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Transient Expression of a Translation Initiation Factor Is Conservatively Associated with Embryonic Gene Activation in Murine and Bovine Embryos¹

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ABSTRACT

In the present study the abundance of mRNAs for eukaryotic translation initiation factors eIF-1A (formerly known as eIF-4C), -2 α , -4A, -4E, and -5 was examined in in vivo-derived mouse embryos throughout preimplantation development using a semiquantitative reverse transcription-polymerase chain reaction assay. Although the mRNA profile for each gene is unique, only mRNA for eIF-1A transiently increases during embryonic gene activation (EGA) at the 2-cell stage, and this was confirmed by an independent hybridization-based assay. In in vitro-developed bovine embryos, mRNA for eIF-1A was transiently detected at the 8-cell stage, when the major activation of the genome occurs in this species. As in the mouse, detection in 8-cell bovine embryos was sensitive to the transcriptional inhibitor α -amanitin. It was also observed at the same time relative to cleavage in embryos cultured in defined medium under a reduced oxygen environment, and in medium supplemented with serum and somatic cells in 5% CO₂ in air. Neither the chronology of early cleavage divisions nor the yield of bovine blastocysts differed in these culture media. Our results suggest that transient expression of eIF-1A in the mouse and cow is a conserved pattern of gene expression associated with EGA in mammals.

INTRODUCTION

The activation of embryonic transcription, also known as embryonic gene activation (EGA), represents the beginning of the transition from oogenetic to embryonic control of development. During this transition the embryo begins to synthesize its own mRNA and then protein, replenishing that which it inherited from the mother in the egg, in order to develop beyond early cleavage stages [1]. Classically, the timing of EGA has been described as species specific and has been found to range from the 2-cell stage in the mouse to the 8- to 16-cell stage in the cow and rabbit [2]. However, recent studies have indicated a greater conservation in the molecular basis of EGA in mammals, if not vertebrates in general, than initially apparent.

In mammals, EGA appears to commence gradually. In the mouse, rabbit, and cow, the species-specific major activation of the genome is in fact preceded by an initial minor degree of embryonic transcription. This activity be-

gins at the end of the first cell cycle in the mouse and rabbit, and in the cow it has been reported as early as the 2-cell stage [3–7]. Reporter gene studies in the mouse and rabbit also suggest that the major activation of the genome is accompanied by the acquisition of a transcriptionally repressive environment, defined by the requirement for enhancer sequence elements to promote transcription [8–10]. This change may be mediated by changes in chromatin structure as evidenced by alterations in the composition and acetylation of histones in chromatin during EGA in the mouse [11]. Treatment of mouse embryos with inhibitors of histone deacetylase, which results in histone hyperacetylation, can also stimulate or block the repression of markers of EGA [12, 13]. In both the mouse and cow, somatic histone H1 subtype is assembled onto chromatin at or near the timing of the major activation of the genome in each species, and this process is dependent on both embryonic transcription and DNA replication [14, 15]. The switching of linker histone subtypes at the time of EGA has also been described in the amphibian, *Xenopus laevis* [16].

In the mouse, EGA at the 2-cell stage is characterized by two bursts of transcriptional activity, with the constitutive activation of most genes late in the second cell cycle preceded by the transient synthesis of a group of α -amanitin-sensitive polypeptides [17–20]. Based on high-resolution two-dimensional gel electrophoresis, this group of proteins is composed of approximately 38 polypeptides of the 1500 expressed at this time [20]. To identify systematically the genes encoding the transiently expressed markers of EGA, we recently implemented the mRNA differential display method [21] as adapted for preimplantation mouse embryos [22]. By this approach, mRNA for the mouse homologue of the human translation initiation factor eIF-1A (formerly known as eIF-4C) was identified, with the transient increase observed at the level of both mRNA and protein [13]. The identification of eIF-1A has been paralleled by the discovery of products of other transiently expressed genes, including 1) U2afbp-rs, the mouse homologue of the human U2af 35 kDa mRNA splicing factor [23] and 2) hsp70.1, a member of the multigenic hsp70 family of heat shock proteins [18, 24, 25]. Although seemingly diverse, the involvement of all of these molecules in the regulation of gene expression suggests that their coordinated expression may be of functional significance during the transition from oogenetic to embryonic control of development. In the present study we investigated whether the types of genes that are transiently expressed during EGA in mammals are conserved both within and across species by examining the expression of translation initiation factor mRNAs in early murine and bovine embryos.

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MATERIALS AND METHODS

In Vitro Fertilization and Oocyte and Embryo Collection and Culture

Fully grown germinal vesicle-staged (GV) murine oocytes, obtained from eCG-primed 6-wk-old CF-1 mice, were denuded of cumulus cells as previously described [26]. For murine oocyte collection, 0.2 mM 3-isobutyl-1-methyl xanthine was included in all handling medium to inhibit germinal vesicle breakdown [26]. The collecting medium was bicarbonate-free minimal essential medium (Earle's salts) supplemented with pyruvate (100 µg/ml), gentamicin (10 µg/ml), polyvinylpyrrolidone (3 mg/ml), and 25 mM Hepes, pH 7.2 (MEM/PVP). GV oocytes were maintained in culture in MEM/PVP in which the Hepes was replaced by 25 mM sodium bicarbonate, at 37°C in an atmosphere of 5% CO₂ in air.

