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Expression of Translation Initiation Factor IF2 is Regulated During Osteoblast Differentiation

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Abstract We isolated and characterized a cDNA for the N-terminal half of the eukaryotic initiation of translation factor 2 (cIF2) during a screen of chicken osteoblast cDNAs. The apparent size of the message for this protein, ~5.6 kb, is slightly larger in size than that for human IF2 (hIF2). There is a high degree of sequence similarity between the human and chicken N-terminal portions of the protein that extends to the encoding nucleotide sequence. The tissue specific expression pattern for cIF2 and hIF2 are similar, being moderately abundant in brain, liver, and skeletal muscle, and detectable in kidney, chondrocytes, and freshly isolated osteoblasts. The ratio of message for cIF2 to that of β-actin was 0.10 and 0.18 for liver and brain. Message levels peak in osteoblasts between 8 and 12 days of culture, coinciding with high levels of matrix protein synthesis. At peak expression, the ratio of cIF2:β-actin for 8 day osteoblasts was 0.76. Treatment of osteoblast cultures with cycloheximide markedly reduces the level of cIF2 message indicating that novel protein synthesis is required for its expression. Hybridization of RNA samples from either chicken osteoblasts or a human osteoblast cell line with a probe for a subunit of human eukaryotic initiation of translation factor 2 (eIF2α), the housekeeping initiation factor, indicates that levels of eIF2 remain low. With hIF2, cIF2 represents the only other vertebrate homolog of IF2 for which a major portion of the coding sequence has been identified. This is the first report of regulated expression for a eukaryotic IF2 and is the first demonstration of its abundance in osteoblasts. J. Cell. Biochem. 81:700–714, 2001. © 2001 Wiley-Liss, Inc.

Key words: initiator tRNA; eukaryotic translation; protein synthesis; translation initiation

A major control point for regulation of protein synthesis is initiation of translation. Translation initiation requires the formation of a complex composed of the 5′ end of the mRNA to be transcribed with an IF2·GTP·Met-tRNA$_i^{Met}$ complex and ribosomes [Pain, 1996]. The binding of tRNA$_i^{Met}$ to the initiation codon, AUG, occurs in an IF2 dependent manner, correctly positioning the Met-tRNA$_i^{Met}$ for accurate initiation of translation [Kozak, 1999].

The bacterial form of IF2, bIF2, is a monomer of approximately 97 kDa that possesses a GTP binding site as well as GTPase activity [Sacerdot et al., 1984; Severini et al., 1991]. Its importance in initiation results from the fact that it only recognizes fMet-tRNA and it is the only tRNA binding factor that can bind the 30S ribosome [Kozak, 1999]. Two domains in the N-terminus of bIF2 are required for optimal binding to the 30S and 50S ribosomal subunits [Moreno et al., 1999] while the C-terminal half of the protein, containing the GTP binding site, is required for catalytic activity [Cenatiempo et al., 1987; Laalami et al., 1991]. The recycling of bIF2 requires GTP hydrolysis since the GDP·bIF2 complex has much lower affinity for the initiation complex and dissociates to allow elongation to occur [Luchin et al., 1999]. bIF2 does not form a stable complex with GTP·Met-tRNA$_i^{Met}$ in the absence of ribosomes or message [Maitra et al., 1982].

The eukaryotic form of IF2, eIF2, is a heterotrimer. It is composed of α, β, and γ subunits which are 36, 38, and 52 kDa in size respectively for human eIF2 [Ernst et al., 1987; Pathak et al., 1988; Gaspar et al., 1994]. It was originally identified as the eukaryotic counterpart to bIF2. The activity of eIF2 is regulated by phosphorylation of serine 51 in the alpha subunit by specific kinases [Dever, 1999];

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phosphorylation of serine 51 inactivates eIF2 thereby preventing translation initiation. Although the rate of transcription of the message for eIF2α does not change, the stability of the immature message for eIF2α can be altered, thereby increasing the number of messages available for translation [Cohen et al., 1990]. These regulatory mechanisms tie the availability of active eIF2 to the level of protein synthesis required by the cell. The beta subunit may contain the RNA binding site while the gamma subunit contains three binding sites for GTP. GTP hydrolysis is also required for recycling of eIF2. The hydrolysis of GTP, via the activity of another translation factor eIF2B, is required for cycling of eIF2 between a bound state, where it is associated with other factors and RNA to form an initiation complex, and an unbound state that occurs once initiation has taken place. The formation of a functional initiation complex requires the association of the 40S ribosome with eIF2·GTP·Met-tRNA$_i^{Met}$ to form a 43S preinitiation complex which then binds mRNA to form the 48S initiation complex [Pain, 1996; Kleijn et al., 1998].

The initial identification of bIF2 only in prokaryotes and eIF2 only in eukaryotes led to the proposal that prokaryotes and eukaryotes used different proteins with differing modes of regulation to initiate translation. With the recent identification of homologs of bIF2 in eukaryotes (yeast IF2, yIF2 or previously FUN12) [Bussey et al., 1995], (human IF2 or hIF2) [Lee et al., 1999], and archaebacteria (aIF2) [Bult et al., 1996], however, it is apparent that IF2 represents a highly conserved translation initiation factor [Lee et al., 1999]. yIF2 has been shown to have the same function as bIF2; that is, it promotes the binding of Met-tRNA$_i^{Met}$ to ribosomes [Choi et al., 1998].

We have now identified a cDNA for the N-terminus of the IF2 homolog in chicken, referred to as cIF2, and used it to characterize cIF2 expression in chicken tissues and cultured osteoblasts.

METHODS

Cell Isolation and Culture

Osteoblasts were isolated from the periosteal surface of tibias from 2 to 3 week old White Leghorn chickens [for details see Gay et al., 1994; Stains and Gay, 1998]. The periosteal membrane was gently removed, the bones split lengthwise and the marrow cavity flushed from the bones prior to sequential enzymatic digestion. Scraping of the outside surface of the bones produced bone chips that were plated as the source of osteoblasts on either fibronectin-coated polystyrene tissue culture plates or glass tissue culture dishes. Culturing was performed in Dulbecco's modified Eagle's medium supplemented with 3.7 g/l sodium bicarbonate, 0.05 g/l ascorbate, 100 U penicillin, 100 μg streptomycin, and 10% heat inactivated fetal bovine serum. The medium was completely removed and replaced 48 h after plating, thereafter, half the medium was replaced every other day. On Day 6 β-glycerophosphate (10 mM) was added to the cultures to permit mineralization.

Immortalized human fetal osteoblasts (hFOB 1.19) were also used [Harris et al., 1995]. To examine rapidly proliferating cells, confluent cultures containing $\sim 10^7$ cells were split 1:10 in Dulbecco's minimal essential media/Ham's F12 (DMEM/F12 1:1 containing 15 mM HEPES, L-glutamine and pyridoxine hydrochloride; Gibco BRL, Rockville, MD) with 10% fetal bovine serum and 1% Pen/strep (Sigma, St. Louis, MO), and cultured to approximately 50% confluency at 34°C. This represents about four days of growth. Alternatively, cultures were allowed to reach confluency then held at 39°C in DMEM/F12 with 5 mg/ml ascorbic acid and 10^{-8} M menadione (vitamin K) for 48 h. These cells were obtained from Dr. Henry J. Donahue at the M.S.Hershey Medical Center (Hershey, PA) with permission of Dr. Thomas C. Spelsberg (Mayo Clinic, Rochester, MN). K562 cells, a human T lymphoblast cell line, were also cultured to provide RNA as a positive control for the eIF2α probe. These cells were grown in Hepes buffered RPMI 1640 supplemented with 10% fetal calf serum and Pen/Strep.

