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Expression of RUNX2 Isoforms: Involvement of Cap-Dependent and Cap-Independent Mechanisms of Translation

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Abstract RUNX2, a major regulator of skeletogenesis, is expressed as type-I and type-II isoforms. Whereas most eukaryotic mRNAs are translated by the cap-dependent scanning mechanism, translation of many mRNAs including type-I and type-II RUNX2 mRNAs has been reported to be initiated by a cap independent internal ribosomal entry site (IRES). Since the dicistronic plasmid assay used to demonstrate IRES has been questioned, we investigated the presence of IRES in RUNX2 mRNAs using dicistronic plasmid and mRNA assays. Our results show that the dicistronic plasmid assay cannot be used to demonstrate IRES in RUNX2 mRNAs because the intercistronic region of dicistronic plasmids containing the 5′-UTRs of both RUNX2 mRNAs operates as a cryptic promoter. In dicistronic mRNA transfection studies the 5′-UTRs of both RUNX2 mRNAs exhibited no IRES activity. When transfected into osteoblastic cells, monocistronic reporter mRNA preceded by the 5′-UTR of type-II RUNX2 (Type-II-FLuc-A100) was translated to a high degree only in the presence of a functional cap (m^7GpppG); in contrast, luciferase mRNA preceded by the 5′-UTR of type-I RUNX2 mRNA (Type-I-FLuc-A100) was translated poorly in the presence of either m^7GpppG or a nonfunctional cap (ApppG). Notably, in transfected cells inhibitors of cap-dependent translation suppressed the translation of m^7GpppG-capped Type-II-FLuc-A100, but not ApppG-capped reporter mRNA preceded by the IRES-containing hepatitis C virus (HCV) 5′-UTR. Our study demonstrates that type-II RUNX2 mRNA is translated by the cap-dependent mechanism. Although efficient translation of type-I RUNX2 mRNA appears to require a process other than cap-dependent, the mechanism of type-I RUNX2 mRNA translation remains to be resolved. J. Cell. Biochem. 99: 1108–1121, 2006. © 2006 Wiley-Liss, Inc.

Key words: type-1 RUNX2; type-II RUNX2; internal ribosomal entry site; cap-dependent translation; 4E-BP1; RNA Aptamer-1

Runt-related transcription factors (RUNXs) are a family of proteins with a common 128 amino acids long DNA binding region known as the 'runt' domain [Kagoshima et al., 1993]. The runt domain mediates binding of RUNX proteins to the consensus sequence PyGPyGGT of target genes [Wheeler et al., 2000]. The three members of the RUNX family, RUNX1, RUNX2, and RUNX3, play important roles in hematopoiesis and osteogenesis in mammals [Westendorf and Hiebert, 1999]. Two promoters (proximal and distal) controlling the expression of each RUNX gene generate two different mRNAs (proximal promoter: type-I mRNA and distal promoter: type-II mRNA) that differ at their 5′-regions but are identical throughout the remaining sequence [Levanon and Groner, 2004]. Alternate splicing and exon skipping have been shown to generate multiple forms of the same species. Apart from the common runt domain, all three RUNX proteins have extensive homology in other regions as well [Levanon et al., 1994].

RUNX2 has been shown to be required for osteoblast differentiation [Banerjee et al., 1997; Ducy et al., 1997; Komori et al., 1997], bone formation [Komori et al., 1997; Mundlos et al., 1997; Otto et al., 1997; Ducy et al., 1999], and chondrocyte maturation [Inada et al., 1999; Kim et al., 1999; Enomoto et al., 2000; Yoshida et al.,

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2004]. Mutations in the RUNX2 gene cause cleidocranial dysplasia, a disorder characterized by multiple developmental abnormalities of the skeleton [Mundlos, 1999]. Differentiation of mesenchymal cells to different cell types (osteoblasts, chondrocytes, myoblasts, and adipocytes) appears to require expression of RUNX2 and cell type-specific transcription factor(s) [Nakashima et al., 2002; Wada et al., 2002; Elango, unpublished data].

The two isoforms of RUNX2 have different amino termini (type-I: MRIPV; type-II: MASNSLFSAVTPCQQSFFW) as well as distinct 5′-UTRs (type-I 5′-UTR: 1,015 nucleotides) [Ogawa et al., 1993] and type-II 5′-UTR: 210 nucleotides [Fujiwara et al., 1999; Drissi et al., 2000], whereas the remaining coding sequence (1,527 nucleotides) and 3′-UTR (3,961 nucleotides) of both mRNAs are identical. In situ studies of developing mouse calvaria and early skeletogenesis have shown that type-I RUNX2 mRNA is expressed in undifferentiated mesenchymal cells, preosteoblasts, and osteoblasts, while type-II RUNX2 mRNA expression is restricted to later stages of osteoblast differentiation [Park et al., 2001; Choi et al., 2002]. These results suggest that the two RUNX2 isoforms have distinct functions in osteogenesis. In a recent study of type-II RUNX2 knockout mice, Xiao et al. [2004] concluded that type-II RUNX2 is necessary for osteoblastic maturation and endochondral bone formation, whereas the type-I RUNX2 isoform is sufficient for early osteoblast differentiation and formation of intramemranous bone.

Expression of the two RUNX2 mRNAs in osteoblastic cells is differentially regulated at the transcriptional level by bone morphogenetic protein 2 [Banerjee et al., 2001] and tumor necrosis factor α [Gilbert et al., 2002]. Absence of RUNX2 protein expression in undifferentiated mesenchymal cells expressing the RUNX2 mRNAs [Tsuji et al., 1998; Xiao et al., 1998; Gori et al., 1999; Wang et al., 1999; Satomura et al., 2000; Park et al., 2001; Sudhakar et al., 2001; Choi et al., 2002], indicates that the RUNX2 mRNAs are dormant in these cells. Thus RUNX2 gene expression is regulated at both transcriptional and translational levels in cells of the osteoblast lineage. Regulation of gene expression at the translational level allows cells to respond rapidly to environmental signals.

The 5′-terminus of all nuclear encoded eukaryotic mRNAs contains a 7-methyl guanosine (m^7GpppN), termed cap. Most eukaryotic mRNAs are translated by the cap-dependent scanning mechanism, which requires the binding of the cap-binding protein eukaryotic initiation factor 4E (eIF4E) to the cap structure [Kozak, 2002]. This scanning mechanism is inefficient when the 5′-UTR is long and highly GC rich [Kozak, 1988, 1996]. Some eukaryotic mRNAs and viral RNAs, which contain long and highly structured GC-rich 5′-UTRs, are reported to be translated by a cap-independent internal ribosomal entry site (IRES) mechanism [Hellen and Sarnow, 2001]. Studies using promoter-containing dicistronic plasmids in a dicistronic assay have led to the conclusion that both type-I and type-II RUNX2 mRNAs are translated by the cap-independent IRES mechanism [Xiao et al., 2003]. However, the intercistronic region of a dicistronic plasmid can act as a cryptic promoter and generate monocistronic mRNA. Thus the validity of the dicistronic assay employing a promoter-containing dicistronic plasmid but without a promoterless dicistronic plasmid control has been criticized [Han and Zhang, 2002]. In this report, using promoterless monocistronic as well as dicistronic plasmids, we demonstrate that the dicistronic assay used to demonstrate the presence of IRES in the 5′-UTRs of type-I and type-II RUNX2 mRNAs is flawed. Our results show that the 5′-UTRs of type-I and type-II RUNX2 mRNAs, when present as the intercistronic region in dicistronic plasmids, operate—either independently or in conjunction with the upstream sequence—as cryptic promoters. Neither 5′-UTR exhibited any IRES activity when m^7GpppG-capped dicistronic mRNAs containing the respective 5′-UTRs as intercistronic region were transfected into cells. Finally, in monocistronic mRNA transfection experiments we observed that the translation of type-II RUNX2 mRNA was suppressed by cap-binding protein inhibitors (4E-BP1 and RNA Aptamer-1). The present study therefore demonstrates that type-II RUNX2 mRNA is translated by the cap-dependent mechanism, although the translation mechanism of type-I RUNX2 mRNA remains to be established.