Metaphase II-arrested (MII) oocytes for study and in vitro insemination were collected from superovulated CF-1 female mice 15–17 h after hCG in bicarbonate-free MEM/PVP, as previously described [27]. These were fertilized in vitro essentially as described previously [28] in Whitten's medium containing 15 mg/ml BSA, for 3 h at 37°C, in an atmosphere of 5% CO₂:5% O₂:90% N₂. In vivo-developed embryos were collected from superovulated CF-1 female mice mated to B6D2F1/J males (Jackson Laboratories, Bar Harbor, ME). Unless otherwise stated in the figure legends, embryos at the 1-cell, mid 2-cell, 4-cell, 8-cell/morula, and blastocyst stages were flushed from either oviducts or uteri at 24–25, 41–44, 65–67, 75–77, and 92–96 h post-hCG, respectively. In vitro-fertilized oocytes and flushed embryos were cultured in KSOM plus amino acids [29] at 37°C in the same atmosphere as used for fertilization. Prior to lysis, oocytes and 1-cell embryos were denuded of any adherent cumulus cells by repeated pipetting in collection or culture medium containing 0.1% hyaluronidase.

Bovine cumulus-oocyte complexes were isolated from slaughterhouse ovaries provided by ABEL Laboratories (Guelph, ON, Canada) and matured in vitro and fertilized as described previously [30]. Embryos were cultured to the blastocyst stage in media microdrops under heavy paraffin oil at 38.5°C by either of two methods: 1) coculture in groups of 25 with bovine epithelial cell vesicles in 50-µl drops of T-199 medium in the presence of 10% (v:v) newborn calf serum (Cansera, Toronto, ON, Canada) in an atmosphere of 5% CO₂ in air [30] or 2) culture in groups of 50 in 25-µl drops of synthetic oviduct fluid medium (SOFM) supplemented with 0.5 mM citrate (sodium citrate-trisodium salt; c-SOFM) in an atmosphere of 5% CO₂:10% O₂:85% N₂ [31]. On Day 3 of culture, an equivalent volume of fresh gas- and temperature-equilibrated medium was added to each culture drop. To select developmentally synchronized embryos, dishes dedicated for synchronization at a specific stage were checked every 3 h for embryos that had cleaved to that stage. Newly cleaved embryos were transferred to separate drops for further development. Cleavage to the 2-cell, 4-cell, 8-cell, and greater than 8-cell stage occurred over 26–40, 32–46, and 46–60 h and by 95–100 h postinsemination, respectively, as described previously [32].

Semiquantitative Reverse Transcription-Polymerase Chain Reaction Assay

Relative changes in the representative abundance of mRNA transcripts were determined using a semiquantita-

TABLE 1. Primers used for RT-PCR and the size of diagnostic amplification products.

Gene primers ^a	Primer sequence		Product size (bp)
eIF-1A	5'	5'-AAGAAGTCTGAAGGCCTATG-3'	170
	3'	5'-CAGAGAAGCTTGAATGTAGC-3'	
eIF-4A	5'	5'-GCTGGACACTCTTTGTGACTTG-3'	190
	3'	5'-ACCCTGATCGGAATCCCTCAT-3'	
eIF-5	5'	5'-GAGATTCGTGTCAAAGCAGAGC-3'	367
	3'	5'-GGACTCCCTAGATGTCTCATTG-3'	
eIF-4E	5'	5'-TTTTGCTCCACTCCATGCAGGA-3'	525
	3'	5'-TGGATGGGACCGCTTTTCTACT-3'	
eIF-2α	5'	5'-GCAGTCTCAGACCCATCTATCT-3'	309
	3'	5'-GATGACTGCCATAGCCTGATTG-3'	
α-globin	5'	5'-GCAGCCACGGTGGCGAGTAT-3'	257
	3'	5'-GTGGGACAGGAGCTTGAAT-3'	

^a For eIF-1A, the 5' and 3' primers correspond to base pair positions 69–88 and 219–238, respectively, of partial mouse cDNA for eIF-1A (GenBank accession no. U28419 [13]) and to bp 507–526 and 657–676, respectively, of the human eIF-4C clone (GenBank accession no. L18960 [35]) for eIF-4A, the 5' and 3' primers correspond to base pair positions 816–837 and 982–1003, respectively, in the mouse eIF-4A1 cDNA clone (EMBL accession no. X12507 [36]) for eIF-5, the 5' and 3' primers correspond to base pair positions 1329–1350 and 1448–1469, respectively, in the rat eIF-5 cDNA clone (GenBank accession no. L11651 [37]) for eIF-4E, the 5' and 3' primers correspond to base pair positions 1047–1068 and 1550–1572, respectively, in the mouse eIF-4E cDNA clone (GenBank accession no. M61731 [38]) for eIF-2α, the 5' and 3' primers correspond to base pair positions 493–514 and 780–801, respectively, in the rat eIF-2α cDNA clone (GenBank accession no. J02646 [39]) for α-globin, the 5' and 3' primers correspond to bp 241–260 and 555–567, respectively, in the rabbit α-globin genomic clone [40].

tive reverse transcription-polymerase chain reaction (SQ-RT-PCR) assay, which uses an exogenously added globin mRNA as an internal standard [33]. This assay can be used to compare the relative representation of one mRNA among different samples but not the absolute representation of one mRNA to that of another [29, 33]. RNA isolation, RT, PCR, and amplification product quantification were performed on murine embryos as described previously [33] and for bovine embryos also as previously described [34]. PCR-amplified cDNA was quantified in bovine experiments using capillary electrophoresis and in murine experiments by Cerenkov counting of radiolabeled products separated on agarose gels.

In experiments using murine embryos, PCR was routinely done on a volume of cDNA equivalent to that of 3–5 embryos from a reverse-transcribed pool of mRNA from 50–100 embryos. In experiments on bovine embryos, pools of 10–20 embryos were reverse transcribed, and PCR was performed on the equivalent of 2 embryos for eIF-1A and 1 embryo for the α-globin gene. In all experiments, amplification of the globin standard was performed on parallel samples.