Cycloheximide (ICN Biomedicals, Inc., Aurora, OH) was prepared as a 20 mg/ml stock in water and stored at 20°C. On the appropriate day in culture, this stock was diluted to a final concentration of 20 μg/ml into the medium of the cultured osteoblasts. The osteoblasts were incubated in the presence of cycloheximide for 24 h and total RNA isolated using TRIzol reagent (Gibco BRL).

cDNA Preparation and Cloning

Isolation of poly A$^+$ RNA for preparation of cDNA was performed using a polyA Spin Kit (NEB, Beverly, MA). cDNA was produced using

a cDNA synthesis kit from Promega (Madison, WI). Selection of poly A^+ RNA was performed using an oligo-dT linker that contained an XbaI site and double-stranded cDNA products less than 400 bp in length were removed using a Sephacryl S400 column. An EcoRI linker adaptor (sequence of the longer primer of the adaptor pair is 5′-GAATTCCGTTGCTGTCG-3′) was added to the 5′ end of the cDNA, and the isolated products were phosphorylated and digested with XbaI. This produced a pool of polyA$^+$ derived cDNAs that contained an EcoRI site at the 5′ end and an XbaI site at the 3′ end. A portion of this cDNA pool was ligated into pUC19 (obtained from NEB), digested with EcoRI and XbaI, and used to transform library efficient competent DH5α bacteria (Gibco BRL).

To identify individual cloned cDNAs, bacterial colonies were transferred from plates to filters (Schleicher and Schuell, Keene, NH) and the plates returned to 37°C so colonies would regenerate. Filters were processed according to manufacturer instructions for colony screening. The original purpose of this cloning was to obtain a cDNA for chicken NCX1 so filters were probed using fragments taken from the canine NCX1 cDNA (obtained from Dr. Kenneth Philipson, UCLA) [Nicoll et al., 1990]. Two canine NCX1 fragments were used: a Hind III-XbaI fragment of 3.7 kb which contains the entire coding sequence and an Ava I 5′-specific fragment that contains the coding sequence for the N-terminal half of the NCX1 protein. Colonies that hybridized strongly to both of these probes were selected for further screening and sequencing by the Nucleic Acid Sequencing Facility at the Pennsylvania State University. Stocks of plasmids used in this study were prepared using either a Qiagen Plasmid Maxi kit (Valencia, CA) or a StrataPrep EF Plasmid kit (Stratagene, La Jolla, CA).

The isolation of poly A^+ RNA from cultured cells, and chicken and human osteoblasts, was performed using TRIzol reagent and the total RNA was held at −80°C in isopropanol prior to determination of concentration and the isolation of poly A^+ RNA. At least three independent osteoblast isolations were performed to establish osteoblast cultures for RNA analysis. Isolation of poly A^+ RNA for northern blot analysis was performed using the MicroPure Poly A kit from Ambion (Austin, TX). A large scale isolation of polyA$^+$ RNA from 8 plates of osteoblasts cultured for 8 days was performed to generate RNA for northern blot controls. This polyA$^+$ RNA was isolated using a 5′-3′ Kit obtained from PGC Scientifics (Gaithersburg, MD). Poly A^+ RNA isolated from chicken brain and liver was obtained from Clontech (Palo Alto, CA), and poly A^+ RNA from chicken kidney, heart, and skeletal muscle was performed using TRIzol reagent following the manufacturer's suggested protocol.

Total growth plate cells isolated from proximal tibia of immature White Leghorn chickens, three to four weeks of age, were obtained from the lab of Dr. Roland M. Leach at the Pennsylvania State University [for isolation see Rosselot et al., 1992]. These cells are a mixture of proliferating and hypertrophic chondrocytes; they were frozen at −80°C immediately following isolation. RNA isolation was performed using 1 ml TRIzol solution for each aliquot of 4×10^7 cells. Isolation of RNA from freshly isolated osteoblasts was performed using bone chips not used for plating that were placed directly into TRIzol. RNA was held in isopropanol and handled in the same manner as that for cultured osteoblasts.

Analysis of cIF2 Expression

Samples of polyA$^+$ RNA were run on formaldehyde gels (1% agarose in 1X MOPS buffer containing 0.22 M formaldehyde in both the gel and buffer [Tsang et al., 1993]). Molecular weight markers obtained from Gibco BRL were also run on each gel. Gels were blotted to GeneScreen Plus nylon membrane(NEN, Boston, MA) in 10X SSC overnight, and the blots baked at 80°C for 2 h after air drying.

Probes were synthesized using the Prime-a-Gene Labeling system from Promega and α^{32}P-dCTP (3000 Ci/mmol; NEN). For hybridizations with both IF2 and β-actin probes, probes were prepared at the same time to the same specific activity. The hIF2 fragments used as probes were isolated from a hIF2 cDNA clone obtained from Dr. Thomas E. Dever (NICHHD, Bethesda, MD) [Lee et al., 1999]. The hIF2 probes were a 2.6 kb BamH I-Kpn I fragment containing most of the hIF2 coding sequences, and a 1.4 kb Hind III-Kpn I fragment containing the 3′ half of the coding sequences. A human eIF2α clone [Ernst et al., 1987] was supplied by Dr. Scot R. Kimball (M.S.Hershey Medical Center, Hershey, PA) with the kind permission of Dr. J.W. Hershey (UC Davis, CA). The eIF2α probe used was a 1.4 kb EcoRI fragment. The β-

actin probe used was a 1.076 kb mouse β-actin fragment obtained from Ambion (Austin, TX). Pre-hybridization and hybridization were performed in glass bottles in a hybridization oven (VWR Scientific). Hybridization was at 42°C in 50% formamide/6X SSC/ 0.5% SDS containing 100 μg/ml sonicated salmon sperm DNA. Washes were performed at high stringency in 0.1X SSC/0.1% SDS at 55°C. Blots were exposed to Kodak XAR film at −80°C with an intensifying screen. The developed films were scanned and individual bands analyzed using ImageQuaNT (Molecular Dynamics, Sunnyvale, CA). Southern blots that were rehybridized, were stripped of the previous probes using sequential treatment of 0.4 M NaOH and 0.1X SSC/0.1% SDS/0.2M Tris-HCl, pH 7.5 for 30 min each at 42°C. Northern blots that were rehybridized, were stripped of the previous probes using one to three treatments of 0.01X SSC/0.01% SDS that was heated to boiling, poured into a hybridization bottle containing the blot, and incubated for 20 min at 75°C. Blots were considered stripped of previous probes if no radioactivity was detected using the most sensitive scale on the Geiger counter. In addition, an overnight exposure of these stripped blots to film did not produce any signal, and reprobing of blots was separated temporally by at least several weeks.

In addition to northern blot analysis, relative quantitative RT-PCR was performed. Using 2.5 μg of total RNA isolated from cultured chicken osteoblasts, first strand synthesis was performed with random decamers using a RETROscript kit from Ambion in a volume of 20 μl. One microliter of each cDNA mixture was then used in amplification reactions using displayTAQ FL DNA polymerase (PGC), the reaction buffer supplied with the polymerase, and primer pairs specific for cIF2 and 18S standards. The sequence of the cIF2 forward primer is 5′-ATATTGATATTGATGCCCTTGC-3′ and that of the cIF2 reverse primer is 5′-CCCGCTTAGGTAAAATATTTC-3′. The size of the fragment produced by the cIF2 primer pair is 386 bp. Following RETROscript kit recommendations, preliminary experiments were performed to define the number of cycles that would produce amplification within a linear range so that band intensity could be quantitated. In addition, alternate 18S internal standard primers and competimers, supplied by Ambion, in a ratio of 1:9 primer to competimer were used in each reaction to permit quantitation. This primer pair produces a band of 324 bp. Preliminary experiments were performed using a range of primer:competimer dilutions under the conditions that produced amplification within a linear range for the cIF2 primer pair. Amplification was performed in a DeltaCycler I thermocycler (Ericomp, San Diego, CA) using 30 repeats of a cycle consisting of 95°C for 1 min, 60°C for 1 min, 72°C for 1 min in a reaction volume of 50 μl. Ten microliters of each amplification reaction were run on 2% agarose gels (0.5X TBE buffer), stained with ethidium bromide and destained to remove excess dye before analysis. An image generated by the Eagle Eye II (Stratagene) was analyzed using ImageQuaNT (Molecular Dynamics).

