation (Fig. 6C). Dual cap- and IRES-dependent translation has been reported for *Cbfa2/Runx1* [Pozner et al., 2000] and may exist for *Cbfa1/Runx2* as well. Finally, we observed cell-type specific differences in translation of the UTR2 and UTR1, with undifferentiated C3H10T$_{1/2}$ and MC3T3-E1 pre-osteoblasts translating UTR2 > UTR1. This suggests that there are both cell type differences in regulating IRES activity, as well as differences in IRES regulation of UTR1 and UTR2, consistent with the presence of several types of cellular IRESs [Creancier et al., 2000, 2001; Stoneley et al., 2000; Vagner et al., 2001].

We have previously reported [Xiao et al., 1998] that the 5′-UTR1 has a low abundant transcript containing a 103 bp "mini-intron" (designated UTR1u) (Fig. 1B). The biological significance of this unspliced transcript is not certain, but it is the predominant form expressed in tissues and cell types that do not express high levels of the Type II isoform [Xiao et al., 1998]. We now show that the inclusion of the "mini-intron" significantly suppresses translation of both monocistronic (Fig. 6A) and bicistronic constructs (Figs. 6B,C and 8). These finding indicate that the inclusion of this mini-intron may further impede expression of *Cbfa1/Runx2* Type II isoform in some cell types. Interestingly, NIH3T3 were able to translate UTR1u more efficiently than MC3T3-E1 (Fig. 8), suggesting the possibility of the presence of mechanisms in some cell types to compensate for the inhibition induced by the mini-intron.

The potential significance of *Cbfa1/Runx2* translation regulation is likely derived from the observation that cellular IRES-containing mRNAs often encode regulatory proteins whose expression is tightly regulated [Creancier et al., 2000, 2001; Vagner et al., 2001]. Internal ribosome entry allows translation of cellular mRNAs in situations where the cap-dependent translation machinery is attenuated, as can occur during stress, apoptosis, G2-to-M transition, and during development [Clemens and Bommer, 1999; Creancier et al., 2000, 2001; Stoneley et al., 2000; Werner, 2000; Hennecke et al., 2001; Vagner et al., 2001]. Maintaining

tight control of the levels of *Cbfa1/Runx2* during different stages of cell growth and differentiation as well as during cell stress may be important in the maintenance of skeletal elements, as it is with other regulatory gene products. A precedent for this is seen in genes that display IRES-dependent translational control. For example, during embryonic development, the 5′-UTR of c-myc regulates protein expression in a spatial-temporal manner through dual cap- and IRES-dependent mechanisms [Creancier et al., 2001]. In addition, translation initiation of fibroblast growth factor 2 is specifically regulated during differentiation, growth, and stress by IRES-dependent translational mechanisms [Creancier et al., 2000]. The mechanisms of IRESs regulation are poorly understood, [Johannes et al., 1999; Vagner et al., 2001; Fernandez et al., 2002], but *trans*-acting proteins bound to the 5′-UTRs are likely involved in targeting the ribosome in mRNAs having IRES activity [Vagner et al., 1996]. Identifying such IRES-promoting proteins in cells involved in skeletalgenesis will be necessary to fully understand the role that IRES dependent activity plays in sustaining the expression of *Cbfa1/Runx2*.

Regardless, we found that the mRNAs from the Type I and II isoforms with different translational potential are also differentially expressed due to differential use of the P1 and P2 promoter. While it is well known that *Cbfa1/Runx2* is expressed in early stages of embryonic development in mesenchymal precursors that later develop into bone, teeth, and cartilage [Satake et al., 1995; Banerjee et al., 1997, 2001; Komori et al., 1997; Mundlos et al., 1997; Xiao et al., 1998; D'Souza et al., 1999; Jiang et al., 1999; Bronckers et al., 2001; Xiao et al., 2001], differences in expression of the *Cbfa1/Runx2* Type I and II mRNAs have not been well characterized. In the current studies, we found that the Type II isoform is the predominant form expressed during embryogenesis, whereas the Type I isoform has a more limited expression during embryogeneis (Fig. 3). Both isoforms have a restricted expression in the adult, with high levels of Type II expression in bone and testis (Fig. 3). We also detected low levels of Type II *Cbfa1/Runx2* in skeletal muscle (and C2C12 pre-myoblasts) and expression of both isoforms in the lung (the later possibly representing the presence of tracheal cartilage). The different expression patterns of the two isoforms suggest a predominant role for the Type II isoform and the P1 promoter in early stages of skeletalgenesis. We also detected differences in expression of the splice variants of UTR1. UTR1d1 is the predominant splice variant that is expressed in bone and during embryogenesis, while UTR1u, consistent with its ability to impede translation, is largely limited to tissues and cells that do not express the processed transcripts.