Primers for the amplification of eIF-1A, eIF-4A, eIF-5, eIF-2α, eIF-4E, and α-globin were designed using known sequence information (Table 1) [13, 35–40]. The basic program for amplification of eIF-1A, eIF-4A, eIF-5, and α-globin in murine experiments was an incubation of 95°C for 1 min, followed by a cycle program of 95°C for 10 sec and 60°C for 15 sec; the last cycle was followed by a 6-min extension at 60°C. Primers for eIF-2α required an annealing temperature of 66°C, which was also used for the extension phase. The eIF-4E primers required a three-step cycling program of 95°C for 15 sec, 66°C for 30 sec, 72°C for 30 sec. This was also preceded by a 1-min incubation at 95°C and was followed by a 5-min extension at 72°C.

The basic program for amplification of bovine cDNA consisted of 94°C for 10 min followed by a cycle program of 94°C for 1 min, a transcript-specific annealing temperature (59°C and 55°C for eIF-1A, and α -globin, respectively) for 30 sec, and 72°C for 1 min. This program was followed by a 5-min extension at 72°C. RT-PCR products were visualized by separation on 2% agarose gels in single-strength TAE buffer (40 mM Tris-acetate, 1 mM EDTA) containing 0.5 μ g/ml ethidium bromide.

Representative PCR-Amplified cDNA Blots

The method of Brady and Iscove [41] was used to make representative cDNA libraries of embryos that were then probed to quantify the abundance of a gene of interest [42]. What follows is a brief description of this procedure; for further details and validation of this method, see Brady and Iscove [42]. Zonae pellucidae of mouse eggs and embryos were removed by brief acid treatment as previously described [43]. Aliquots of 3–5 mouse embryos, in a minimal volume (1–2 μ l), were lysed in 8.5 μ l ice-cold reverse transcriptase/lysis buffer containing 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂, 20 mM dNTP, 0.1 mg/ml, dT₂₄ primer, 100 U/ml Prime RNase Inhibitor (5 Prime-3 Prime Inc., Boulder, CO), and 0.5% Nonidet P-40 in diethylpyrocarbonate (DEPC)-treated water. Embryo lysates were heated to 65°C for 1 min and then incubated at room temperature for 3 min to allow annealing of the dT₂₄ primer. After addition of 0.5 μ l (100 U) of Superscript Reverse Transcriptase II (Gibco-BRL, Burlington, ON, Canada), the lysates were incubated for 15 min at 37°C. Reverse transcription reactions were terminated by heating for 10 min at 65°C. A homopolymer deoxyadenosine tail was added to the 3' end of the first strand cDNA by adding 8.5 μ l of double-strength terminal deoxy-transferase (TdT) buffer (Gibco-BRL) containing 200 μ M dATP, followed by 0.7 μ l (10 U) of TdT enzyme (Gibco-BRL), and incubating the sample for 15 min at 37°C. Reactions were terminated as described above. Reaction products not used immediately for PCR were stored at –70°C.

PCR was performed on 4 μ l of lysed, reverse-transcribed, and deoxyadenosine-tailed embryo cDNA product in 50- μ l reactions containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 1 mM of each dNTP, 5 U of *Taq* polymerase (Perkin Elmer Cetus, Foster City, CA), and 3.3 μ g of PCR primer (5'-CAGGCCG-T₂₃) modeled after that of Brady and Iscove [41]. Each PCR reaction was amplified through 25 cycles of 94°C for 1 min, 42°C for 2 min, and 72°C for 6 min. After addition of a further 5 U of *Taq* polymerase, the reactions were amplified through 25 cycles of 94°C for 1 min, 42°C for 1.5 min, and 72°C for 4 min. PCR products were stored at –20°C.

Amplified cDNA was purified using the Wizard PCR prep DNA purification system (Promega, Madison, WI) and reconstituted in 50 μ l of DEPC-treated water. The concentration of cDNA was determined by measuring the absorbance at 260 nm; it typically varied between 20 and 120 ng/ml. After purification, approximately 300 ng of each cDNA sample was run on a 1.5% agarose gel to determine the size distribution of products, which ranged from 200 to 300 base pairs (bp) (data not shown). Only cDNA samples showing similar product size distribution were slot blotted together as a developmental series. Slot blots were prepared by diluting equivalent quantities of cDNA (300 ng) in 300 μ l of 0.3 N NaOH. The samples were heated at 65°C for 30 min, after which 125 μ l of 20-strength SSC (single-

strength SSC is 0.15 M sodium chloride and 0.015 M sodium citrate) was added; the samples were blotted onto Hybond-N (Amersham, Arlington Heights, IL) that had been previously wetted with 10-strength SSC [44]. After loading, the slots were washed once with 500 μ l of 10-strength SSC. Blots were rinsed once with double-strength SSC, UV cross-linked for 1 min at 1.2×10^6 μ J in a UV Stratalinker (Stratagene, La Jolla, CA), and air dried.

The mRNA differential display cDNA clone identified as eIF-1A (formerly known as eIF-4C [13]) was ligated into the pCR vector (Invitrogen, San Diego, CA). Full-length mouse β -actin cDNA was in pBluescript (Stratagene). Plasmid containing cDNA inserts that were to be used as probes for hybridization was isolated from transformed DH5 α (Gibco-BRL) or One Shot (Invitrogen) bacteria using the Wizard Miniprep DNA purification system. Cloned cDNA inserts were restriction digested from plasmids for 1 h at 37°C, and after electrophoresis in a 1.5% agarose gel, the released inserts were purified with a GeneClean II kit (BIO 101 Inc., La Jolla, CA) according to specifications recommended by the manufacturer. Cloned cDNA (10–25 ng) was radiolabeled with [α -³²P]dCTP by random primer labeling [45]. Unincorporated radiolabeled dCTP was removed using TE Select-D G-25 spin columns (5 Prime-3 Prime, Inc.). The specific activity of the probe ranged from 0.5 to 1×10^9 cpm/ μ g. Hybridization was performed overnight at 65°C according to the method of Church and Gilbert [46]; probe concentration was 2 – 3×10^6 cpm/ml. Blots were imaged on a PhosphoImager and the data quantified using the ImageQuant (Molecular Dynamics, Sunnyvale, CA) software package. Blots were stripped for re-probing by boiling for 10 min in 0.5% SDS.