Sequence Analysis Information

BLAST searches were performed to identify nucleotide and protein sequence matches to the chicken cDNA inserts [Altschul et al., 1997]. Alignments between cIF2 and other IF2 homologs were performed using the DIALIGN 2 program [Morgenstern et al., 1996; 1998].

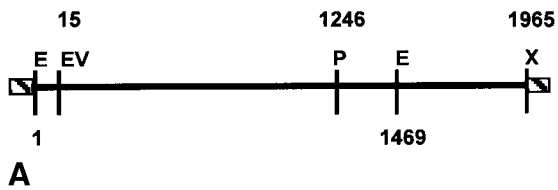
RESULTS

Identification of Chicken IF2 Sequences

Four cDNA clones representing messages expressed in chicken osteoblasts after eight days in culture were identified by hybridization to the canine NCX1 cDNA probe. Three of these cDNAs represented novel messages that appear to encode integral or transmembrane proteins. The fourth cDNA clone (Fig. 1) was identified by sequence match at both the nucleotide and amino acid levels to two cDNA sequences in GenBank. One cDNA was identified as KIAA0741 (Accession Number AB018284) submitted by the Kazusa DNA Research Institute in Japan. This cDNA was isolated from human brain but an identity was not included with the original sequence submission [Nagase et al., 1998]. The other nucleotide sequence, for a cDNA clone isolated from human testis (Accession Number AF078035) submitted by a group at NIH, was nearly identical to KIAA0741 and identified as encoding hIF2 [Lee et al., 1999]. This latter cDNA clone had been isolated by sequence homology to FUN12, the yeast IF2 homolog (Accession Number P39730) [Sutrave et al., 1994], and by function as a translation initiation factor [Choi et al., 1998]. A third

cDNA sequence corresponding to hIF2 was isolated from a T-lymphocyte library and submitted in July 1999 by a British group (Accession Number AJ006776) [Wilson et al., 1999]. The translation product for this hIF2 cDNA differs from that submitted by Lee et al. [1999] by only four amino acids while the translation product for the cDNA submitted by Nagase et al. [1998] differs by only six amino acids. Although this fourth chicken cDNA clone had short regions of significant sequence similarity to other proteins, the extensive sequence similarity to hIF2 indicated it was the chicken homolog of IF2.

The size of the cIF2 cDNA fragment is 1965 nucleotides and the translation produce for this insert is 655 amino acids in length (Fig. 1A and B). A comparison of the cIF2 amino acid sequence with that for any of the hIF2 cDNA clones (Fig. 1C) suggests that this partial cDNA sequence begins with amino acid 13 so that only a small portion of the 5′ end of the message is missing. In addition, the cDNA is truncated at the 3′ end although the insert sequence ends at