In conclusion, *Cbfa1/Runx2* Type I and II isoforms have complex 5′-UTRs that impart IRES activity in vitro. Translational control by IRES-dependent activity likely enables rapid adjustments of *Cbfa1/Runx2* protein production without new mRNA synthesis, as well as provides a secondary level for controlling the amount of *Cbfa1/Runx2* proteins in concert with transcriptional regulation. Further studies are needed to confirm the role of IRES-dependent translation in vivo and the relative roles of cap-and IRES-dependent control of *Cbfa1/Runx2* translation. Nevertheless, dual transcriptional and translational control mechanisms provide a means to fine-tune *Cbfa1/Runx2* expression across a wide range of cellular conditions during skeletalgenesis, where the amount of *Cbfa1/Runx2* gene products may be an important determinant of their biological effects.

## ACKNOWLEDGMENT

We thank Cristy McGranahan for secretarial support.

## REFERENCES

Banerjee C, McCabe LR, Choi JY, Hiebert SW, Stein JL, Stein GS, Lian JB. 1997. Runt homology domain proteins in osteoblast differentiation: AML3/CBFA1 is a major component of a bone-specific complex. J Cell Biochem 66:1–8.

Banerjee C, Javed A, Choi JY, Green J, Rosen V, van Wijnen AJ, Stein JL, Lian JB, Stein GS. 2001. Differential regulation of the two principal *Runx2/Cbfa1* n-terminal isoforms in response to bone morphogenetic protein-2 during development of the osteoblast phenotype. Endocrinology 142:4026–4039.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254.

Bronckers AL, Engelse MA, Cavender A, Gaikwad J, D'Souza RN. 2001. Cell-specific patterns of *Cbfa1* mRNA and protein expression in postnatal murine dental tissues. Mech Dev 101:255–258.

Clemens MJ, Bommer UA. 1999. Translational control: The cancer connection. Int J Biochem Cell Biol 31:1–23.

Creancier L, Morello D, Mercier P, Prats AC. 2000. Fibroblast growth factor 2 internal ribosome entry site (IRES) activity ex vivo and in transgenic mice reveals a stringent tissue-specific regulation. J Cell Biol 150:275–281.

Creancier L, Mercier P, Prats AC, Morello D. 2001. c-myc Internal ribosome entry site activity is developmentally controlled and subjected to a strong translational repression in adult repression in adult transgenic mice. Mol Cell Biol 21:1833–1840.

D'Souza RN, Aberg T, Gaikwad J, Cavender A, Owen M, Karsenty G, Thesleff I. 1999. *Cbfa1* is required for epithelial-mesenchymal interactions regulating tooth development in mice. Development 126:2911–2920.

Dever TE. 2002. Gene-specific regulation by general translation factors. Cell 108:545–546.

Drissi H, Luc Q, Shakoori R, Chuva De Sousa Lopes S, Choi JY, Terry AM, Jones S, Neil JC, Lian JB, Stein JL, van Wijnen AJ, Stein GS. 2000. Transcriptional autoregulation of the bone related *CBFA1*/*RUNX2* gene. J Cell Physiol 184:341–350.

Ducy P, Karsenty G. 1995. Two distinct osteoblast-specific *cis*-acting elements control expression of a mouse osteocalcin gene. Mol Cell Biol 15:1858–1869.

Ducy P, Starbuck M, Priemel M, Shen J, Pinero G, Geoffroy V, Amling M, Karsenty G. 1999. A *Cbfa1*-dependent genetic pathway controls bone formation beyond embryonic development. Genes Dev 13:1025–1036.

Fernandez JM, Yaman I, Sarnow P, Snider MD, Hatzoglou M. 2002. Regulation of internal ribosomal entry site-mediated translation by phosphorylation of the translation initiation factor eIF2$\alpha$. J Biol Chem 277:19198–19205.

Fujiwara M, Tagashira S, Harada H, Ogawa S, Katsumata T, Nakatsuka M, Komori T, Takada H. 1999. Isolation and characterization of the distal promoter region of mouse *Cbfa1*. Biochim Biophys Acta 1446:265–272.