Cloning and Sequencing of Bovine eIF-1A cDNA Fragment

Primers for eIF-1A, designed after the murine clone isolated by differential display [13], were used to amplify the homologous bovine sequence from cDNA prepared from 48-h cultures of bovine epithelial cell vesicles [47]. This amplification yielded a 170-bp product comparable to that in the mouse. This product was cloned using the Invitrogen TA cloning kit according to the manufacturer's instructions. Double-stranded plasmid DNA was isolated and manually sequenced using a T7 sequencing kit (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions. The cloned sequence was used in an alignment search of the nonredundant GenBank database (<http://www.ncbi.nlm.nih.gov>) using the BLAST algorithm [48] and subsequently submitted to GenBank (accession no. AF051848).

Statistical Analysis

Data were analyzed using the SigmaStat (Jandel Scientific, San Rafael, CA) software package. Parametric analysis of differences in the means between two or more populations were tested using a one-way ANOVA followed by multiple pair-wise comparisons using Bonferroni's method. Differences of $p \leq 0.05$ were considered significant.

RESULTS

Translation Initiation Factor mRNA Profiles during Murine Preimplantation Development

In our initial publication, the transient increase in eIF-1A expression during EGA in the mouse was shown in in

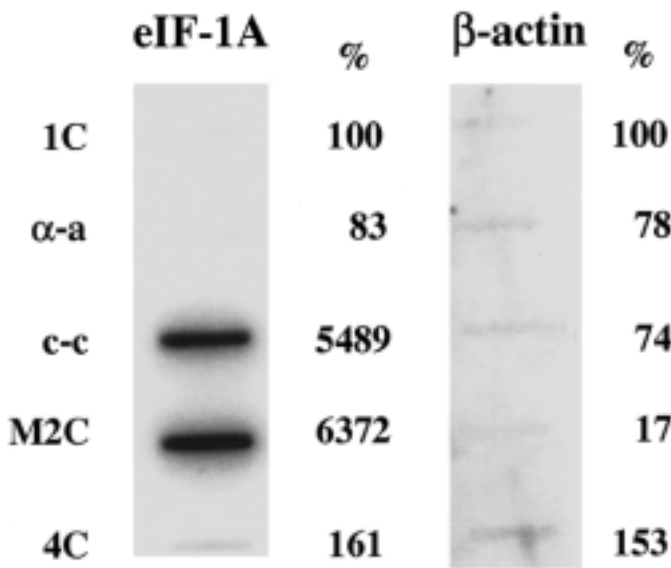


FIG. 1. Changes in the relative amounts of eIF-1A and β -actin transcripts determined by transcript-specific hybridization to representative cDNA from mouse 1-cell to 4-cell embryos. Copy DNA representative of the mRNA pool at each stage of development was slot blotted on a nylon membrane and probed with gene-specific probes for eIF-1A and β -actin. In vivo-developed 1-cell embryos (1C) were isolated 24-h post-hCG and sampled or cultured either in the presence of α -amanitin (24 μ g/ml; α -a) or in its absence (control culture; c-c), from 23 h to 48 h post-hCG. Mid 2-cell (M2C) or 4-cell (4C) embryos were flushed from oviducts at 44 and 66 h post-hCG, respectively. The percentage values refer to the intensity of the hybridization signal; the signal obtained for the 1-cell stage was set as 100%. The experiment was performed three times and a representative example is shown.

vivo-developed embryos and was demonstrated to be α -amanitin sensitive in cultured embryos using an SQ-RT-PCR assay. To confirm the validity of this assay and extend our original study, the expression of eIF-1A mRNA was first examined in early cleavage stage embryos by an independent hybridization-based assay (Fig. 1) and then in all preimplantation stages by SQ-RT-PCR (Fig. 2). Hybridization of gene-specific probes to representative cDNA generated by PCR represents an alternative method for the quantification of mRNAs in small numbers of cells or embryos [41, 42]. In this assay the intensity of the hybridization signal is proportional to the representation of that transcript within the cDNA population. The original mouse cDNA clone identified as eIF-1A was hybridized to slot-blotted representative cDNA from three independent series of in vivo- and in vitro-developed mouse embryos (Fig. 1). All experiments confirmed the transient increase in eIF-1A mRNA at the 2-cell stage in both in vivo- and in vitro-developed embryos. In in vitro-derived embryos, this increase was always inhibited by pretreatment from the 1-cell stage with α -amanitin, although this treatment did not inhibit cleavage to the 2-cell stage. Stripping and reprobing of the same blots, from two experiments, for β -actin (with the order of probing and stripping for eIF-1A or β -actin being reversed in each trial) revealed a different expression profile from that for eIF-1A, with weak hybridization in all stages. This is consistent with previous reports describing a low abundance of this mRNA during this period of development [33, 49]. Although the hybridization intensity for β -actin was virtually identical in successive experiments, the magnitude of the increase in eIF-1A hybridization intensity from the 1-cell to 2-cell stages varied from approx-