A

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gcaaaagatgatattgatattgatgcccttgctgctgagatagaaggtgcaggcgctgcaaaagaacaagagcctcagaaatccaaaggcaaaaagaaaaaa
 A  K  D  D  I  D  I  D  A  L  A  A  E  I  E  G  A  G  A  A  K  E  Q  E  P  Q  K  S  K  G  K  K  K  K
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 E  K  K  K  Q  D  F  D  E  D  D  I  L  K  E  L  E  E  L  S  I  E  A  Q  G  G  K  A  D  R  E  P  S  T
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 G  K  V  N  T  V  G  E  G  E  D  Y  V  I  G  N  S  W  D  T  R  H  L  C  P  R  K  V  S  L  C  C  G  L
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 G  L  L  S  E  V  S  G  Y  F  L  L  Y  L  F  K  C  Y  S  K  C  Q  F  E  I  F  Y  P  K  R  V  F  G  D
gatcatcattcaaagtcaatcagcataaattttaactgtgggctgagtgtagcacattcacagtaatagttactgcttttctaacttcgtcttcagtgagag
 D  H  H  S  K  S  I  S  I  N  F  N  C  G  L  S  V  A  H  S  A  V  I  V  T  A  F  L  T  S  S  S  V  R
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 E  V  F  L  D  I  S  E  C  C  N  S  Q  K  H  V  V  F  M  A  D  L  S  L  N  Y  Q  V  E  N  D  N  E  D
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ttggaacaagaaagaaaagaaagaagg
 L  E  Q  E  R  K  E  R  R
```

B

Fig. 1. Identification of the chicken homolog of hIF2. **A:** A schematic representation of the cIF2 cDNA insert in pUC19 with location of key restriction enzyme sites used for the generation of probes for Southern and northern blots. E = EcoRI; EV = EcoR V; P = Pst I; X = XbaI. The hatched bars represent pUC19 vector. **B:** Nucleotide sequence and putative translation product for the N-terminus cIF2 cDNA clone. **C:** Sequence alignment of yeast FUN12 (Y; Acc.No. P39730), human IF2 (H; Acc.No. AF078035) and chicken IF2 (C; Acc.No. AJ144637) sequences. The alignment is given for the translation product of each cDNA over the length of the chicken cDNA. Amino acids shown in capital letters represent aligned sequences.

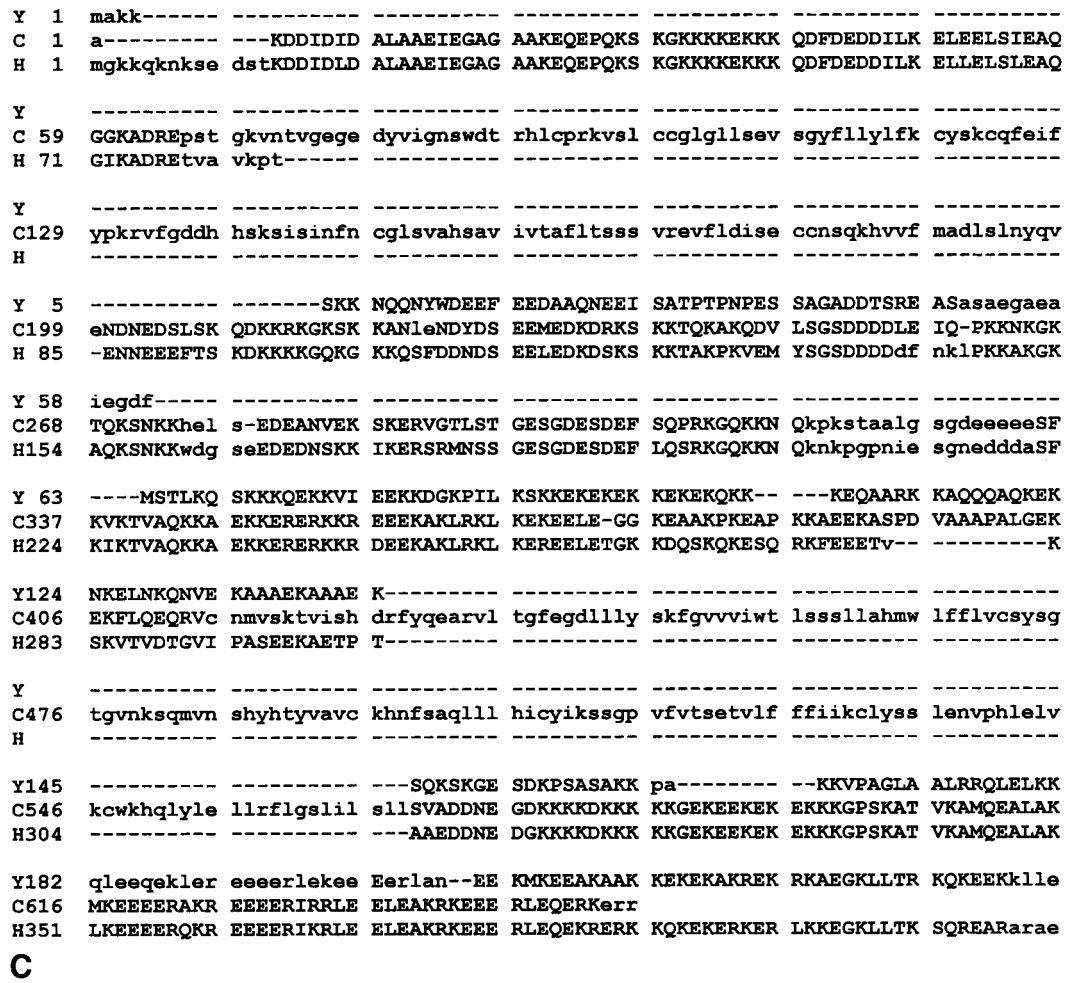

Fig. 1. (*Continued*)

an oligo-dT-XbaI linker sequence. The nucleotide sequence in the hIF2 cDNA just downstream of this sequence is A-rich which indicates that the oligo-dT portion of the poly A^+ selection primer annealed in this location because of its sequence similarity to the poly A tail. In fact, during the screening of cDNA clones for NCX1, colonies containing a plasmid with a 1.5 kb EcoRI fragment were isolated from nearly every filter. This observation predicted the abundance of this message in cultured osteoblasts.

Conservation of Sequence Among Eukaryotic IF2s

A sequence alignment of the putative translation product of cIF2 with hIF2 shows a high degree of conservation over three regions between these two vertebrate IF2 homologs (Fig. 1C). The regions in cIF2 and hIF2 that show alignment are amino acids 2–65, 200–414, and 569–652 of cIF2 with amino acids 14–77, 85–303, and 304–416 of hIF2. The degree of identity between these pairs is 95.2, 61.8, and 90.5%. This high degree of conservation is also seen at the nucleotide level. An alignment of the nucleotide sequences that encode these same regions shows 79.0, 68.5, and 78.9 % sequence identities.

cIF2 shows a higher degree of sequence similarity with hIF2 than with yIF2. Three regions of yIF2 (amino acids 5–49, 63–144, and 145–245) align within two of the same regions of cIF2 that align with hIF2 (amino acids 200–414 and 569–652) although the alignment is not as extensive and the sequence is not as conserved. Sequence identity for these three regions of yIF2 with their corresponding regions in cIF2 are 18, 33, and 27%.

The N-terminal half of IF2, defined as the sequence up to the GTP-binding domain that is centrally located in hIF2, is the least conserved

portion of the protein. For hIF2 the GTP-binding domain begins at amino acid 638. A comparison of the N-terminal sequence of hIF2 with the same region of yIF2 (amino acids 1–411) shows that the two are 33% identical [Lee et al., 1999]. The same authors reported the sequence identity of the bIF2 (bacterial IF2) N-terminal portion of the protein (amino acids 1–397) as only 23% while aIF2 (archaebacterial IF2) showed virtually no identity since the N-terminal domain consists of only 23 amino acids. The N-terminal portion of cIF2, therefore, extends at least to amino acid 655 since a GTP-binding domain motif is not identified in the cIF2 amino acid sequence. Alignments of the cIF2 translation product with the N-terminal regions of bIF2, yIF2, and hIF2 show 16.3, 17.7, and 24.6% identity respectively.

IF2 is Abundantly Expressed in Cultured Chicken Osteoblasts and Expressed in a Tissue Specific Manner

Figure 2A represents the initial detection of cIF2 in osteoblast polyA$^+$ RNA. RNA was isolated from chicken osteoblasts cultured for 8 days on glass and polyA$^+$ RNA was purified and used for northern analysis. This represents a separate but similar preparation than that used to generate the pool of cDNAs used for cloning. It is evident that at this point in their differentiation in culture the osteoblasts are expressing high levels of message for cIF2 since the level of message is nearly half that of β-actin. The cIF2 probe and the β-actin probe were labeled to the same specific activity ($\sim 4 \times 10^8$ cpm/μg) and the exposure time for the two lanes is the same.

To determine whether or not the chicken IF2 gene might be a single copy gene, chicken genomic DNA was digested to completion and subjected to Southern blot analysis using the 1.5 kb cIF2 cDNA EcoRI fragment as a probe (Fig. 2B). Under stringent wash conditions this probe hybridizes to a single EcoRI band of approximately 5 kb, a single Hind III band slightly larger than this, and a single high molecular weight Xho I band greater than 23 kb in size. No other bands were evident upon longer exposure of the blot suggesting that the cIF2 message arises from a single gene, as is known to occur in yeast [Choi et al., 1998]. The cIF2 probe used on this blot contains an EcoRI site in the linker adaptor at the 5′ end of the insert and ends at an internal EcoRI site at nucleotide 1469. The

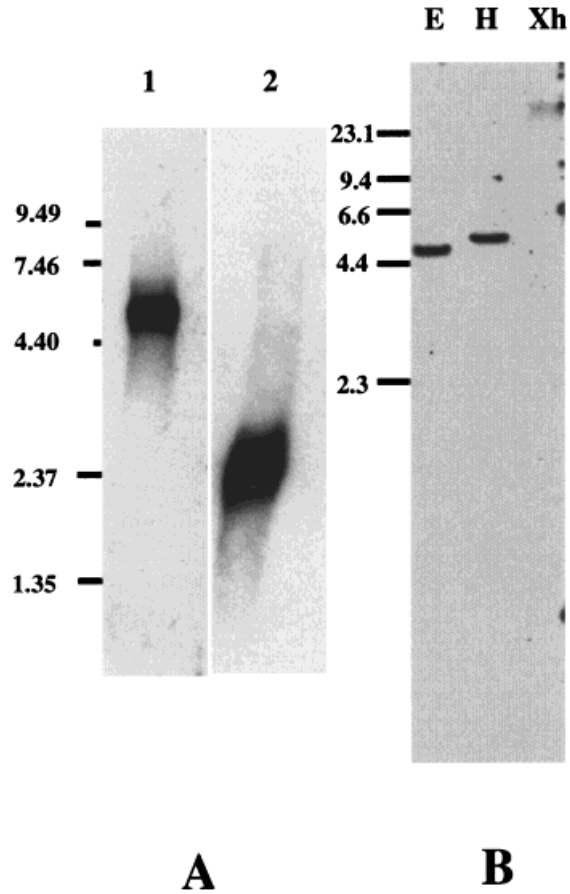

