FLGα, a germ cell specific transcription factor involved in the coordinate expression of the zona pellucida genes

Li-fang Liang, Selma M. Soyal and Jurrien Dean*

Laboratory of Cellular and Developmental Biology, NIDDK, National Institutes of Health, Bethesda, MD 20892, USA

*Author for correspondence (e-mail: jurrien@helix.nih.gov)

SUMMARY

The mouse zona pellucida is composed of three glycoproteins, ZP1, ZP2 and ZP3, encoded by single-copy genes whose expression is temporally and spatially restricted to oocytes. All three proteins are required for the formation of the extracellular zona matrix and female mice with a single disrupted zona gene lack a zona and are infertile. An E-box (CANNTG), located approximately 200 bp upstream of the transcription start sites of Zp1, Zp2 and Zp3, forms a protein-DNA complex present in oocytes and, to a much lesser extent, in testes. It has been previously shown that the integrity of this E-box in Zp2 and Zp3 promoters is required for expression of luciferase reporter genes microinjected into growing oocytes. The presence of the ubiquitous transcription factor E12 in the complex was used to identify a novel basic helix-loop-helix protein, FIGα (Factor In the Germine alpha) whose expression was limited to oocytes within the ovary. The ability of FIGα to transactivate reporter genes coupled to each of the three mouse zona promoters in heterologous 10T½ embryonic fibroblasts suggests a role in coordinating the expression of the three zona pellucida genes during oogenesis.

Key words: FLGα, basic helix-loop-helix transcription factor, zona pellucida, oocyte-specific gene expression, mouse

INTRODUCTION

The complexity of many biological systems requires temporal and spatial orchestration of multiple gene products. Not infrequently, the absence of a single component precludes the formation of important structures. The three zona pellucida proteins, ZP1, ZP2, ZP3, form an extracellular matrix that surrounds growing oocytes, ovulated eggs and pre-implantation embryos. This structure is critical for ordinal-specific fertilization, the subsequent block to polyspermy and passage of the early embryo through the oviduct prior to implantation (Yanagimachi, 1994). The assembly of the zona matrix requires coordinate synthesis of the three proteins (Bleil and Wassarman, 1980; Shimizu et al., 1983) and the absence of either ZP2 or ZP3 in vitro prevents zona formation (Tong et al., 1995). Mice in which the Zp3 gene has been genetically disrupted, express only ZP1 and ZP2 proteins and zonae pellicdae are not observed within their ovaries. Although oocytes can grow, mature and be ovulated, no 2-cell embryos are recovered after mating and no litters have been born to homozygous mutant females. Thus, the absence of the zona matrix effectively blocks fertilization in vivo (Liu et al., 1996; Rankin et al., 1996).

The mouse zona proteins are encoded by single copy genes, Zp1, Zp2, Zp3 (Kinloch et al., 1988; Chamberlin and Dean, 1989; Liang et al., 1990; Epifano et al., 1995a), located on chromosomes 19, 7 and 5, respectively (Lunsford et al., 1990; Epifano et al., 1995a). Nevertheless, the expression of the mouse Zp1, Zp2 and Zp3 genes is precisely regulated and restricted to a two-week growth phase of oogenesis (Philpott et al., 1987; Liang et al., 1990; Epifano et al., 1995b). Throughout the reproductive life of the mouse, groups of resting oocytes are recruited to initiate growth under the regulation of an as yet to be identified mechanism. Few if any zona transcripts are detected in resting oocytes. However, once these oocytes begin to grow, all three zona transcripts begin to accumulate, reaching maximum abundance in oocytes of 50-60 μm diameter, where together they represent approximately 1.5% of the total poly(A)+ RNA (Epifano et al., 1995b). The RNA accumulation profiles of ZP1, ZP2 and ZP3 transcripts are coordinate, maintaining the same 1:4:4 ratio throughout oocyte growth, suggesting that the zona genes are regulated, in part, by identical transcription factors binding to the promoters of all three zona genes.