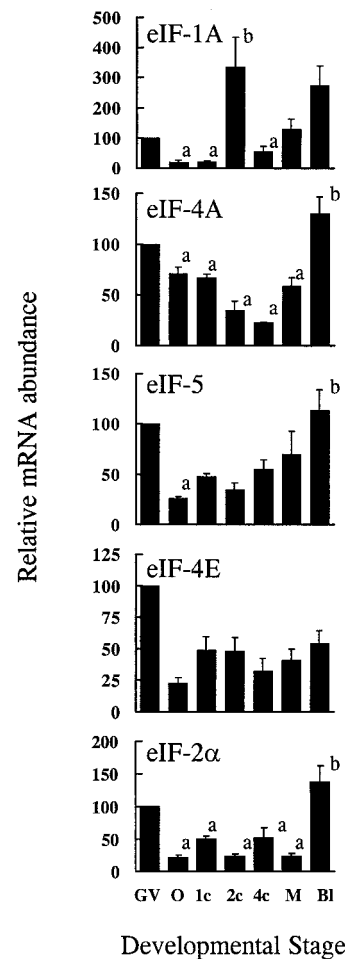


FIG. 2. Changes in the relative abundance of mRNAs for different translation initiation factors in in vivo-developed mouse embryos. RNA was isolated from fully grown, germinal vesicle-intact oocytes (GV), ovulated metaphase II-arrested oocytes (O), and embryos at the 1-cell (1C), mid 2-cell (2C), 4-cell (4C), morula (M), or blastocyst (BI) stages. The relative abundance of translation initiation factor mRNA for eIF-1A, eIF-4A, eIF-5, eIF-4E, and eIF-2 α was expressed relative to levels in GV oocytes arbitrarily set at 100%. The experiment was performed at least three times for each primer set, and the data are expressed as the mean \pm SEM. For each transcript dissimilar superscripts denote significant differences ($p < 0.05$).

imately 17-fold to 59-fold (Fig. 1) to 200-fold. This increase was more pronounced and variable than the mean 16-fold increase in eIF-1A relative abundance detected by SQ-RT-PCR, with individual experiments exhibiting a 4-fold, 23-fold, and 25-fold increase (Fig. 2). Thus, although both methods exhibited variation in the magnitude of changes in eIF-1A mRNA abundance, they both confirmed the existence of a transient expression pattern at the 2-cell stage.

Using SQ-RT-PCR to profile the expression of eIF-1A mRNA throughout early development, it was determined that the expression of this transcript was not confined to early cleavage stages. Like many transcripts whose degradation begins during oocyte maturation [50], the abundance of eIF-1A transcript was less in the MII oocytes as compared to GV oocytes. Furthermore, after the decrease in its abundance between the 2-cell and 4-cell stages, the amount of eIF-1A transcript steadily increased between the 4-cell and blastocyst stages (Fig. 2).

To determine whether other translation initiation factors

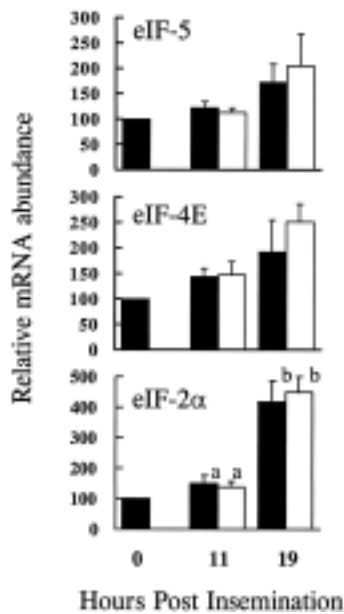


FIG. 3. Changes in the relative abundance of mRNAs for a subset of translation initiation factors during the first cell cycle in in vitro-developed mouse embryos. RNA was isolated from oocytes inseminated in vitro and cultured in the presence (open bars) or absence (solid bars) of 24 $\mu\text{g}/\mu\text{l}$ α -amanitin until 0, 11, or 19 h postinsemination. The relative amounts of transcripts for eIF-5, eIF-4E, and eIF-2 α were expressed relative to levels at time zero, arbitrarily defined as 100%. The experiment was performed at least three times for each primer set, and the data are expressed as the mean \pm SEM. For each transcript, dissimilar superscripts denote significant differences ($p < 0.05$).

were coordinately expressed in a manner similar to that for eIF-1A, SQ-RT-PCR was used to examine the temporal patterns of expression of mRNAs encoding eIF-4A, -5, -4E, and -2 α (Fig. 2). Although at least 10 different factors have been identified in the process of translation initiation in mammals, we selected these four based on the availability of either mouse or rat sequences for primer design and their distinct function (see *Discussion*). As described for eIF-1A, each transcript was assayed for in a minimum of three independent series of in vivo-developed embryos, and the mean values and accompanying standard error are reported. Although each factor displayed a unique mRNA representation profile, none of them displayed a transient increase during the 2-cell stage. All factors displayed a decrease associated with meiotic maturation, namely, a decrease between the fully grown GV oocytes and MII oocytes. The profile for eIF-4A was typical of that of many genes such as β -actin, i.e., a decrease between the oocyte and 2-cell/4-cell stage, followed by an increase. In addition, the representation of mRNA for eIF-5 and -2 α increased significantly from the MII oocyte to the blastocyst stage, although that for eIF-4E did not.