MATERIALS AND METHODS

Cell Culture

Two osteoblast-like osteosarcoma cell lines from rat (ROS 17/2.8 and UMR-106) and a

mouse calvarial preosteoblast cell line (MC3 T3-E1) were cultured as described earlier [Sudhakar et al., 2001].

Plasmids

Promoter-containing monocistronic expression plasmids. The monocistronic firefly luciferase (FLuc) reporter plasmids pGL3-Prom-Type-I and pGL3-Prom-Type-II containing the 5′-UTR of type-I and type-II RUNX2 mRNAs, respectively, were constructed as follows: the 5′-UTRs of type-I and type-II RUNX2 mRNAs were PCR amplified using pCR-II TOPO vectors containing the respective 5′-UTR cDNAs as templates [Sudhakar et al., 2001], a type-specific forward primer with a *Hind*III site (Type-I F: 5′-ATAGGGAGAC CCAAGCTTCT GAAGTTAACA ACGAAAAATT AACGCCAGTC GGAGCAGCC; Type-II F: 5′-GGGAGACCCA AGCTTGTGTG AATGCTT-CAT TCGCCTCACA AACAACCACA GAAC-CAC) and a type-specific reverse primer with a *Nco*I site (Type-I R: 5′-TGTTTTTGGC GTCTTCCATGG TGGCACAACA GCCACAA-GTT AGCGAAGTGG CCG; Type-II R: 5′-TGGCGTCTTC CATGGTGGCA GTCCCTCC-TT TTTTTTCCAG ATAGAACTTG TGCCC-TCTG). The PCR products were digested with *Hind*III and *Nco*I and cloned into *Hind*III/*Nco*I sites of pGL3-Promoter vector (pGL3-Prom; Promega). pGL3-Prom-Globin containing the 5′-UTR of β-globin mRNA [Radosavljevic and Crkvenjakov, 1989] was generated by anne-aling two complementary oligonucleotides (Globin F: 5′-AGCTTACATT TGCTTCTGAC ATAGTTGTGT TGACTCACAA ACTCAGAAAC AGCCAC and Globin R: 5′-CATGGTGGCT GTTTCTGAGT TTGTGAGTCA ACACAACTAT GTCAGAAGCA AATGTA), and then inserting the resulting double-stranded DNA into *Hind* III/*Nco*I sites of pGL3-Promoter vector. pGL3-Prom-HCV, which contains the hepatitis C virus (HCV) 5′-UTR (324 nucleotides), was obtained by PCR amplifying the HCV 5′-UTR with HCV-F (5′-TAGGGCGAAT TGAAGCT-TGG CGACACTCCA CCATAGATCA CTCC-CCTG) and HCV-R (5′-CGGATAGAAT GGC-GCCGGGC CTTTCTTTAT GTTTTTGGCG TC-TTCAGGAT TCGTGCTCAT GGTGCACGGT CTACG) primers using pHCV as a template (a generous gift from Dr. M.G. Katze, Department of Microbiology, University of Washington, Seattle, Washington); the PCR product was digested with *Hind*III/*Nar*I and cloned into *Hind*III/*Nar*I digested pGL3-Promoter.

Promoterless monocistronic expression plasmids. Promoterless monocistronic plasmids pGL3-Basic, pGL3-Basic-Type-I, pGL3-Basic-Type-II, pGL3-Basic-Globin, and pGL3-Basic-HCV were generated by removing the *Sac*I-*Hind*III fragment containing the SV40 promoter from pGL3-Prom, pGL3-Prom-Type-I, pGL3-Prom-Type-II, pGL3-Prom-Globin, and pGL3-Prom-HCV.

Promoter-containing and promoterless dicistronic expression plasmids. Dicistronic plasmids containing *Renilla* luciferase (RL) as the first cistron and FLuc as the second cistron were used in the dicistronic assay. Promoter-containing dicistronic plasmids were constructed using monocistronic phRG-Promoter vector with a SV40 promoter generated by cloning a *Sac*I/*Nco*I fragment from pGL3-Prom into the *Sac*I/*Nco*I sites of phRG-Basic vector. The dicistronic plasmids pRL-Prom-FLuc, pRL-Prom-Type-I-FLuc, pRL-Prom-Type-II-FLuc, pRL-Prom-Globin-FLuc, and pRL-Prom-HCV-FLuc were constructed by cloning the *Hind*III(B)/*Bam*HI cassettes from pGL3-Prom, pGL3-Prom-Type-I, pGL3-Prom-Type-II, pGL3-Prom-Globin, and pGL3-Prom-HCV, respectively, into *Xba*I(B)/*Bam*HI sites of phRG-Promoter. Promoterless dicistronic plasmids pRL-FLuc, pRL-Type-I-FLuc, pRL-Type-II-FLuc, pRL-Globin-FLuc, and pRL-HCV-FLuc were generated as described above for promoter-containing dicistronic plasmids, except that the cloning vector used was phRG-Basic (Promega).

Monocistronic transcription plasmids. *Hind*III(B)/*Xba*I fragments containing the luciferase reporter gene with different 5′-UTRs from the respective monocistronic pGL3-Promoter plasmids (described above) were cloned into *Sac*I(B)/*Xba*I sites of pBluescript II KD+ (Stratagene) to generate pBS-FLuc, pBS-Type-I-FLuc, pBS-Type-II-FLuc, pBS-Globin-Fluc, and pBS-HCV-FLuc. A DNA fragment containing the 3′-UTR of a SV40 late mRNA followed by $d(A/T)_{100}$ was excised from the polyA vector pBS-FLuc-A100 [Elango et al., 2005] with *Xba*I/*Xho*I and cloned into the *Xba*I/*Xho*I sites of pBS-Type-I-Fluc, pBS-Type-II-FLuc, pBS-Globin-FLuc, and pBS-HCV-FLuc to generate pBS-Type-I-FLuc-A100, pBS-Type-II-FLuc-A100, pBS-Globin-FLuc-A100, and pBS-HCV-FLuc-A100. The pBS-FLuc-A100 contains the type-II

restriction endonuclease Esp3I site after the d(A/T) tail, and in vitro transcription of the poly $d(A/T)_{100}$ plasmids linearized with Esp3I will generate RNA with a polyA tail of 100A residues followed by no heterologous sequence [Elango et al., 2005]. Since the 5′-UTR of type-I RUNX2 contains an internal Esp3I site, the *Bam*HI/*Hind*III fragment (upper strand: 5′-GATC-CA_{101}GAGACGA) from pBS-Type-I-Fluc-A100 was replaced by a *Bam*HI/*Hind*III fragment (upper strand: 5′-GATCCA_{100}ATGCATA) containing a *Nsi*I site (obtained by annealing two complimentary oligonucleotides). Linearization of the modified pBS-Type-I-FLuc-A100 with *Nsi*I followed by blunt ending with T4 DNA polymerase and in vitro transcription will result in RNA containing a polyA tail followed by no heterologous sequence.