Fig. 2. Detection of cIF2 sequence in chicken osteoblast RNA and genomic DNA. **A:** Northern blot analysis representing the initial detection of cIF2 in osteoblast A$^+$ RNA. **Lane 1** represents 4 μg of polyA$^+$ RNA isolated from 8 day osteoblasts probed with a 1.5 kb fragment from the cIF2 cDNA clone. **Lane 2** shows the same lane stripped and reprobed with the mouse β-actin probe. The exposure times are identical and the two probes have comparable specific activity. Molecular weight markers in kilobases are shown to the left. **B:** Southern blot of chicken genomic DNA (4 μg per lane) digested to completion with EcoRI (E), Hind III (H) and Xho I (Xh), probed with the same fragment of cIF2 as A. Molecular weight markers in kilobase pairs, generated by a Hind III digestion of lambda DNA, are shown to the left.

bands recognized by this probe, therefore, represent genomic fragments containing coding sequences for cIF2. These genomic bands should also contain 5′ untranslated sequences and portions of the promoter (EcoRI, Hind III, and XhoI) or portions in the C-terminal half of cIF2 (Hind III and XhoI).

The level of cIF2 message was also examined in chicken tissues other than osteoblasts (Fig. 3). Two micrograms of poly A$^+$ RNA from chicken liver and brain, that were available from a commercial source, show differing detectable

levels of cIF2 but these are still well below the high levels evident in 1 μg of A^+ RNA from cultured osteoblasts. The ratio of cIF2 expression to β-actin expression was 0.10 and 0.18 for liver and brain respectively, compared to 0.76 for the osteoblast sample. Poly A^+ RNA isolated in the lab from chicken heart, kidney, and skeletal muscle also showed cIF2 expression (data not shown) but levels of expression were either barely detected (heart and kidney) or within the range of expression for liver and brain (skeletal muscle). Also evident in these chicken samples is the presence of an additional, slightly larger RNA that hybridizes to the cIF2 probe. This band is present in RNA samples where sufficient levels of cIF2 are present to generate a readily detectable signal.

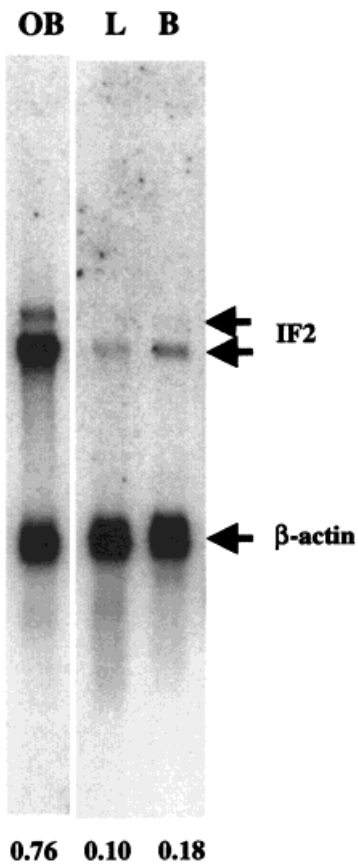

Fig. 3. Tissue specific expression of cIF2. A northern blot containing 1 μg of 8 day osteoblast A^+ RNA, and 2 μg each of liver (L) and brain (B) A^+ RNA was probed with the same cIF2 probe used in Figure 2 then reprobed with the mouse β-actin probe. Also visible in this blot is the slightly larger minor RNA that hybridizes to the cIF2 probe. The value for the ratio of cIF2 to β-actin for the major cIF2 band is given beneath each lane.

The Level of cIF2 mRNA Changes Quantitatively During Osteoblast Differentiation

To determine how levels of cIF2 message change during osteoblast culture, additional polyA^+ samples in larger quantities were run so that the amount of cIF2 message could be determined relative to that of β-actin. This blot was hybridized first with cIF2 and β-actin probes (Fig. 4A), then stripped and rehybridized with hIF2 and β-actin probes (Fig. 4B). The blot hybridized with the cIF2/β-actin combination was exposed to film for 3 days while the blot hybridized with the hIF2/β-actin combination was exposed to film for 5 days. The positions of the IF2 and β-actin bands are shown on both sides of the figure. Quantitation was performed using a scan of the film with ImageQuaNT and the results of this quantitation are presented in Figure 4C.

Although the β-actin band is clearly visible in the chondrocyte sample (Fig. 4A, lane C) there is very little cIF2 message detected (ratio of IF2 to actin is 0.06). Poly A^+ RNA samples from freshly isolated bone chips do not have a detectable signal for cIF2 message (data not shown). For Day 4 osteoblasts, however, there is a strong cIF2 band (Fig. 4A, lane 4); the ratio has increased to 0.37. The highest levels of cIF2 relative to β-actin are detected on Days 8 and 12 (Fig. 4A, lanes 8 and 12) where the ratios are 0.60 and 0.63. By Days 16 and 20 (Fig. 4A, lanes 16 and 20) the level of cIF2 message has dropped dramatically to 0.14 and 0.11 and is approaching the low level seen in chondrocytes. These results indicate that the levels of cIF2 mRNA change during osteoblast culture.

Since the 5′ half of the hIF2 cDNA had such high sequence similarity to the cIF2 cDNA, an hIF2 cDNA probe that contained these sequences was used on the same blot. These results are shown in Figure 4B and C. The hIF2 probe detects the same cIF2-specific major band as well as the larger band detected by the cIF2 probe although this band is only visible, barely, in the 8 day osteoblast sample. Using the ratio of the cIF2 band detected by the hIF2 probe relative to that of β-actin, a similar pattern of expression is seen.

IF2 is the Major Translation Initiation Factor Detected in Cultured Osteoblasts

We examined the expression of hIF2 and eIF2α in hFOB cells, a human osteoblast cell line

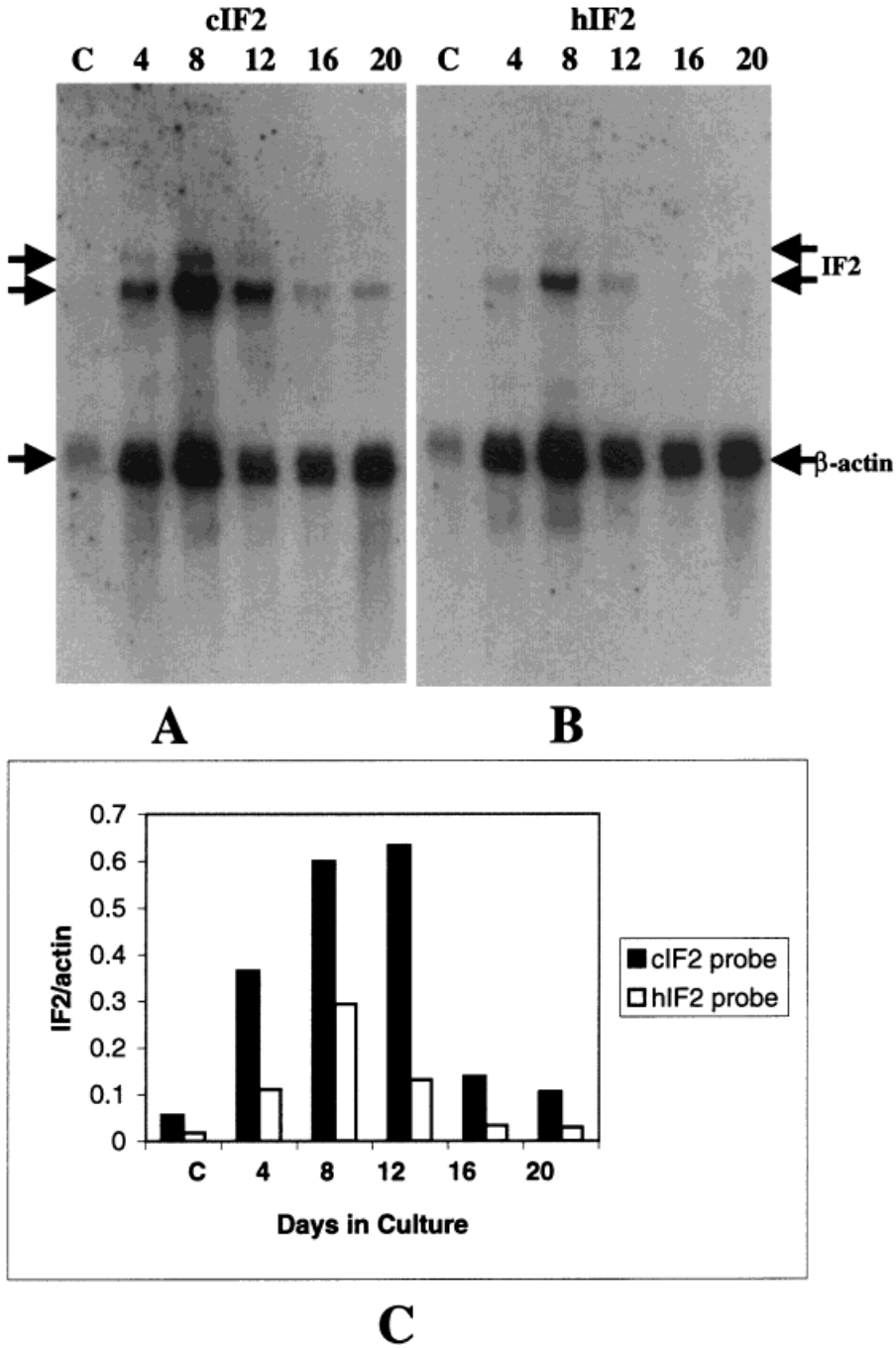

Fig. 4. Levels of cIF2 expression change during osteoblast differentiation in culture. **A:** Northern blot analysis of A^+ RNA isolated from chondrocytes (C), and osteoblasts cultured for 4, 8, 12, 16, and 20 days. The probes used are the 1.5 kb fragment of cIF2 and the mouse β-actin probe. The amount of RNA loaded in each lane is as follows: C, 8 μg; 4 days, 4 μg; 8 days, 1 μg; 12 days, 3 μg; 16 days, 2 μg; and 20 days, 3 μg. **B:** The same blot shown in A stripped and reprobed with a 2.6 kb fragment of hIF2 and the mouse β-actin probe. **C:** Graphical representation of the ratio of IF2 (either seen with the cIF2 probe or the hIF2 probe) to β-actin for the blots shown in A and B. The black bars represent the cIF2 probe to β-actin ratios while the white bars represent the hIF2 probe to β-actin ratios.

[Harris et al., 1995]. These cells are characterized by the presence of a transfected gene for a temperature-sensitive mutant of the large T antigen of SV40 that allows for temperature-dependent control of proliferation. At 34°C, which is the permissive temperature, hFOB cells divide rapidly and these cultures were only allowed to reach 50% confluence to ensure that