To explore the possibility of a common regulatory mechanism, the promoter regions of the three mouse zona genes have been examined by mutagenesis. Although little sequence homology exists among the promoters, a canonical E-box (CANNTG) has been identified approximately 200 bases upstream of the transcription start sites of Zp1, Zp2 and Zp3 (Millar et al., 1991; Epifano et al., 1995a). This conserved DNA element is a known binding site for basic helix-loop-helix (bHLH) transcription factors (Murre et al., 1989b), many of which have been shown to be tissue-specific. Six base pair clustered mutations of the E-box in mouse Zp2, human ZP2 and mouse Zp3 promoters dramatically inhibited luciferase reporter gene activity of plasmids microinjected into the nucleus of growing oocytes, demonstrating that the E-box plays a crucial role in zona gene expression (Millar et al., 1991; Liang and Dean, 1993). Gel mobility shift assays using
synthetic oligonucleotides centered on the Zp2 and Zp3 promoter E-boxes have identified a tissue-specific protein-DNA complex in oocytes, but not in surrounding follicle cells nor in other tissues in female mice. The complex is also present in the testis, albeit in low abundance (Millar et al., 1991). This tissue-specific protein-DNA complex (formerly called ZAP-1) is first detected in oocytes isolated from 19 day gestational mice and the proteins within the complex increase in abundance during oocyte growth (Millar et al., 1993). The appearance of the protein-DNA complex in the oocyte temporally coincides with the detection of ZP2 transcripts in the prenatal ovary (Millar et al., 1993) suggesting that this complex is involved in the activation of the zona pellucida genes.

Most often bHLH transcription factors that regulate tissue-specific gene expression exist as heterodimers composed of a near ubiquitious class A protein [e.g., E12/E47 (Murre et al., 1989a)] and a tissue-specific class B protein [e.g. myoD (Davis et al., 1987)]. While all tissue-specific components share the defining bHLH domains required for heteroduplex formation and DNA binding, unique protein domains involved in gene activation or in interactions with other regulatory proteins have been identified. As more bHLH transcription factors have been reported, they have been grouped into subclasses based on their expression patterns, conservation of their bHLH motifs and biological functions. Basic helix-loop-helix proteins have been associated with the activation of genes involved in myogenesis (Davis et al., 1987), neurogenesis (Villares and Cabrera, 1987), mesoderm formation (Quertermous et al., 1994; Cserjesi et al., 1995; Olson et al., 1996), hematopoiesis (Villares and Cabrera, 1987; Chen et al., 1990) and sex determination (Caudy et al., 1988; Torres and Sanchez, 1989; Parkhurst et al., 1990; Erickson and Cline, 1991). However, to date, none of the described bHLH transcription factors have expression that is restricted to germ cells and none have been implicated in the regulation of oocyte-specific gene expression.

We now report the molecular characterization of FIGα (Factor In the Germline alpha), a novel germ cell-specific bHLH transcription factor that binds as a heterodimer with E12 to the E-box in the promoter region of all three mouse zona pellucida genes and has the ability to transactivate reporter gene constructs in vitro. These data suggest that FIGα has a role in the coordinate, oocyte-specific expression of the three zona pellucida genes, the products of which form an extracellular matrix required for fertilization and early development.