pBS-4E-BP1-A100 containing the cap-binding protein inhibitor 4E-BP1 cDNA was constructed as follows: the coding region of mouse 4E-BP1 was RT-PCR amplified using total RNA from MC3T3-E1 cells, and a forward primer (5′-GTAAAGCCAC CATGGCGGCG GGCAG-CAGCT GCAGCCAGAC TC) and a reverse primer (5′-CAGCATGCAT CTAGATTAAA TG-TCCATCTC AAATTGTGAC TCTTC) synthesized on the basis of the mouse 4E-BP1 sequence [Lin et al., 1995]. After digesting with *Nco*I and *Xba*I, the PCR product was cloned into pBS-FLuc-A100 to obtain pBS-4E-BP1-A100 [Elango et al., 2005].

p4E-Aptamer-1 containing the 86-nucleotide long inhibitor RNA Aptamer-1 was generated in two steps. First, a plasmid containing 70 nucleotides of the 86-nucleotide long aptamer was constructed by annealing two 75-nucleotide long oligonucleotides (sense primer: 5′-AGC-TTCGCTC AATGTTCAAC CAGAGTGAAA CCACTAACGG GTCAGAGCCC CTTCGAC-AGG AGGCTCACAA CAGGC; antisense primer: 5′-TCGAGCCTGT TGTGAGCCTC CT-GTCGAAGG GGCTCTGACC CGTTAGTGGT TTCACTCTGG TTGAACATTG AGCGA), synthesized on the basis of the high affinity RNA Aptamer-1 [Mochizuki et al., 2005] and cloning the resulting fragment into the *Hind*III/*Xho*I sites of pcDNA3. The remaining 16 nucleotides were then added by site-directed mutagenesis using the QuickChange XL site-directed mutagenesis kit (Stratagene), a forward primer (5′-TCACTATAGG GAGACAAGAA TAAACGC-TCA ATGTTCAACC AGAGTG-AAACC ACT) and a reverse primer (5′-GTT-GAACATT GA-GCGTTTATT TCTTGTCTCC CTATAGTGAG TCGTATTAAT TTCG).

Dicistronic transcription plasmids. Dicistronic transcription plasmids used as templates to generate dicistronic mRNAs were constructed as follows: first, the *Xba*I/*Bam*HI DNA fragment containing the SV40 mRNA 3′-UTR from the polyA vector pBS-FLuc-A100 was cloned into the respective monocistronic pGL3-Prom vectors described above to generate pGL3-Prom-SV40, pGL3-Prom-Type-I-SV40, pGL3-Prom-Type-II-SV40, pGL3-Prom-Globin-SV40, and pGL3-Prom-HCV-SV40. Dicistronic transcription plasmids pBS-RLuc-FLuc-A100, pBS-RLuc-Type-I-FLuc-A100, pBS-RLuc-Type-II-FLuc-A100, pBS-RLuc-Globin-FLuc-A100, pBS-RLuc-HCV-FLuc-A100 were then generated by cloning the *Hind*III(B)/*Bam*HI fragment from the respective pGL3-Prom-SV40 plasmids into the *Xba*I(B)/*Bam*HI sites of pBS-RLuc-A100.

All plasmids were sequenced to confirm the sequence and orientation of the inserts.

In Vitro Transcription

All monocistronic and dicistronic transcription plasmids excluding pBS-Type-I-FLuc-A100 and pBS-RLucType-I-FLuc-A100 were linearized with Esp3I; pBS-Type-I-FLuc-A100 was linearized with *Nsi*I and the ends were made blunt with T4 DNA polymerase. Since the RL cDNA contains *Nsi*I site, pBS-RLuc-Type-I-FLuc-A100 was linearized with *Xho*I. In vitro transcription was performed with or without cap analog m^7GpppG (Promega) or ApppG (New England Biolabs) employing T7 RNA polymerase (MEGAscript T7 kit, Ambion, Inc.). Capped monocistronic RL reference mRNA was transcribed from pBS-RLuc-A100. The in vitro transcribed polyA containing RNAs were quantified by absorbance at 260 nm, and the integrity of the transcripts was verified on a formaldehyde-agarose gel. All RNAs were then diluted to 100 μg/ml with yeast tRNA (100 μg/ml) prepared as described previously [Elango et al., 2005]. The plasmid p4E-Aptamer-1 was linearized with *Xho*I and transcription was carried out with all four regular nucleotides and T7 RNA polymerase. The sequence of the in vitro transcribed Aptamer-1 is: 5′-GGGAGACAAG AA-UAAACGCU CAAUGUUCAA CCAGAGUGAA ACCACUAACG GGUCAGAGCC CCUUCGA-CAG GAGGCUCACA ACAGGCucga (last four

nucleotides come from the *Xho*I site used to linearize the plasmid).

DNA Transfection

Cells were plated at a density of 2×10^5 (UMR-106) or 2.5×10^5 (ROS 17/2.8 and MC3T3-E1) cells per well in a six-well plate, grown for 24 h and then transfected in triplicate using lipofectamine (Invitrogen) according to the manufacturer's instructions. After 3 h the transfection medium was replaced with 3 ml of culture medium, and 40 h post-transfection cells were lysed with 0.5 ml passive lysis buffer (Promega) by freeze thawing three times. The lysates were centrifuged, and 20 μl of lysate was used to assay both firefly and RLs using the dual luciferase assay kit (Promega).

mRNA Transfection

All monocistronic and dicistronic mRNA transfections were carried out in triplicate with yeast tRNA as a carrier using an optimized mRNA transfection method described previously [Elango et al., 2005]. In monocistronic mRNA transfections, RL mRNA was used as a reference mRNA. At 16 h post-transfection, the cells were washed with PBS and lysed with 0.5 ml passive lysis buffer (Promega) by freeze thawing two times. The lysates were centrifuged at 14,000 rpm for 5 min in an Eppendorf centrifuge. Both firefly and RL activities were determined using the dual luciferase assay kit as described above.

Northern Blot Analysis

ROS 17/2.8 cells were seeded and transfected with uncapped, m^7GpppG- and ApppG-capped mRNAs containing different 5′-UTRs as described above. After 16 h total RNA was isolated by the guanidinium thiocyanate-phenol-chloroform extraction method [Chomczynski and Sacchi, 1987] using TRI reagent (Molecular Research Center, Inc., OH). RNA samples (10 μg each) were electrophoresed on a 1.0% formaldehyde-agarose gel and transferred to nitrocellulose membrane. Ten nanograms of m^7GpppG-capped FLuc mRNA containing the 5′-UTR of type-II RUNX2 mRNA was used as a marker. The RNA was UV cross-linked to the membrane, after which the membrane was prehybridized, hybridized with random primer ^{32}P-labeled FLuc cDNA insert, washed and exposed to a PhosphorImager screen as described [Sudhakar et al., 2001]. The membrane, without removal of the hybridized probe, was then probed with a 5′-end labeled antisense 18S ribosomal RNA oligonucleotide (5′-GCCGTG-CGTACTTAGACATGCATG) as a control for RNA loading and transfer [Sudhakar et al., 2001].