they represented osteoblasts in an exponential growth phase. In addition, cultures of hFOB cells were allowed to reach confluence at 34°C, then held for two days at 39°C to induce differentiation. The latter cell culture condition provides a clear contrast to the cultures undergoing active cell division at 34°C.

Figure 5A shows A^+ RNA samples of hFOB grown under the two different conditions (labeled 34 and 39), and a sample of chicken osteoblast A^+ RNA run on the same gel for comparison (labeled cOB). The probes used are a 2.6 kb probe for human IF2 and the mouse β-actin probe. There are several things to note. First, the size of the major cIF2 RNA species is larger than that for hIF2. This result confirms the difference in size of the chicken and human messages indicated in the northern blot in Figure 2A. Second, there appear to be two minor bands of considerably larger size than the 4.5 kb major RNA species for hIF2. This result parallels the detection of an additional larger cIF2 RNA species in the chicken tissues and osteoblasts (see Fig. 3). The presence of minor higher molecular weight bands for hIF2 was also reported by Wilson et al. [1999] in a screen of human cell lines for hIF2 expression. These minor RNA species appear to migrate as less

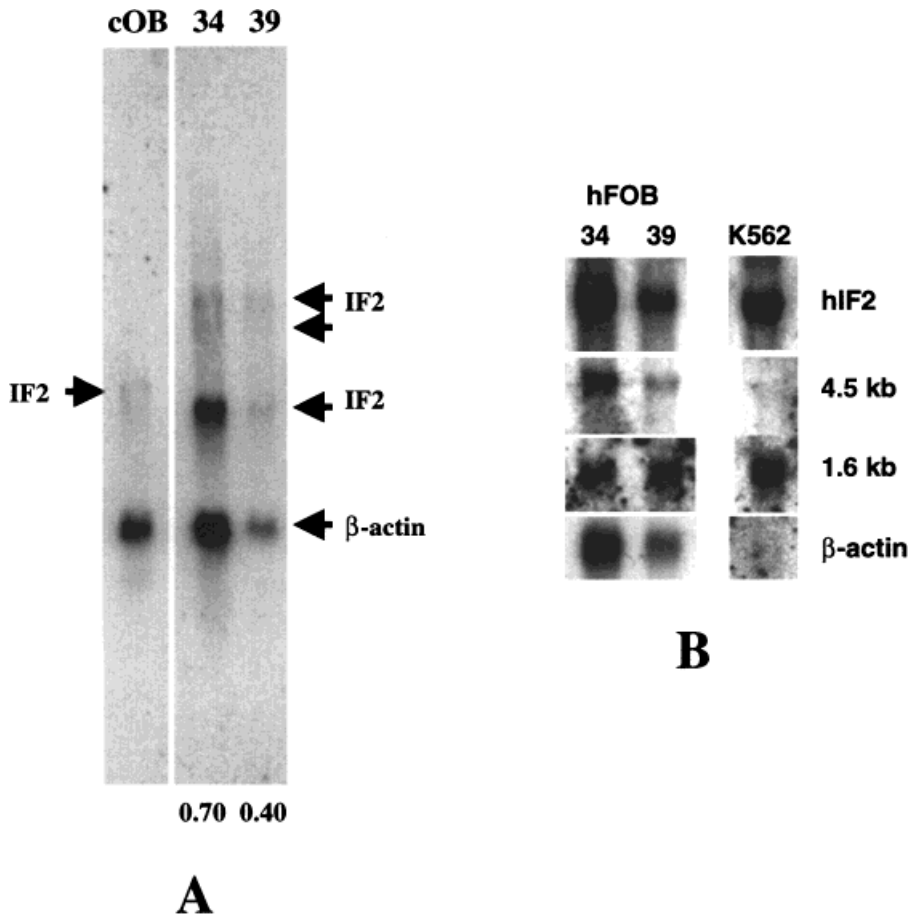

Fig. 5. Variable expression of IF2 may be a general feature of OBs. **A:** Poly A^+ RNA isolated from human fetal osteoblasts grown under differing conditions and from chicken osteoblasts was analyzed by northern blot analysis using a probe for hIF2. Each lane contains 2 μg poly A^+ RNA. Lanes labeled 34 contain samples from hFOBs grown to about 50% confluence at 34°C while lanes labeled 39 contain samples from confluent cells held at 39°C for 48 h. This blot was probed using a hIF2 probe and the mouse β-actin probe. Shown below each panel is the ratio of hIF2 signal to β-actin signal. Shown in the lane labeled cOB for the hIF2 probed blot is a 1 μg sample of chicken osteoblast poly A^+ RNA run on the same gel that shows the slightly larger IF2 message detected in the chicken osteoblasts. The hIF2 probed lanes were exposed to film for 5 days while the cIF2 probed lanes were exposed for 3 days. **B:** RNA samples from hFOB cells grown at either 34°C or 39°C (1.7 and 2 μg polyA$^+$ RNA respectively for lanes labeled hFOB 34 and 39) and exponentially growing K562 cells (5 μg total RNA) were run on formaldehyde gels, blotted, and probed sequentially for messages for human eIF2α, mouse β-actin, then hIF2; the blot was stripped between hybridizations. The human eIF2α cDNA probe detects both the 1.6 and 4.5 kb messages previously reported for eIF2α.

than 7.5 kb. Finally, the level of hIF2 message for the hFOB cells grown under proliferative conditions approximates the peak level of cIF2 message seen in chicken osteoblasts (the ratio of hIF2 to β-actin is 0.70).

As a positive control for eIF2α, considered the housekeeping translation initiation factor [Cohen et al., 1990], total RNA isolated from exponentially growing K562 cells, a human lymphoblast cell line, was run on the same gel with additional samples of A^+ RNA from hFOB cells and blotted and probed at the same time. Individual bands representing signals for eIF2α, hIF2, and β-actin have been isolated for comparison (Fig. 5B). Although the hybridizations were performed separately, the specific activity of the probes, the sizes of the probes (1.4 kb hIF2 probe, 1.4 kb eIF2α probe), and the length of exposures are equal. The order in which hybridizations were performed was eIF2α, β-actin, then hIF2, and the blot was stripped between hybridizations. The human eIF2α cDNA probe detects both the 1.6 and 4.5 kb messages previously reported for both human and mouse cells [Miyamoto et al., 1996; Lee et al., 1999]. It is apparent, however, that the level of message for hIF2 is much higher than that seen for either of the eIF2α messages in both K562 cells and cultured human osteoblasts.

Expression of cIF2 RNA Requires Protein Synthesis

The expression of eIF2, as measured by levels of expression for the alpha subunit, does not require novel protein synthesis [Cohen et al., 1990]. In order to determine whether expression of the message for cIF2 would be affected by a block to protein synthesis, cultured osteoblasts were treated with 20 μg/ml cycloheximide for 24 h on Days 5 through 8. According to our previous results, cultured osteoblasts show increasing levels of message for cIF2 during this time in culture. Under these conditions the cells remain viable and show no difference in appearance to the parallel cultures of untreated osteoblasts. Three separate chicken osteoblast isolations were performed and relative quantitative RT-PCR, under conditions permitting linear amplification and quantitation using 18S standards, was used to examine levels of cIF2 expression.

In Figure 6A the intensity of each RNA-specific cIF2 band produced in the RT-PCR reaction is expressed in terms of pixel intensity. For Days 4 through 8, three separate RNA isolations contribute to each timepoint, while there are two isolations each for Days 12 and 16, and a single 20 day timepoint. For the treated groups on Days 6 and 8, and the untreated group on Day 12, the standard deviation is too small to be depicted on the graph. The pixel intensity of the cIF2 bands for each of the cycloheximide treated cultures on Days 5 through 8 is clearly much lower than that for the corresponding untreated cultures ($P < 0.05$ for Days 5, 7, and 8; $P < 0.10$ for Day 6). This can be seen clearly in Figure 6B from the gel electrophoresis of representative samples of RT-PCR reactions for these same timepoints. If the intensity of the cIF2 bands (upper bands in Fig. 6B) are standardized by expressing them as a ratio relative to the 18S standard bands for each sample, there is also a clearly significant difference between the cycloheximide treated cultures and untreated cultures from Days 5 through 8 (Fig. 6C; $P < 0.05$ for all samples). In addition, the cIF2:18S ratio parallels the pattern of expression seen using the cIF2:β-actin ratio taken from northern blot analysis of polyA$^+$ RNA (see Fig. 4C) over the same culture period using a completely separate osteoblast isolation.