**MATERIALS AND METHODS**

**Gel mobility shift assays**

Whole cell ovarian extracts were prepared by freeze-thaw lysis of ovaries from 7-day-old NIH Swiss female mice (Millar et al., 1991) and oligonucleotides were labeled with \( ^{32}P \)dCTP and \( ^{32}P \)dATP using the Klenow fragment of DNA polymerase I. The double stranded E-box oligonucleotides used in the gel mobility shift assays were: Zp1, CCTGGGTAGTCCAGCTGCAACAGAAAT; mutant Zp1, CCTGGGTAGTCCAGCTGCAACAGAAAT. Zp2, CACTATTCTACCTAGGGAGCCATTTT; mutant Zp2, CACTATTCTACCTAGGGAGCCATTTT; Zp3, TTGATATGAGTCTGAGTCTGCTTTAC; mutant Zp3, TTGATATGAGTCTGAGTCTGCTTTAC; creatine kinase E-box AGCTTC-CAACACCTGCTGCAACAGT. All E-box oligonucleotides were labeled to equivalent specific activities. Monoclonal antibodies to E2A, E2-2, HEB (Santa Cruz Biotechnology, Inc) and E12, E47 (PharMingen), and polyclonal antiserum to myoD bind target proteins and supershift protein-DNA complexes (Shirakawa and Paterson, 1995; Ikin-Ansari et al., 1996). Each antibody (1 μg) or antiserum (1:5 μl) was pre-incubated with ovariect extract for 15 minutes at 4°C prior to the addition of labeled oligonucleotides. Gel mobility shift assays were performed as previously described (Millar et al., 1991; Shirakawa and Paterson, 1995).

**Screening the mouse ovarian CDNA expression library**

A mouse ovarian CDNA expression library was constructed in Lambda ZAP (Stratagene) using 2 μg of ovariect poly(A)+ RNA isolated from 7-day-old mice. Partial human E12 and E47 cDNAs (Murre et al., 1989a) were subcloned into the FLAG-fusion protein expression vector pAR(ARI)59/60 (Blair and Rutter, 1992) and recombinant fusion proteins were purified by affinity chromatography using anti-FLAG antibodies (IBI). 4.6×10^6 plaques of the amplified library were screened with FLAG-E12 and FLAG-E47 recombinant proteins, labeled with \( ^{35}S \)ATP by heart muscle protein kinase (Armand et al., 1994). Positive clones were plaque-purified and the sequence of the insert cDNA was determined on both strands (Eripiano et al., 1995b). Near full-length FIGα cDNA was obtained using the same RNA and 5'-RACE (Life Technologies, Inc.) according to the manufacturer’s protocol. Two independent reactions were sequenced to confirm fidelity of the PCR products. The GenBank accession number of the FIGα cDNA is U91840.

**FIGα-specific polyclonal antibody production**

Polyclonal anti-FIGα serum was produced by immunizing female Balb/c mice with a synthetic FIGα peptide (residues 156 to 170) conjugated to KLH carrier (Harlow and Lane, 1988). An initial intraperitoneal immunization of 100 μg (peptide) in complete Freund’s adjuvant (0.5 ml) was followed 3 weeks later by an identical immunization in incomplete Freund’s adjuvant. Antisera were obtained 2 weeks later.

FIGα cDNA was subcloned into pET28c (Novagen, Inc) and recombinant FIGα expressed in BL21(DES)pLysS cells was purified by N++-Agarose column chromatography (Qiagen, Inc.) according to the manufacturer’s instructions. Recombinant FIGα and E12 (Shirakawa and Paterson, 1995) protein (0.5 μg) were separated by SDS-PAGE and assayed by western blot using antibodies to FIGα (1:500) and E12 (1:500; Shirakawa and Paterson, 1995) and an Enhanced Chemiluminescence Kit (Amersham Life Sciences, Inc.) according to the manufacturer’s instructions.

**RT-PCR and northern blot analyses**

Total RNA was isolated from organs using RNazol B (Cinna/Biotex Laboratories) and poly(A)+ RNA was isolated using an Oligotex mRNA isolation kit (Qiagen). Approximately 1 μg of total RNA from each tissue was analyzed by RT-PCR (Perkin-Elmer RT-PCR) according to the manufacturer’s protocol using oligonucleotides that span intron 1 of the FIGα gene (CTGAGGAGCTGAGACTAAGAAGCTG; TGGAGACCTTCGCTTCCAG) and intron 8 of the Hprt gene.