Geoffroy V, Corral DA, Zhou L, Lee B, Karsenty G. 1998. Genomic organization, expression of the human CBFA1 gene, and evidence for an alternative splicing event affecting protein function. Mamm Genome 9:54–57.

Geoffroy V, Kneissel M, Fournier B, Boyde A, Matthais P. 2002. High bone resorption in adult aging transgenic mice overexpressing *Cbfa1*/*Runx2* in cells of the osteoblastic lineage. Mol Cell Biol 22:6222–6233.

Hennecke M, Kwissa M, Metzger K, Oumard A, Kroger A, Shirmbeck R, Reimann J, Hauser H. 2001. Composition and arrangement of genes define the strength of IRES-driven translation I bicistronic mRNAs. Nucleic Acids Res 29:3327–3334.

Jiang H, Sodek J, Karsenty G, Thomas H, Ranly D, Chen J. 1999. Expression of core binding factor Osf2/*Cbfa-1* and bone sialoprotein in tooth development. Mech Dev 81:169–173.

Jimenez MJ, Balbin M, Lopez HM, Alvarez H, Komori T, Lopez-Otin C. 1999. Collagenase 3 is a target of *Cbfa1*, a transcription factor of the runt gene family involved in bone formation. Mol Cell Biol 19:4431–4442.

Johannes G, Carter MS, Eisen MB, Brown PO, Sarnow P. 1999. Identification of eukaryotic mRNAs that are translated at reduced cap binding complex eIF4F concentrations using a cDNA microarray. Proc Natl Acad Sci USA 96:13118–13123.

Kim IS, Otto F, Zabel B, Mundlos S. 1999. Regulation of chondrocyte differentiation by *Cbfa1*. Mech Dev 80:159–170.

Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki A, Deguchi K, Shimizu Y, Bronson RT, Gao YH, Inada M, Sato M, Okamoto R, Kitamura Y, Yoshiki S, Kishimoto T. 1997. Targeted disruption of *Cbfa1* results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell 89:755–764.

Lengner CJ, Drissi H, Choi JY, van Wijnen AJ, Stein JL, Stein GS, Lian JB. 2002. Activation of the bone-related *Runx2*/*Cbfa1* promoter in mesenchymal condensations and developing chondrocytes of the axial skeleton. Mech Dev 114:167–170.

Mathews DH, Sabina J, Zuker M, Turner DH. 1999. Expanded sequence dependence of thermodynamic parameters improves prediction of RNA secondary structure. J Mol Biol 288:911–940.

Morris DR, Geballe AP. 2000. Upstream open reading frames as regulators of mRNA translation. Mol Cell Biol 20:8635–8642.

Mundlos S, Otto F, Mundlos C, Mulliken JB, Aylsworth AS, Albright S, Lindhout D, Cole WG, Henn W, Knoll JH, Owen MJ, Mertelsmann R, Zabel BU, Olsen BR. 1997. Mutations involving the transcription factor CBFA1 cause cleidocranial dysplasia. Cell 89:773–779.

Ogawa E, Maruyama M, Kagoshima H, Inuzuka M, Lu J, Satake M, Shigesada IY. 1993. PEBP2/PEA2 represents a family of transcription factors homologous to the products of the *Drosophila* runt gene and the human AML1 gene. Proc Natl Acad Sci USA 90:6859–6863.

Otto F, Thornell AP, Crompton T, Denzel A, Gilmour KC, Rosewell IR, Stamp GW, Beddington RS, Mundlos S, Olsen BR, Selby PB, Owen MJ. 1997. *Cbfa1*, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. Cell 89:765–771.

Pestova TV, Kolupaeva VG, Lomakin IB, Pilipenko EV, Shatsky IN, Agol VI, Hellen CU. 2001. Molecular mechanisms of translation initiation in eukaryotes. Proc Natl Acad Sci USA 98:8635–8642.

Pozner A, Goldenberg D, Negreanu V, Le SY, Elroy-Stein O, Levanon D, Groner Y. 2000. Transcription-coupled translation control of AML1/RUNX1 is mediated by cap- and internal ribosome entry site-dependent mechanisms. Mol Cell Biol 20:2297–2307.

Quarles LD, Yohay DA, Lever LW, Caton R, Wenstrup RJ. 1992. Distinct proliferative and differentiation states of murine MC3T3-E1 cells in culture: An in vitro model of osteoblast development. J Bone Miner Res 7:683–692.