RESULTS

The Dicistronic Assay Employed to Demonstrate IRESs in Type-I and Type-II RUNX2 mRNAs Is Flawed

Using MC3T3-E1 cells stably transfected with SV40 promoter-containing dicistronic plasmids, Xiao et al. [2003] reported that both type-I and type-II RUNX2 mRNAs are translated by the IRES mechanism. The dicistronic assay is performed to reveal the presence of an IRES in the 5′-UTR of viral and cellular mRNAs [Hellen and Sarnow, 2001]. In this assay it is presumed that when cells are transfected with a promoter containing dicistronic plasmid, a dicistronic mRNA is transcribed from the promoter-cloned upstream of the first cistron. While the first cistron of the dicistronic mRNA is translated by a cap-dependent scanning mechanism, the second cistron is assumed to be translated only when preceded by an IRES-containing UTR. The validity of these assumptions has been questioned on the basis of results obtained from dicistronic assays performed with both promoterless and promoter-containing dicistronic plasmids [Han and Zhang, 2002]. The 5′-UTRs of some cellular mRNAs suggested to contain IRESs by the dicistronic assay have been found to operate as cryptic promoters when placed as the intercistronic region in a promoterless dicistronic plasmid [Han and Zhang, 2002]. It should be noted that monocistronic transcripts can also be generated from dicistronic plasmids by aberrant splicing [Van Eden et al., 2004]. In the previous study by Xiao et al. [2003], no experiments were carried out to eliminate the possibility that monocistronic mRNAs might have been generated from the dicistronic plasmids containing the 5′-UTRs of type-I and type-II RUNX2 mRNAs as the intercistronic region.

To verify whether the 5′-UTRs of type-I and type-II RUNX2 mRNAs contain IRES elements, we performed the dicistronic assay with SV40 promoter-containing as well as promoterless dicistronic plasmids (Fig. 1). Both types of

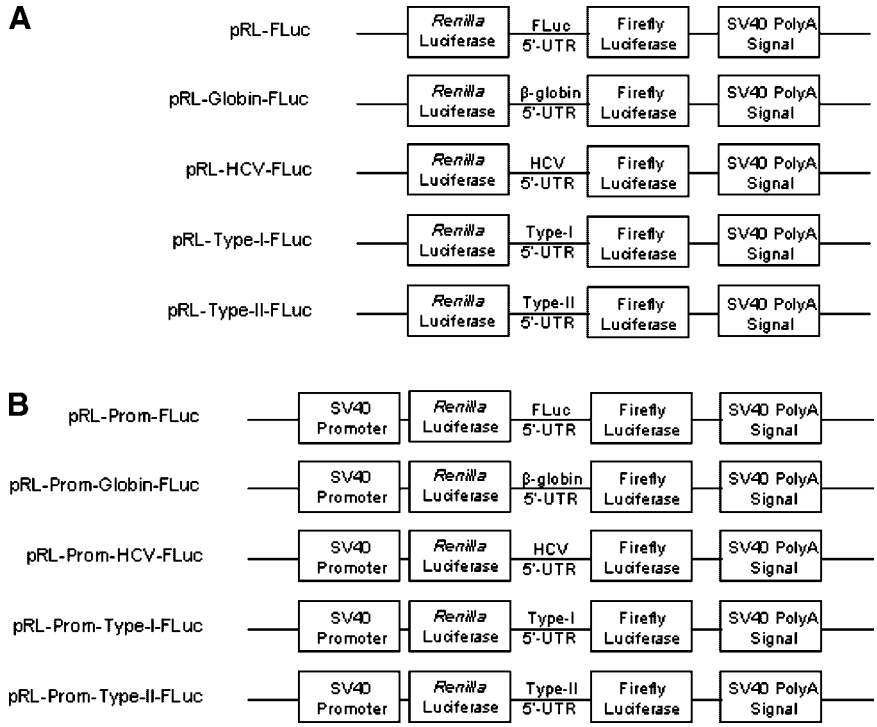

Fig. 1. Schematic representation of dicistronic plasmids. **A**: Promoterless dicistronic plasmids. **B**: SV40 promoter-containing dicistronic plasmids. RL, *Renilla* luciferase; FLuc, firefly luciferase; HCV, hepatitis C virus; Type-I, type-I RUNX2; Type-II, type-II RUNX2. The lengths of the different intercistronic 5′-UTRs are not drawn to scale.

plasmids contained the RL gene as the first cistron followed by a 5′-UTR as the intercistronic region and the FLuc gene as the second cistron. All promoter-containing plasmids had a SV40 promoter upstream of the RL gene. The 5′-UTRs of β-globin and FLuc mRNAs were used as negative controls: β-globin mRNA, with a 52-nucleotide long 5′-UTR, is known to be translated by the cap-dependent scanning mechanism [Lockard and Lane, 1978] while translation of FLuc mRNA is expected to be cap-dependent on the basis of a short unstructured 5′-UTR sequence (35 nucleotides long). The IRES-containing 5′-UTR of HCV RNA [Tsukiyama-Kohara et al., 1992] was used as a positive control.

Osteoblastic cell lines (ROS 17/2.8 and UMR-106) and preosteoblasts (MC3T3-E1) were transfected with promoterless or promoter-containing dicistronic plasmids, and the firefly and RL enzymes expressed were assayed as described under Materials and Methods. Figure 2 shows results from UMR-106 cells; similar results were obtained in ROS 17/2.8 and MC3T3-E1 cells. As expected, very little of the first cistron (RL) was detected in cells transfected with promoterless dicistronic plasmids compared to cells transfected with promoter-containing dicistronic plasmids. On the other hand, the second cistron (FLuc) was expressed by all promoterless as well as promoter-containing plasmids. Expression of the second cistron by promoterless dicistronic plasmids clearly invalidates the use of the dicistronic plasmid assay to demonstrate IRES activity in 5′-UTRs. Surprisingly, promoterless dicistronic plasmids expressed more of the second cistron than did the promoter-containing dicistronic plasmids (Fig. 2). Lower expression level of the second cistron by the promoter-containing vectors raises the possibility that in these vectors two promoters (SV40 and cryptic) present in close proximity may compete for transcription factors, whereas a single cryptic promoter in the promoterless dicistronic plasmids may function without competition.

The cDNAs Corresponding to the 5′-UTRs of Type-I RUNX2 and HCV RNA Exhibit Cryptic Promoter Activities

Expression of the second cistron by promoterless dicistronic plasmids (see above) suggested

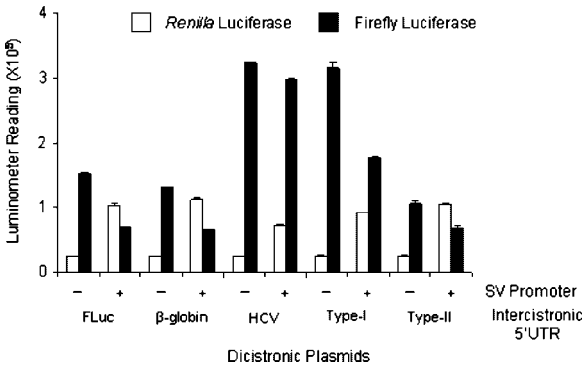

Fig. 2. The dicistronic plasmid assay in UMR-106 cells transfected with promoterless and SV40 promoter-containing dicistronic plasmids. Cells were transfected in triplicate with 1 μg of dicistronic plasmids (Fig. 1), and after 40 h *Renilla* and firefly luciferase activities (mean ± SD) were measured as described under Materials and Methods.