α -Amanitin-Insensitive Increase in Murine eIF-2 α mRNA Abundance during the First Cell Cycle

In repeated experiments on in vivo-developed embryos, the representation of mRNA for eIF-5, -4E, and -2 α consistently increased between the MII oocyte and 1-cell embryo stages. To test whether these increases represented early transcription of these genes, MII oocytes were fertilized and cultured in vitro in the presence or absence of 24 $\mu\text{g}/\text{ml}$ α -amanitin, followed by RNA isolation for SQ-RT-PCR (Fig. 3). The efficacy of this treatment for inhibiting RNA

polymerase II-dependent transcription in early murine embryos has been previously described [51, 52] and was demonstrated in the present study for transcription of eIF-1A (Fig. 1). Changes observed in in vivo-developed embryos (Fig. 2) were also observed in embryos derived in vitro (Fig. 3). However, only the increase observed in the abundance of mRNA for eIF-2 α by 19 h postinsemination was statistically significant. This 4-fold increase in the mean representation of this mRNA relative to that for embryos at 11 h postinsemination was observed in the presence or absence of α -amanitin, suggesting that it did not depend on transcriptional activity. Instead this increase could have been a manifestation of eIF-2 α mRNA polyadenylation during this time in development (see *Discussion*).

Transient Expression of eIF-1A in Cleavage-Synchronized In Vitro-Produced Bovine 8-Cell Embryos

Given that the timing of the maternal-to-zygotic transition is species specific, we wished to determine whether the transient expression of eIF-1A remained correlated with the major activation of embryonic transcription in a species such as the cow, where it begins much later. In bovine embryos, EGA has definitely occurred by the 8-cell stage (for review see [2, 53]). Since transient expression of eIF-1A at the 2-cell stage in the mouse is approximately confined to a 10-h window beginning within 1 h of cleavage, and since development of in vitro-derived bovine embryos is normally highly asynchronous, it was first necessary to plot the chronology of early bovine embryogenesis. Developmental progression to the 2-cell, 3- to 4-cell, 8-cell, and $>$ 8-cell stages was evaluated under two culture environments, namely 1) serum-supplemented T-199 medium with bovine epithelial cells in a 5% CO_2 in air (20% O_2) atmosphere, or 2) synthetic oviductal fluid medium with citrate (c-SOFM) in a reduced O_2 atmosphere (5% CO_2 :10% O_2 :85% N_2). Although the former has represented the traditional method for bovine embryo production, recent developments have favored a shift to defined media that is preferable for experimental manipulations (e.g., α -amanitin treatment). In both culture environments, progression from the 2-cell to 4-cell and from 4-cell to 8-cell stages occurred within 10 h, respectively, the approximate duration of the second and third cell cycles. Progression from the 8-cell stage to the $>$ 8-cell stage required approximately 50 h (Fig. 4). There was a slight, 5–10%, increase in the proportion of embryos cleaving to the 2- and 4-cell stages in defined medium/reduced O_2 conditions that was statistically significant ($p < 0.05$). However, this difference diminished with progression to the 8-cell stage and disappeared with subsequent development. Approximately half of the embryos that had cleaved beyond the 8-cell stage by 126 h postinsemination formed blastocysts by 8 days postinsemination, and there was no significant difference in blastocyst yield between the two culture environments (serum/coculture vs. defined medium/reduced O_2 mean \pm SEM: 66 \pm 27% vs. 44 \pm 13%, $n = 3$).

Amplification of bovine oviduct epithelial cDNA using murine eIF-1A oligonucleotide primers resulted in a comparably sized product of 170 bases whose nucleotide sequence (accession no. AF051848) was 95% conserved with the mouse sequence (data not shown). To examine the expression of eIF-1A at the 8-cell stage, RNA was isolated from small pools of synchronously dividing embryos collected as described in *Materials and Methods*. In four independent experiments on series of blastocysts (developed

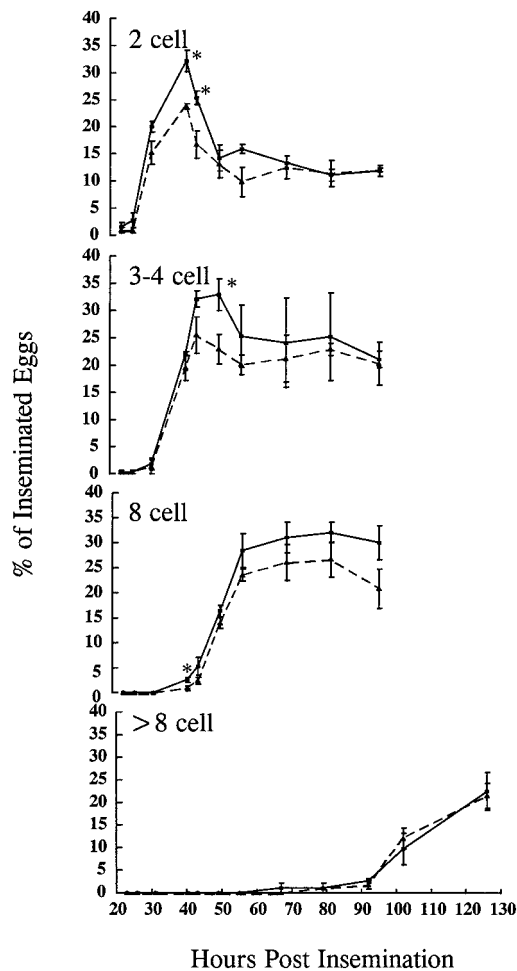


FIG. 4. Chronology of early cleavage divisions in in vitro-derived bovine embryos. Bovine embryos were produced by in vitro maturation and fertilization of cumulus-oocyte complexes and cultured in serum-supplemented medium with bovine epithelial cells under 5% CO₂ in air (broken lines) or chemically defined c-SOFM under reduced O₂ tension (solid lines). Beginning at 23 h postinsemination (hpi), pools of embryos were examined at 3- to 8-h intervals until 55 hpi and subsequently at 12-h intervals until 126 hpi, and the percentage that had cleaved to the 2-cell, 3- to 4-cell, 8-cell, or > 8-cell stage was determined. Beyond 92 hpi, only the proportion cleaving beyond the 8-cell stage was determined. The plotted values represent the mean percentage at each stage \pm SEM in 3 experiments comparing equal numbers of embryos in each culture environment, which ranged from 60 to 100 per experiment. Asterisks denote time points at which differences were significant ($p < 0.05$).