DISCUSSION

We have identified a partial cDNA for the chicken homolog of hIF2, a human translation initiation factor, and used it to characterize cIF2 expression in chicken osteoblasts. This cDNA encodes nearly the entire N-terminal half of the protein but is missing a small portion of the 5′ end of the message and ends at an internal site that is upstream of the coding sequence for the highly conserved GTP-binding domain. While a 4.5 kb message for IF2 has been detected in other vertebrates, either human or rabbit [Henrion et al., 1997; Lee et al., 1999; Wilson et al., 1999], the cIF2 message is clearly larger, perhaps by as much as a kilobase. The recently cloned Drosophila IF2 homolog, dIF2, is also larger in size, as a 5.3 kb message is detected in Drosophila embryos and adults [Carrera et al., 2000]. An alignment of cIF2 with yIF2 (yeast) and hIF2 shows a high degree of conservation among eukaryotic IF2s. Not surprisingly, the degree of sequence similarity between the N-terminal portions of cIF2 and hIF2 is higher than that between cIF2 and yIF2 [Choi et al.,

1998]. The message for cIF2 is expressed at very high levels in cultured osteoblasts, and at lower levels in other chicken tissues. The level of expression for the IF2 message also appears to be regulated in chicken osteoblasts during differentiation in culture.

yIF2 was found to be a single copy gene, and essential in yeast, since fun12Δ strains that carried a disrupted IF2 gene showed defects in translation initiation and exhibited slow growth. This defect could be corrected by transforming the yeast with a plasmid containing the intact IF2 gene. In addition, yIF2 was dependent on the same translation initiation factors as eIF2 and was required for efficient translation initiation in addition to eIF2. Finally, yIF2 was found to function at the initiation step in translation, facilitating the binding of Met-tRNA$_i^{Met}$ to ribosomes. Thus, yIF2 appears to be a general translation factor, working in association with eIF2 to insure efficient binding of initiator tRNAs to message associated with ribosomes [Choi et al., 1998]. In keeping with the high degree of sequence identity observed for IF2s, studies in yeast have found that both hIF2 and aIF2 (archaebacterial IF2) [Bult et al., 1996] can compensate for the loss of yIF2 in fun12Δ strains [Lee et al., 1999].

A consistent observation for IF2 expression in higher eukaryotes is the tissue-specific nature of its expression. In a study by Henrion et al. [1997] that examined the stability of maternal transcripts in rabbit embryos prior to zygotic genome expression using differential display, a band was identified that decreased in intensity between the single cell and 8–16 cell stage of embryogenesis. One of the clones generated from this band, Li9, hybridized to a 4.5 kb band in RNA isolated from several tissues of adult rabbits. Li9 has since been identified as a portion of the rabbit homolog of IF2. The translation product for this clone represents an 89 amino acid segment corresponding to amino acids 833–921 of hIF2 and having 98.9% identity to it. In human tissues the highest level of hIF2 expression was in skeletal muscle and testis, and it was readily detectable in ovary, small intestine, prostate, heart, and pancreas [Lee et al., 1999]. Variable levels of hIF2

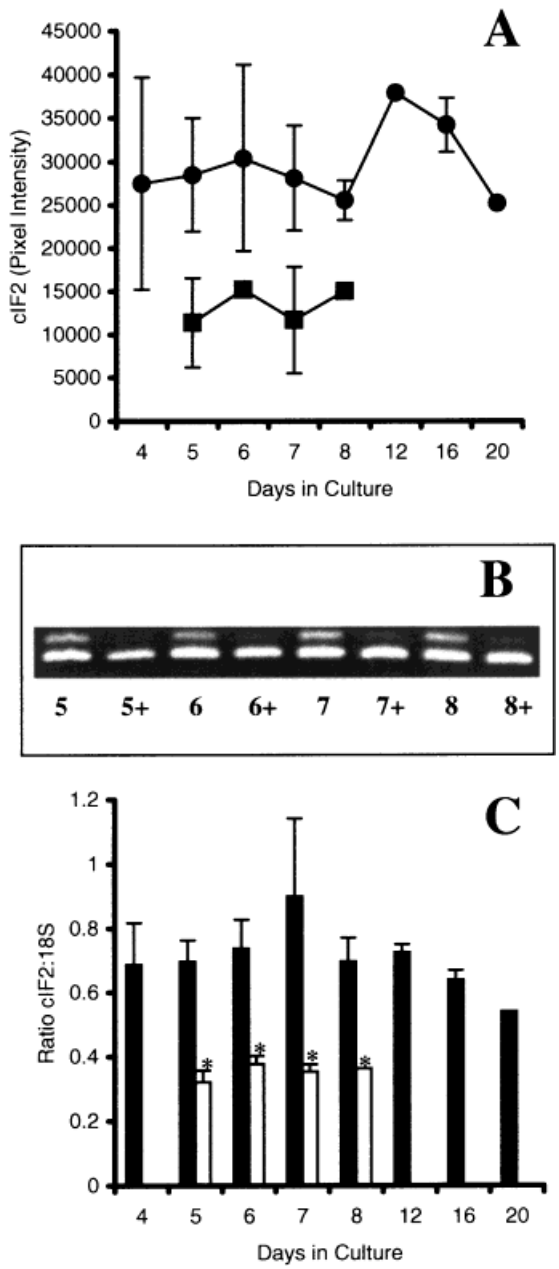

Fig. 6. The level of cIF2 message is severely reduced by treatment with cycloheximide. **A:** Comparison of the level of message for cIF2 in treated (■) and untreated (●) chicken osteoblasts. Levels of cIF2 message were measured by determining the pixel intensity in the cIF2 band as a result of performing RT-PCR on samples of total RNA from cultured chicken osteoblasts. The results of three separate isolations are shown for Days 4 through 8. The standard deviation for each timepoint containing two or more samples is shown. (The standard deviation for Day 12 is too small to be drawn to scale and only one sample is shown for Day 20.) **B:** Representative samples of RT-PCR for cIF2 (upper band) and 18S standard (lower band) for Days 5 through 8 in culture. The plus symbol indicates treatment with cycloheximide. **C:** Statistical analysis of cIF2 mRNA levels in treated (open bars) and untreated (black bars) chicken osteoblasts. In this graph levels of cIF2 mRNA are expressed as a ratio of the intensity of the cIF2 signal to that of 18S in each sample, and standard deviation is indicated for each day except Day 20. Significant differences are indicated by an asterik ($P < 0.05$).

message have also been detected in a panel of human tumor cell lines derived from a variety of tissues [Wilson et al., 1999]. The level of cIF2 expression in chicken tissues is also tissue-specific and similar in pattern to that of hIF2 with the possible exception of the osteoblasts, since bone or bone cells represent a tissue source not yet examined for expression of translation initiation factors.

Until recently the translation initiation factors were thought to be clearly defined along evolutionary lines; prokaryotes would have IF2 while archaebacteria and eukaryotes would have eIF2. As a result of the cloning of eukaryotic homologs of bIF2, however, it now appears that the picture for archaebacteria and eukaryotes is more complex since both have eIF2 and IF2. Lee et al. [1999] have proposed that the IF2 homologs in archaebacteria and eukaryotes function via molecular mimicry, by binding to the ribosomal A site, to direct binding of the eIF2·GTP·Met-tRNA$_i^{Met}$ ternary complex to the P site on the ribosomes. The binding of IF2 may make the binding of the eIF2 complex to ribosomes with an occupied A site more efficient than the binding to unoccupied ribosomes. This may explain why archaebacteria and eukaryotes have two initiation of translation factors, eIF2 and IF2, that apparently differ in function.