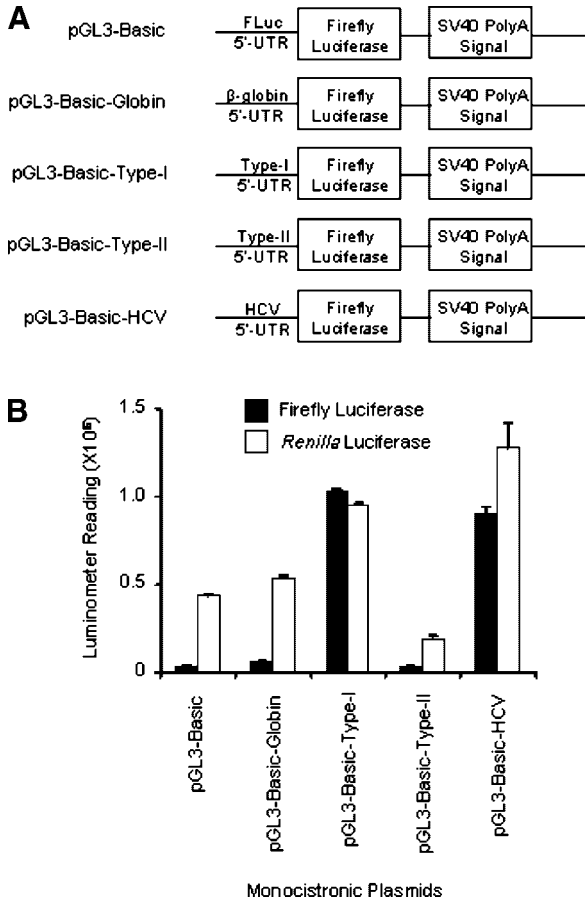

Fig. 3. Cryptic promoter activity of cDNAs containing type-I RUNX2 mRNA and HCV RNA 5′-UTRs. **A**: Schematic representation of promoterless monocistronic plasmids containing the 5′-UTRs of different RNAs. FLuc, firefly luciferase; Type-I, type-I RUNX2; Type-II, type-II RUNX2; HCV, hepatitis C virus. The lengths of the different 5′-UTRs are not drawn to scale. **B**: Firefly luciferase expression by promoterless monocistronic plasmids. ROS 17/2.8 cells were cotransfected in triplicate with 1 μg of promoterless monocistronic plasmids, and 0.1 μg of the reference plasmid phRG-Promoter containing *Renilla* luciferase cDNA; after 40 h firefly and *Renilla* luciferase activities (mean ± SD) were measured as described under Materials and Methods. Firefly and *Renilla* luciferase activities are presented as absolute values (luminometer reading, $10^6\times$) to show that the expression level of the reference reporter varies with the cotransfected plasmid.

that the intercistronic 5′-UTRs may contain a cryptic promoter or that the intercisronic region, together with the upstream sequence, may function as a putative cryptic promoter. To test this hypothesis we generated promoterless monocistronic plasmids containing the 5′-UTRs of FLuc (pGL3-Basic), type-I RUNX2 (pGL3-Basic-Type-I), type-II RUNX2 (pGL3-Basic-Type-II), β-globin (pGL3-Basic-Globin), and HCV (pGL3-Basic-HCV) RNAs and carried out transient transfection studies in ROS 17/2.8 cells; phRG-Promoter containing the RL gene was used as a reference plasmid. As shown in Figure 3, pGL3-Basic-Type-I and pGL3-Basic-HCV expressed about 10-fold higher levels of luciferase enzyme compared to that expressed by the control promoterless plasmid pGL3-Basic. On the other hand, pGL3-Basic-Type-II and pGL3-Basic-Globin expressed low levels of luciferase comparable to the control plasmid. These results demonstrate that the cDNAs corresponding to the 5′-UTRs of type-I RUNX2 and HCV RNAs are capable of acting as cryptic promoters, whereas the cDNAs of the 5′-UTRs of globin and type-II RUNX2 mRNAs are not. Accordingly, the cDNAs of the 5′-UTRs of type-I RUNX2 and HCV mRNAs are likely to act as cryptic promoters in dicistronic plasmids with no contribution from the upstream sequence; in contrast, the short unstructured cDNAs of the 5′-UTRs of type-II RUNX2, β-globin and FLuc mRNAs may require the upstream sequence of the dicistronic plasmid to function as cryptic promoters or in aberrant splicing. The RL expression by the reference reporter plasmid phRG-Promoter varied among the transfections, suggesting modulation of RL expression by the cotransfected plasmids.

The 5′-UTRs of Type-I and Type-II RUNX2 mRNAs Display No IRES Activity in Dicistronic Reporter Transcripts

Since monocistronic mRNA can be generated from dicistronic plasmids due to cryptic

promoter or splice site(s) present in the intercistronic region, it has been suggested to use dicistronic mRNAs rather than dicistronic plasmids in in vitro or in vivo translation assays to demonstrate the presence of an IRES in a 5′-UTR [Kozak, 2001]. In the present study we performed dicistronic mRNA transfection experiments to determine whether IRES activity is present in the 5′-UTRs of type-I and type-II RUNX2 mRNAs. When ROS 17/2.8 cells were transfected with m^7GpppG-capped dicistronic transcripts (RLuc-FLuc-A100, RLuc-Globin-FLuc-A100, RLuc-Type-I-FLuc-A100, RLuc-Type-II-FLuc-A100, and RLuc-HCV-FLuc-A100) containing different 5′-UTRs as the intercistronic region, only the dicistronic transcript bearing the HCV-5′-UTR displayed IRES activity; no IRES activity was exhibited by other 5′-UTRs, including the 5′-UTRs of type-I and type-II RUNX2 mRNAs (Fig. 4). The expression level of the first cistron (RL) varied among the five dicistronic mRNAs tested, the lowest expression being observed with the type-I RUNX2 dicistronic mRNA and the highest with the globin dicistronic mRNA. This result suggests that the sequence and length of the intercistonic region of a dicistronic mRNA affect the translation of the first cistron.