in serum/coculture) and cleavage-synchronized 8-cell embryos cultured in either a serum/coculture environment or defined medium, eIF-1A mRNA could be detected only at the 8-cell stage at 14 h postcleavage, not at early or later time points. Treatment of embryos in defined medium/reduced O₂ environment with 24 μ g/ml α -amanitin, begun upon cleavage to the 8-cell stage, inhibited the appearance of the transcript at 14 h postcleavage. In only 1 of 4 experiments was eIF-1A detected in bovine blastocysts developed in a serum/coculture environment, 8 days postinsemination. Although the apparent absence of eIF-1A transcript at other time points at the 8-cell stage was surprising, the amplification of exogenously supplied α -globin indicated that the recovery of RNA and efficiency of reverse transcription were comparable for all samples (Fig. 5). Since the detection of eIF-1A in 8-cell embryos was an all-

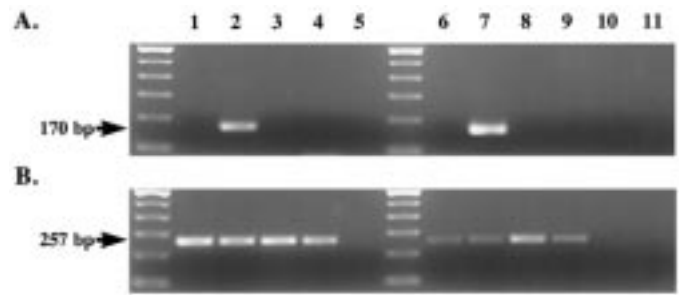


FIG. 5. Transient expression of eIF-1A in bovine preattachment embryos at the 8-cell stage. Representative ethidium bromide-stained gels showing amplification of A) a 170-bp product representing eIF-1A and B) a 257-bp product representing the exogenously supplied α -globin transcript from bovine embryos produced in vitro by coculture with somatic cells in serum-supplemented medium (lanes 1–5) or defined medium in a reduced O₂ tension environment (lanes 6–11). In 4 independent experiments, eIF-1A was reproducibly detected in 8-cell embryos synchronous with respect to their last cleavage at 14 h postcleavage (hpc; lanes 2 and 7), but not at earlier (3 hpc; lanes 1 and 6) or later (24 hpc, lane 9; 45 hpc, lane 3) time points. No eIF-1A could be detected in Day 8 blastocysts in 3 of 4 experiments (lane 4) or in 8-cell embryos treated in defined medium with 24 μ g/ μ l of α -amanitin from 0 to 14 hpc (lane 8). Neither eIF-1A nor α -globin was detected in negative control reactions on water (lanes 5 and 11) or in an aliquot of negative control reverse transcription reactions performed on blank messenger affinity paper product used to isolate bovine embryo mRNA (lane 10).

or-nothing phenomenon, no relative abundance histogram could be generated.

DISCUSSION

In the present study we demonstrate that the transient expression of eIF-1A, associated with EGA in the mouse at the 2-cell stage, is conserved in the cow at the 8-cell stage, when the major activation of the genome occurs in this species. Transient eIF-1A expression in the mouse was also found to be unique to this gene and was not observed for the translation initiation factors eIF-2 α , -4A, -4E, and -5. Translation is a major control point for the regulation of gene expression during development [54], and the regulation of translation is realized mostly through translation initiation mechanisms, specifically those involved in the recruitment of mRNA or initiator (methionyl) tRNA [55]. With the exception of eIF-5, which catalyzes the association of ribosomal subunits to form an active ribosome [56], the initiation factors examined in the present study all functioned in mRNA and initiator tRNA recruitment.

Of the factors investigated, mRNA for eIF-4E was the only one not to increase during development. This suggests that by the end of the preimplantation development, eIF-4E mRNA is either present in all cells in limiting amounts, or preferentially expressed in a subset of cells. As a component of eIF-4F (along with eIF-4A), eIF-4E protein binds to the 5' methyl-cap of mRNAs during the process of mRNA recruitment and is recognized as an early downstream target of growth regulatory molecules including insulin, platelet-derived growth factor, epidermal growth factor, and tumor necrosis factor [55]. Relative to other initiation factors, eIF-4E protein is normally present in limiting amounts in the cytoplasm [57, 58]. Its overexpression in NIH 3T3 cells, rat embryonic fibroblasts, and HeLa cells can lead to aberrant growth and oncogenic transformation [59, 60]. Assuming that expression of eIF-4E protein is directly correlated with the abundance of its mRNA, as ap-

pears to be the case for eIF-1A [13], a spatially restricted expression would permit eIF-4E to induce differentiation of specific cell lineages. Consistent with this, the microinjection of mRNA for eIF-4E into the ectodermal explants taken from early *Xenopus laevis* embryos leads to mesoderm induction [61].

Changes in protein synthesis associated with the maturation and fertilization of amphibian, sea urchin, and starfish eggs are regulated through the eIF-4F complex as well as the guanine nucleotide exchange factor for eIF-2, eIF-2B [62–64]. Although in the present study the abundance of mRNA for all of the factors decreased with meiotic maturation, mRNA for eIF-4A appeared to be the most stable, reaching its low point only by the 4-cell stage. Protein for eIF-4A is the component of the eIF-4F complex that possesses RNA helicase activity [56]. Such activity would be useful to unmask oogenetic mRNAs for translation prior to the activation of the embryonic genome, assuming that the stability of eIF-4A mRNA is paralleled at a protein level.