eIF2 expression is commonly assessed using a cDNA probe for the alpha subunit. Human eIF2α is a single copy gene [Humbelin et al., 1989] but in human and mouse tissues a probe for eIF2α hybridizes to a 1.6 and a 4.5 kb band [Miyamoto et al., 1996]. The 1.6 kb message is more abundant and differs from the 4.5 kb message only by having a shortened 3′ untranslated region. A substantial increase in eIF2α message precedes the rapid increase in protein synthesis required for T lymphocyte activation [Mao et al., 1992]. It is the stabilization of the primary transcript (4.5 kb message) that accounts for the increase in eIF2α expression, as much as 50-fold in 24 h [Cohen et al., 1990]. Based on the results of their characterization of eIF2α mRNA expression, Cohen et al. [1990] classified eIF2α as a housekeeping gene. In these cells, message for eIF2α is always being transcribed at a steady low level and new protein synthesis is not required for the increase in message levels. In fact, treatment with cycloheximide to block protein synthesis results in an increase in the level of messages of both sizes. Cell proliferation requires high levels of protein synthesis and this may be the simplest explanation for the increase in eIF2 expression.

In their study of hIF2, Lee et al. [1999] used the same multiple tissue northern blots of human A$^+$ RNA to assess levels of message for both hIF2 and eIF2α (see Fig. 1C of that reference). Their results indicate that for the sixteen tissues sampled, which did not include either osteoblasts or bone, the abundance of the 4.5 kb message for eIF2α appears to be proportional to the abundance of hIF2 while the 1.6 kb message is always readily detected. These results support the proposal that eIF2 is the principal eukaryotic translation initiation factor and that IF2 may be expressed only in certain tissues at times when there is a distinctive demand for protein synthesis. Wilson et al. [1999] also concluded from their study that IF2 was a general translation initiation factor and would not likely be involved in the translation of a specific subset of messages. Thus, on the basis of these considerations, levels of IF2 would not be expected to exceed those of eIF2 and the predicted cellular response to an increased need for protein synthesis would be an increase in the availability of message for eIF2 rather than an increase in IF2 message.

Since high levels of IF2 message were detected in both human and chicken osteoblasts, it was important to determine whether levels of eIF2 in human osteoblasts would follow the same pattern of expression relative to IF2 demonstrated for other human tissues. The human eIF2α cDNA probe detected both the 4.5 and the 1.6 kb band in RNA isolated from a human fetal osteoblast cell line but the signals for these bands are much weaker than that of the hIF2 band. A strong signal for hIF2 is also readily seen in 5 μg total RNA from K562 cells, although RNA from these cells was intended to demonstrate high levels of eIF2. Wilson et al. [1999] reported a strong signal for hIF2 in A$^+$ RNA isolated from K562 cells but a sequential hybridization with an eIF2α probe was not performed to determine levels of eIF2. A direct comparison is possible here because the same lane has been probed for both hIF2 and eIF2α messages, using probes of the same size labeled to the same specific activity, and the films have been exposed for the same length of time.

The finding here that cIF2, unlike eIF2, requires novel protein synthesis to achieve high levels of expression, clearly separates cIF2 from the class of housekeeping genes. For the

cultured chicken osteoblasts, both northern blot and RT-PCR analyses indicate that high levels of cIF2 expression are evident during cell proliferation, up to Day 6, and during matrix secretion, Days 8 through 12. The stages of proliferation and differentiation of these cultured chicken osteoblasts have been well characterized in our laboratory [Gay et al., 1994; Stains and Gay, 1998; Luan et al., 2000]. The observation that cultured human osteoblasts also exhibit higher levels of hIF2 expression than eIF2, under conditions where the levels of hIF2 can be compared directly with those of eIF2, also serves to distinguish the roles of hIF2 and eIF2 in this cell type. A modified role for IF2 is also supported by the recent findings on the *Drosophila* IF2 homolog [Carrera et al., 2000]. The cDNA for dIF2 was identified using the yeast two-hybrid approach in which the *Drosophila* protein vasa, an RNA helicase involved in pattern formation and germ cell development, was the bait. In *Drosophila*, dIF2 message is expressed throughout development in both germline and somatic tissues. In contrast to the yeast fun12Δ strains that grew poorly, however, homozygous dIF2 mutants did not survive past the first or second instar larval stage indicating that dIF2 is essential for viability in *Drosophila*.

One implication of our results is that in certain cells IF2 may play more than an auxiliary role in translation initiation and may, in fact, be a required factor in translation initiation. This view is not necessarily incompatible with the model proposed by Lee et al. [1999] and Wilson et al. [1999] in which IF2 functions with eIF2 to make the binding of Met-$tRNA_i^{Met}$ more efficient. Clearly, in actively dividing human osteoblasts and K562 cells the levels of hIF2 message are much higher than those of eIF2. In chicken osteoblasts, however, high levels of cIF2 expression are not only associated with actively dividing cells. By 8 to 12 days in culture, when the cIF2 message peaks, the osteoblast cultures are already confluent, are synthesizing extracellular matrix (ECM) components, and are preparing for matrix mineralization [Luan et al., 2000]. This suggests that the heavy demand for the synthesis of matrix components, and other proteins necessary for matrix formation and mineralization, may require more efficient translation initiation. Our results provide some unexpected observations about a newly identified eukaryotic translation initiation factor, IF2, and also underscore the importance of studying the regulation of protein synthesis in a variety of cell types.

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