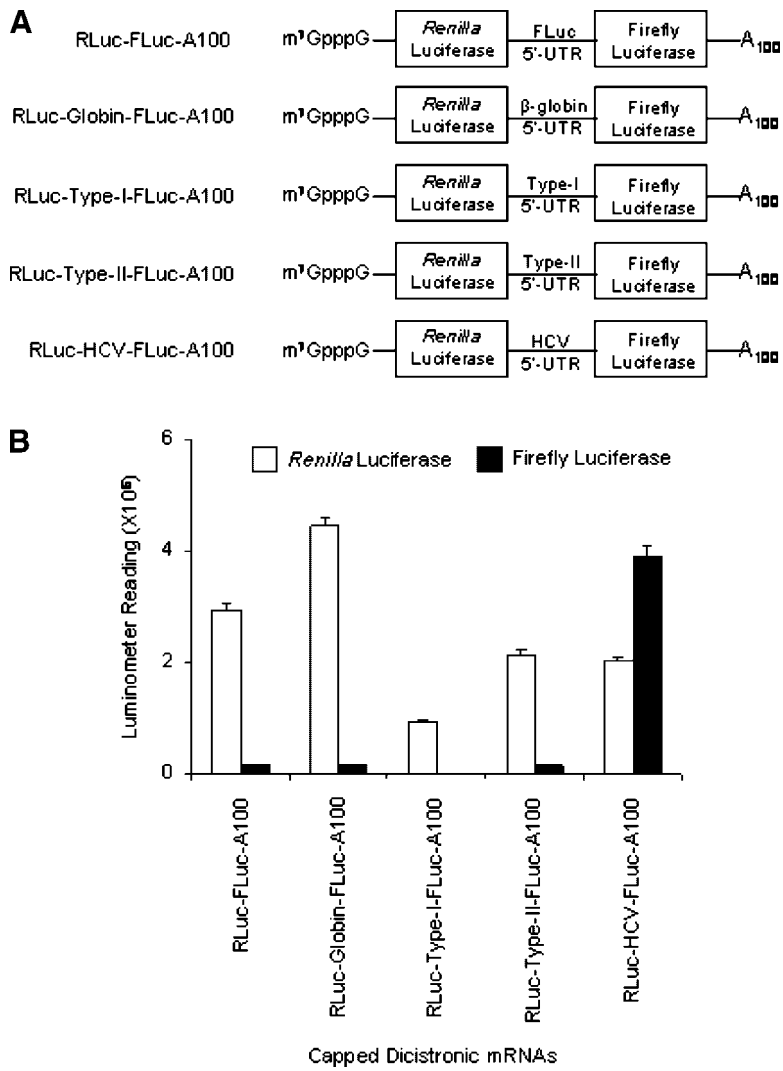

Fig. 4. The dicistronic mRNA assay in ROS 17/2.8 cells transfected with m^7GpppG-capped dicistronic mRNAs. **A**: Schematic representation of m^7GpppG-capped dicistronic mRNAs. A100 denotes a polyA tail of 100 nucleotides. RLuc, *Renilla* luciferase; FLuc, firefly luciferase; Type-I, type-I RUNX2; Type-II, type-II RUNX2; HCV, hepatitis C virus. The lengths of the different intercistronic 5′-UTRs are not drawn to scale. **B**: Translation of dicistronic mRNAs in transfected cells. Cells were transfected in triplicate with 500 ng of capped dicistronic mRNAs, and after 16 h *Renilla* and firefly luciferase activities (mean ± SD) were measured as described under Materials and Methods.

The Presence of m^7GpppG-cap Confers a High Level of Translation to Luciferase mRNA Preceded by the 5′-UTR of Type-II RUNX2 mRNA in Transfected Cells

Translation of mRNAs by the cap-dependent scanning mechanism requires the functional cap m^7GpppN at the 5′-terminus of mRNA; in contrast, IRES-dependent mRNA translation does not require a cap at the 5′-end [Hellen and Sarnow, 2001]. To determine whether translation of type-I and type-II RUNX2 mRNAs in cells is cap dependent, we performed transfection studies using uncapped and capped monocistronic FLuc mRNAs. Osteoblast-like cells (ROS 17/2.8) were transfected with Type-I-FLuc-A100 and Type-II-FLuc-A100 containing, respectively, the 5′-UTRs of type-I and type-II RUNX2 mRNAs; mRNAs were either uncapped, or capped with m^7GpppG or the nonfunctional cap analog ApppG. Reporter mRNAs containing the 5′-UTRs of β-globin mRNA (Globin-FLuc-A100) and HCV RNA (HCV-FLuc-A100) were used as controls for cap-dependent and cap-independent translation. Capped RL mRNA was cotransfected as a reference mRNA in all transfections. ROS 17/2.8 cells were used because both type-I and type-II RUNX2 mRNAs are translated in these cells [Sudhakar et al., 2001].

At 16 h post-transfection Type-II-FLuc-A100 and Globin-FLuc-A100 were translated to a high degree in the presence of m^7GpppG-cap, with minimal translation observed in the absence of a cap (Fig. 5A). Translation of the two mRNAs was also markedly reduced in the presence of the nonfunctional ApppG-cap. Uncapped and ApppG-capped HCV-FLuc-A100 mRNAs were translated to a greater extent than uncapped and ApppG-capped Type-II-FLuc-A100 and Globin-FLuc-A100, consistent with a cap-independent mechanism of HCV RNA translation. Nonetheless, the presence of m^7Gppp G-cap conferred a higher degree of translation to HCV-FLuc-A100 than did ApppG-cap or no cap. This observation is consistent with the findings of a previous study in which the m^7GpppG-cap was shown to enhance translation of HCV RNA [Wiklund et al., 2001]. Under all conditions (m^7GpppG-cap, ApppG-cap or no cap) Type-I-FLuc-A100 was translated to a lower extent than the other mRNAs tested; the presence of m^7GpppG-cap enhanced translation of Type-I-FLuc-A100 but to a very limited degree. The cotransfected RL mRNA (reference mRNA) was translated to the same degree in all transfections (results not shown).

Northern blot analysis of RNA from cells transfected with capped and uncapped mRNAs revealed that a small percentage of FLuc transcripts remained intact 16 h post-transfection (Fig. 5B). Based on the levels of intact mRNAs at 16 h, it is evident that for Globin-FLuc-A100, Type-II-FLuc-A100, and HCV-FLuc-A100 the differences in translation observed among m^7GpppG-capped, ApppG-capped, and uncapped mRNAs were not due to variations in mRNA stability. The amounts of Type-I-FLuc-A100 remaining 16 h post-transfection were lower than those of other mRNAs tested. To assess further whether the reduced levels of luciferase expressed by Type-I-FLuc-A100 (see above) were related to mRNA instability, we compared luciferase expression and mRNA levels of Type-I-Fluc-A100 and Type-II-FLuc-A100 at 4 h post-transfection. Figure 5C shows that in the presence of either m^7GpppG-cap or ApppG-cap, the translation of Type-I-FLuc-A100 at 4 h was lower than that of Type-II-Fluc-A100, despite the presence of intact Type-I-FLuc-A100 mRNA. As observed at 16 h post-transfection, translation of Type-I-FLuc-A100 at 4 h was enhanced by m^7GpppG-cap.

Taken together these results suggest that translation of type-II RUNX2 mRNA, like β-globin mRNA, is cap-dependent. The presence of cap confers only a very limited degree of translation to type-I RUNX2 mRNA. The low level of m^7GpppG-capped Type-I-FLuc-A100 translation in our experiments suggests that translation of type-I RUNX2 mRNA involves a mechanism other than the cap-dependent scanning mechanism.