Quarles LD, Siddhanti SR, Medda S. 1997. Developmental regulation of osteocalcin expression in MC3T3-E1 osteoblasts: Re-examination of the role of E-box *cis*-acting promoter elements. J Cell Biochem 65:11–24.

Ryoo HM, Lee MH, Javed A, Kim HJ, Shin HI, Choi JY, Rosen V, Stein JL, van Wijnen AJ, Stein GS, Lian JB. 1998. Transient expression of *Cbfa1* in response to bone morphogenetic protein-2 and transforming growth factor-beta 1 in C2C12 myogenic cells: A necessary milestone but not sufficient for osteoblast differentiation. Bone 23:S173.

Satake M, Nomura S, Yamaguchi-Iwai Y, Takahama Y, Hashimoto Y, Niki M, Kitamura Y, Ito Y. 1995. Expres-

sion of the runt domain-encoding PEBP2α genes in T cells during thymic development. Mol Cell Biol 15:1662–1670.

Stewart M, Terry A, Hu M, O'Hara M, Blyth K, Baxter E, Cameron E, Onions DE, Neil JC. 1997. Proviral insertions induce the expression of bone-specific isoforms of PEBP2alphaA (CBFA1): Evidence for a new myc collaborating oncogene. Proc Natl Acad Sci USA 94:8646–8651.

Stoneley M, Subkhankulova T, LeQuesne JP, Coldwell MJ, Jopling CL, Belsham GJ, Willis AE. 2000. Analysis of the c-myc IRES; a potential role for cell-type specific trans-acting factors and the nuclear compartment. Nucleic Acids Res 28:687–694.

Sudhakar S, Katz MS, Elango N. 2001. Analysis of type-I and type-II RUNX2 protein expression in osteoblasts. Biochem Biophys Res Commun 286:74–79.

Takeda S, Bonnamy JP, Owen MJ, Ducy P, Karsenty G. 2001. Continuous expression of *Cbfa1* in nonhypertrophic chondrocytes uncovers its ability to induce hypertrophic chondrocyte differentiation and partially rescues *Cbfa1*-deficient mice. Genes Dev 15:467–481.

Thirunavukkarasu K, Mahajan M, McLarren KW, Stifani S, Karsenty G. 1998. Two domains unique to osteoblast-specific transcription factor *Osf2*/*Cbfa1* contribute to its transactivation function and its inability to heterodimerize with Cbfb. Mol Cell Biol 18:4197–4208.

Ueta C, Iwamoto M, Kanatani N, Yoshida C, Liu Y, Enomoto-Iwamoto M, Ohmori T, Enomoto H, Nakata K, Takada K, Kurisu K, Komori T. 2001. Skeletal malformations caused by overexpression of *Cbfa1* or its dominant negative form in chondrocytes. J Cell Biol 53: 87–100.

Vagner S, Touriol C, Galy B, Audigier S, Gensac MC, Amalric F, Bayard F, Prats H, Prats AC. 1996. Translation of CUG- but not AUG-initiated forms of human fibroblast growth factor 2 is activated in transformed and stressed cells. J Cell Biol 135:1391–1402.

Vagner S, Galy B, Pyronnet S. 2001. Irresistible IRES. Attracting the translation machinery to internal ribosome entry sites. EMBO Rep 2:893–898.

Werner R. 2000. IRES elements in connexin genes: A hypothesis explaining the need for connexins to be regulated at the translational level. IUBMB Life 50: 173–176.

Xiao ZS, Thomas R, Hinson TK, Quarles LD. 1998. Genomic structure and isoform expression of the mouse, rat and human *Cbfa1*/*Osf2* transcription factor. Gene 214:187–197.

Xiao ZS, Liu SG, Hinson TK, Quarles LD. 2001. Characterization of the upstream mouse *Cbfa1*/Runx2 promoter. J Cell Biochem 82:647–659.

Zeng C, Van Wijnen A, Stein J, Meyers S, Sun W, Shopland L, Lawrence J, Penman S, Lian J, Stein G, Hiebert S. 1997. Identification of a nuclear matrix targeting signal in the leukemia and bone-related AML/CBF-α transcription factors. Proc Natl Acad Sci USA 94:6746–6751.

Zuker M, Mathews DH, Turner DH. 1999. Algorithms and thermodynamics for RNA secondary structure prediction: A practical guide. In: Barciszewski J, Clark BFC, editors. RNA biochemistry and biotechnology. NATO ASI Series. Kluwer Academic Publishers, Boston, USA. pp 11–43.