After the meiotically associated decrease in mRNA content, the abundance of the eIF-2 α transcript exhibited a significant α -amanitin-insensitive increase during the first cell cycle. Protein for eIF-2 α is the GTP-binding regulatory subunit of eIF-2, involved in methionyl-tRNA recruitment [56]. Similar increases in mRNA content have been previously reported for the transcription factor Sp1 and a cyclin-dependent kinase, cdk4, during the first cell cycle and likely reflect polyadenylation of these messages [28, 65]. Polyadenylation could increase the representation of a message after reverse transcription with an oligo-dT primer by increasing the probability that such a primer will anneal to the transcript [28]. If in fact mRNA for eIF-2 α is polyadenylated during the first cell cycle in the mouse, this may reflect its recruitment for translation as may be the case for Sp1 [54, 65]. However, the regulation of eIF-2 α is likely to be complex. In lymphocytes, the abundance of eIF-2 α mRNA is rapidly regulated through the synthesis of antisense transcripts driven by an opposing promoter downstream of the sense promoter for this gene [66, 67].

The transient expression of eIF-1A mRNA observed at the 2-cell stage in the mouse was confirmed in the present study by two independent assays [33, 42]. Although the extent of increase in this transcript differed between methods, this may have reflected differences in the sensitivity of these methods to low points in mRNA content and adenylation. Along with eIF-2, eIF-1A was recently demonstrated to play a role in the catalysis of methionyl-tRNA recruitment [68, 69]. However, unlike eIF-2 α and eIF-4E, whose respective functions are negatively and positively regulated by phosphorylation, eIF-1A is one of the few translation initiation factors that is not posttranslationally modified [70]. Since transient transcription of eIF-1A in mouse embryos is accompanied by changes in the relative rate of synthesis of this protein [17], its activity may be a direct reflection of the abundance of its mRNA. This type of regulation would make eIF-1A particularly sensitive to the effects of mRNA degradation initiated with egg maturation. The transient expression of eIF-1A prior to the activation of most constitutively expressed genes may thus represent a compensatory mechanism by the embryo to meet its protein synthesis requirements during the maternal-to-zygotic transition.

The transient α -amanitin-sensitive detection of eIF-1A transcripts in bovine embryos at the 8-cell stage correlates with the major activation of the embryonic genome at this time [53]. Its expression also occurred at the same time in

bovine embryos cultured under wholly distinct environments, suggesting that it may represent a fundamental feature of early development under conditions that support growth. Our inability to detect this transcript in 8-cell-stage bovine embryos at time points other than 14 h postcleavage was likely due to scarcity of this mRNA and the fact that experiments were performed on smaller pools of reverse-transcribed embryos than experiments in the mouse. However, it should also be noted that in the mouse the transient expression of eIF-1A occurs within a 10-h window and that the time points examined at the 8-cell stage in the cow were spaced by 10 h or more. A quantitatively minor transcriptional activity has been reported in the cow beginning at the 2-cell stage [5, 6]. In preliminary experiments we uncovered no evidence of a transient expression pattern in 2-cell or 4-cell embryos (data not shown). It is possible that eIF-1A is not one of the first genes to be transcribed during this period in the cow. In the mouse, transcription of eIF-1A is accompanied by its translation [13]; and during the minor activation of the genome at the 1-cell stage, transcription and translation are uncoupled [71]. In the cow an uncoupling between transcription and translation during early cleavage stages may also exist. Bovine embryos transfected with reporter genes at the 1-cell stage express detectable amounts of reporter gene protein only after 42 h of culture around the 4- to 8-cell stage or later. In those experiments, reporter protein was also detected in arrested 1-cell embryos at this time [72, 73].

Conservation in the transient expression of eIF-1A in the mouse and cow suggests that the mechanisms regulating its expression, and EGA in general, are conserved. These mechanisms may involve alterations in chromatin structure. In the mouse, transient expression of eIF-1A mRNA, as well as of the protein marker of EGA, the transcription-requiring complex, is dependent on the preceding and succeeding rounds of DNA replication that remodel chromatin [13, 19]. Evidence that chromatin is being restructured at this time comes from studies demonstrating the transient and unique localization of RNA polymerase II and selected isoforms of acetylated H2A, H3, and hyperacetylated H4 at the nuclear periphery of mouse 2-cell embryos [12, 74]. Localization of acetylated H2A and H3 at the periphery of 2-cell nuclei also requires DNA replication, although it is independent of ongoing transcription or of cytokinesis [74]. Interestingly, during bovine embryogenesis, intense uridine incorporation, indicative of transcriptional activity, has been reported at the nuclear periphery at the 8-cell stage but not in earlier- or later-staged embryos [75]. Alterations in chromatin structure likely modulate transcriptional activity by permitting or restricting transcription factor access to genomic regulatory elements.

In summary, the conservation of eIF-1A transient expression during EGA in the mouse and cow suggests that the mechanisms regulating the onset of embryonic transcription are broadly conserved in mammals, despite chronological differences in the timing of this event. This expression pattern could therefore serve as a reliable indicator of embryo normalcy during the maternal-to-zygotic transition and of the capacity of culture environment to support this transition. It may also serve as a useful indicator of nuclear reprogramming following nuclear transfer for evaluation of both nuclear and cytoplasmic competence to initiate a developmental program. In the future it will be interesting to determine whether transient expression of eIF-1A, as well as the other genes observed in the mouse, is a general feature of EGA in lower vertebrates and inverte-

brates and to uncover the functional significance of this phenomenon.

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