The Cap-Binding Protein Inhibitors 4E-BP1 and RNA Aptamer-1 Suppress the Translation of Reporter mRNA Containing the 5′-UTR of Type-II RUNX2

The cap-dependent scanning mechanism of translation requires the binding of the cap-binding protein eIF4E to the m^7GpppN-cap at the 5′-end of the mRNA. Through association with eIF4G of the translation initiation complex, eIF4E directs the translation machinery to the 5′-end of mRNA. Three eIF4E binding proteins (4E-BP1, 4E-BP2, 4E-BP3) have been shown to modulate the amount of eIF4E

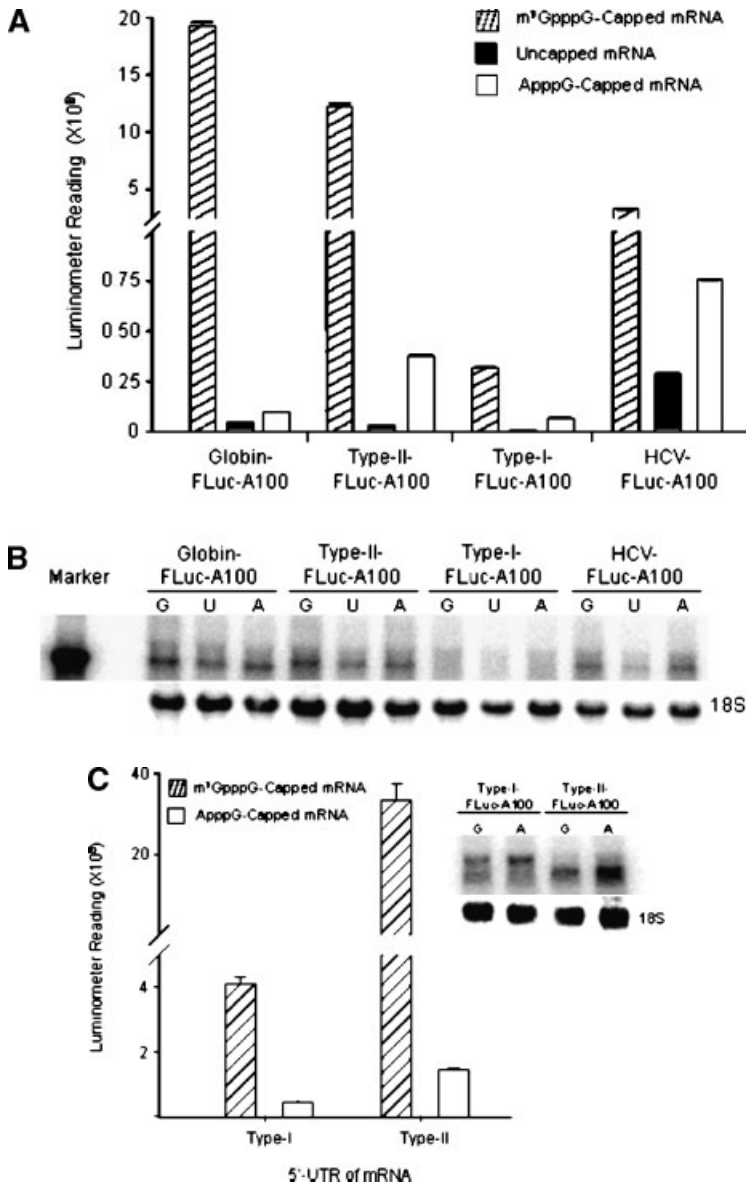

Fig. 5. Translation of firefly luciferase mRNA containing the 5′-UTR of type-II RUNX2 mRNA in transfected cells: enhancement by m^7GpppG-cap. **A**: Firefly luciferase expression from transfected monocistronic mRNAs (16 h post-transfection). In vitro transcribed m^7GpppG-capped, uncapped and ApppG-capped firefly luciferase mRNAs (100 ng) containing the 5′-UTRs of β-globin, type-II RUNX2, type-I RUNX2, and hepatitis C virus RNAs were cotransfected into ROS 17/2.8 cells with 50 ng of capped *Renilla* luciferase mRNA. A100 denotes a polyA tail of 100 nucleotides. FLuc, firefly luciferase; Type-II, type-II RUNX2; Type-I, type-I RUNX2; HCV, hepatitis C virus. Transfections were carried out in triplicate in six-well plates, and firefly and *Renilla* luciferase activities (mean ± SD) were measured 16 h post-transfection as described under Materials and Methods. The reference reporter (*Renilla* luciferase) activity was about 16×10^6 (luminometer reading) in all transfections (not shown). **B**: Northern blot analysis of m^7GpppG-capped (G), uncapped (U), and ApppG-capped (A) transcripts remaining at 16 h post-transfection. Transfections were performed as in (A); after 16 h total RNA was isolated, and 10 μg RNA samples were subjected to Northern blot analysis as described under Materials and Methods. Marker, 10 ng of m^7GpppG-capped Type-II-FLuc-A100. 18S, 18S ribosomal RNA. **C**: Firefly luciferase expression from transfected monocistronic mRNAs (m^7GpppG- and ApppG-capped Type-I-FLuc-A100 and Type-II-FLuc-A100), and Northern blot analysis of transcripts (4 h post-transcription). The activity of cotransfected reference reporter *Renilla* luciferase was about 3×10^6 (luminometer reading) in all transfections (not shown).

available for translation initiation; by competing with eIF4G for the binding site on eIF4E, the three 4E-BPs inhibit the eIF4E–eIF4G interaction and the recruitment of the translation machinery to the mRNA 5′-end [Richter and Sonenberg, 2005]. In addition to the 4E-binding proteins, an 86-nucleotide long RNA aptamer (Aptamer-1) with a high affinity for the

cap-binding protein has recently been reported to inhibit cap-dependent, but not IRES-dependent, translation by interfering with eIF4E binding to the cap [Mochizuki et al., 2005]. Aptamers are short nucleic acids or peptide ligands that have been engineered to inhibit the activity of specific proteins.

To confirm that type-II RUNX2 mRNA is translated by the cap-dependent mechanism, we tested the effects of 4E-BP1 and RNA Aptamer-1 in ROS 17/2.8 cells on the translation of transfected m^7GpppG-capped FLuc mRNA containing the 5′-UTR of type-II RUNX2 mRNA (Type-II-FLuc-A100). m^7GpppG-capped FLuc mRNA containing the 5′-UTR of β-globin mRNA (Globin-FLuc-A100) and ApppG-capped FLuc mRNA with HCV 5′-UTR (HCV-FLuc-A100) were used as controls for cap- and IRES-dependent translation, respectively. RL mRNA was cotransfected as a reference mRNA. Results shown in Figure 6A,B demonstrate that translation of m^7GpppG-capped reporter mRNAs containing the 5′-UTRs of type-II RUNX2 and β-globin mRNAs were suppressed by coexpressed 4E-BP1 and RNA Aptamer-1; translation of the reference reporter was similarly reduced by the inhibitors. In contrast, the translation of ApppG-capped HCV-FLuc-A100 was not inhibited by either 4E-BP1 or RNA Aptamer-1, but rather was slightly enhanced (Fig. 6C); enhancement of IRES-dependent translation could be due to increased availability of the translation machinery under conditions of suppressed cap-dependent translation. These results confirm that translation of type-II RUNX2 mRNA is cap-dependent.

DISCUSSION

Cell culture studies have shown that both type-I and type-II RUNX2 mRNAs are expressed in osteoblastic precursors and mature osteoblast-like cells as well as in nonosteoblastic cells [Tsuji et al., 1998; Xiao et al., 1998; Gori et al., 1999; Wang et al., 1999; Satomura et al., 2000; Park et al., 2001; Sudhakar et al., 2001; Choi et al., 2002]. In contrast, RUNX2 protein expression is more cell-type specific: type-I protein is expressed in early and mature osteoblasts, whereas type-II RUNX2 protein is expressed only in mature osteoblasts [Sudhakar et al., 2001]. Despite a half-life of 2 h [Gilbert et al., 2002], RUNX2 mRNAs are polysome free in preosteoblasts and polysome associated in osteoblasts, suggesting that RUNX2 mRNAs are dormant in osteoblast precursors and translated during osteoblast differentiation [Sudhakar et al., 2001]. These observations have led us to hypothesize that RUNX2 gene expression is translationally regulated. This hypothesis is supported by the finding that RUNX2 protein expression is increased without an increase in RUNX2 mRNA level in human preosteoblasts induced to differentiate by dexamethasone treatment [Prince et al., 2001]. Nothing is known about the mechanisms

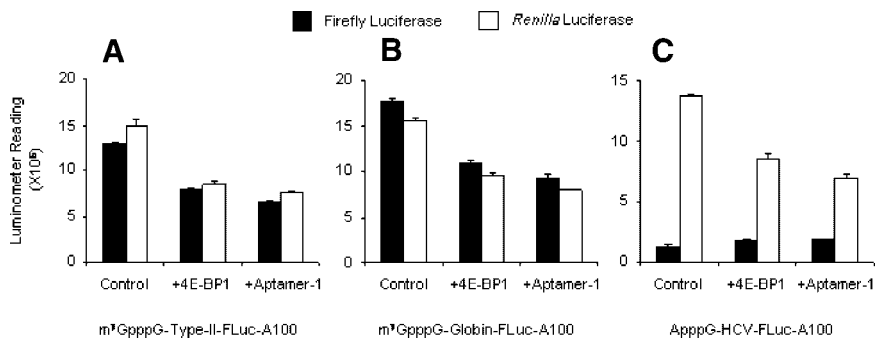

Fig. 6. Translation of m^7GpppG-capped firefly luciferase mRNA containing the 5′-UTR of type-II RUNX2 mRNA: suppression by inhibitors of cap-dependent translation. **A**: ROS 17/2.8 cells were cotransfected with m^7GpppG-capped firefly luciferase mRNA containing the 5′-UTR of type-II RUNX2 mRNA (Type-II-FLuc-A100, 100 ng) and m^7GpppG-capped *Renilla* luciferase mRNA (reference mRNA, 50 ng) with or without inhibitors (m^7GpppG-capped 4E-BP1 mRNA, 300 ng; RNA Aptamer-1, 1 μg). Transfections with (**B**) m^7GpppG-capped firefly luciferase mRNA containing the 5′-UTR of globin mRNA (Globin-FLuc-A100, 100 ng) and (**C**) ApppG-capped firefly luciferase mRNA containing the 5′-UTR of HCV RNA (HCV-FLuc-A100, 100 ng) were used to examine the effects of inhibitiors on translation of mRNAs known to be translated by cap- and IRES-dependent mechanisms, respectively. Transfections were carried out in triplicate in six-well plates, and luciferase activities (mean ± SD) were measured 16 h post-transfection as described under Materials and Methods. Control, no inhibitor.

controlling cell-type specific translation of RUNX2 mRNAs.

The 5′-UTR of type-I RUNX2 mRNA is long (1,015 nucleotides) with a high G + C content (69%), whereas type-II RUNX2 mRNA has a short 5′-UTR (210 nucleotides) with a low G + C content (47%). These features of the 5′-UTRs suggested to us that type-II RUNX2 mRNA would be translated by the cap-dependent translation mechanism, but the complex secondary structure of the type-I RUNX2 mRNA 5′-UTR would be inhibitory to this mechanism of translation.

Based on data from the dicistronic plasmid assay with promoter-containing dicistronic plasmids, Xiao et al. [2003] reported that type-I and type-II RUNX2 mRNAs are translated by the cap-independent IRES mechanism. No experiments were presented to demonstrate that the IRES activity observed was not due to cryptic promoter activity of the UTRs or aberrant splicing [Han and Zhang, 2002; Van Eden et al., 2004]. In the present study we evaluated the dicistronic plasmid assay using promoter-containing and promoterless dicistronic plasmids, promoterless monocistronic plasmids, and dicistronic mRNAs. The results of our experiments (Figs. 2 and 3) demonstrate that cDNA of the 5′-UTR of type-I RUNX2 mRNA acts as a cryptic promoter when used as the intercistronic region of a dicistronic plasmid. The results further suggest that the putative IRES activity of the 5′-UTR of type-II RUNX2 mRNA observed in the dicistronic plasmid assay is due to either cryptic promoter activity of the intercistronic region (5′-UTR) and upstream sequence or aberrant splicing. Moreover, neither type-I nor type-II RUNX2 mRNA 5′-UTR exhibited IRES activity in the dicistronic mRNA transfection assay (Fig. 4). Thus the dicistronic plasmid assay cannot be used to implicate translation of RUNX2 mRNAs by the IRES-dependent mechanism.

Monocistronic mRNA transfections revealed that m^7GpppG-capped type-II reporter mRNA was translated to a high degree, whereas translation was greatly reduced in the absence of m^7GpppG-cap (Fig. 5). Translation of m^7GpppG-capped type-II reporter mRNA was also suppressed by inhibitors of cap-dependent translation (Fig. 6). These findings clearly establish that type-II RUNX2 mRNA is translated by the cap-dependent scanning mechanism.

Currently available assays are inadequate to demonstrate whether type-I RUNX2 mRNA is translated by the cap-independent IRES mechanism. In our studies m^7GpppG-capped monocistronic type-I RUNX2 reporter mRNA was translated only to a very limited degree in comparison with type-II RUNX2 reporter mRNA (Fig. 5). This finding suggests that efficient translation of type-I RUNX2 mRNA requires a process other than the cap-dependent mechanism. However, in dicistronic mRNA transfections type-I RUNX2 reporter mRNA did not demonstrate any IRES activity (Fig. 4). Interestingly, when cells were transfected with monocistronic plasmids containing the 5′-UTRs of type-I (pGL3-Promoter-Type-I) or type-II (pGL3-Promoter-Type-II) RUNX2 mRNAs, a much higher level of FLuc reporter was expressed from pGL3-Promoter-Type-I than pGL3-Promoter-Type-II [Xiao et al., 2003; our unpublished results]. A high level of luciferase expression by type-I RUNX2 reporter plasmid but not by type-I reporter mRNA raises the possibility that nuclear factor(s) bound to type-I RUNX2 mRNA during transcription (but absent from in vitro transcribed reporter mRNA) may be essential for translation in the cytoplasm. Alternatively, type-I RUNX2 mRNA could be translated in the nucleus and not in the cytoplasm. Translation within nuclei of mammalian cells has been reported [Iborra et al., 2001] but remains controversial [Dahlberg et al., 2003]. Further investigation will be required to evaluate these two possibilities.

Data from our laboratory and others implicate translation as a control point in RUNX2 gene expression [Prince et al., 2001; Sudhakar et al., 2001]. Temporally distinct patterns of type-I and type-II RUNX2 protein expression during osteoblast differentiation suggest that both 5′- and 3′-UTRs are involved in translational regulation of the two isoforms [Park et al., 2001; Sudhakar et al., 2001; Choi et al., 2002]. The results of the current study demonstrate that the 5′-UTRs of type-I and type-II RUNX2 mRNAs are subject to different mechanisms of translation initiation. Our preliminary experiments confirm a role for the 3′-UTR in translational regulation of RUNX2 gene expression (data not shown). Elucidation of the translational regulation of both RUNX2 isoforms by the 5′- and 3′-UTRs will provide insight into the molecular processes by which the two isoforms

control osteoblast differentiation and skeletogenesis.

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