For northern blot analysis, 2 μg of poly(A)+ RNA from each tissue was electrophoresed in a 1.2% formaldehyde gel, transferred onto nylon membrane and probed with \( ^{32}P \)dCTP by heart muscle protein kinase (Armand et al., 1994). For northern blot hybridization, FIGα cDNA was hybridized in 1.2% formaldehyde gel, transferred onto nylon membrane, and probed with \( ^{32}P \)dCTP random primed labeled FIGα DNA. The final, high stringency wash was 0.1× SSPE, 0.1% SDS at 65°C. Autoradiograms were scanned with a Molecular Dynamics densitometer and quantified using ImageQuant software.

**In situ hybridization**

Ovaries from 12- to 14-day old mice were fixed in 2% paraformaldehyde, 0.1 M sodium cacodylate, pH 7.2 for 5 hours at room temperature, transferred to 70% ethanol, dehydrated and embedded in paraffin (Rankin et al., 1996). The sections were then hybridized with \( ^{35}S \)-labeled sense- and anti-sense synthetic FIGα RNA using previ-
For each transfection, 0.5 cells were incubated overnight until the cells were 60-70% confluent. seeded in each well of six-well (35 mm) tissue culture plates. The fibroblast cells in 2 ml of DMEM with 10% fetal bovine serum were and incubated an additional 36 hours with one refeeding. Cells were on the growing cells. After 5 hours at 37°C, the medium was replaced minutes The mixture was diluted with 0.8 ml of DMEM and overlaid

The protein-DNA complex formed using a 32 P-labeled HEB, E2-2) were used in a gel mobility shift assay (Fig. 1A).

To investigate the composition of this germ cell specific protein-DNA complex, monoclonal antibodies to the three promoters to the multiple cloning site of the luciferase reporter vector pXP1 (Nordeen, 1988). Junctions and mutated sites were confirmed by DNA sequencing.

Transient transfection assays were performed using LipofectAMINE (Life Technologies) according to the manufacturer’s protocol. On the day before transfection, 1.5×10^5 mouse 10T^5 embryonic fibroblast cells in 2 ml of DMEM with 10% fetal bovine serum were seeded in each well of six-well (35 mm) tissue culture plates. The cells were incubated overnight until the cells were 60-70% confluent. For each transfection, 0.5 μg of luciferase reporter plasmid, 0.01 μg of β-galactosidase reporter plasmid, pSV-β-gal (Promega), with/without 0.25 μg pcME12 and/or 0.25 μg pcMFIGTx were mixed and pGEM-1 (Promega) was added to ensure a total of 1 μg of DNA for suspension in 100 μl of Opti-MEM I Reduced Serum Medium (Life Technologies). LipofectAMINE (8 μl in 100 μl of Opti-MEM) was mixed with the DNA solution and incubated at 20°C for 45 minutes The mixture was diluted with 0.8 ml of DMEM and overlaid on the growing cells. After 5 hours at 37°C, the medium was replaced and incubated an additional 36 hours with one refeeding. Cells were then harvested and luciferase and β-galactosidase activities were determined using Dual-Light chemiluminescent reporter gene assay kit (Tropix, Inc.) according to the manufacturer’s instructions. Each data point was the average of three wells and each experiment was repeated 3-6 times.

RESULTS

E12 participates in a protein complex that binds mouse Zp1, Zp2, and Zp3 promoters

The ability of mouse Zp2, mouse Zp3 and human ZP3 promoters to activate reporter genes microinjected into growing mouse oocytes is dependent on the integrity of an E-box located approximately 200 bp upstream of their transcription start sites. The protein complex formed with this E-box is present in oocyte and to a much lesser extent, in testis. Within the ovary, the complex is further localized to oocytes and is not detected in the surrounding granulosa cells (Millar et al., 1991; Liang and Dean, 1993; Epifano et al., 1995a).

To investigate the composition of this germ cell specific protein-DNA complex, monoclonal antibodies to the three known class A basic helix-loop-helix (bHLH) proteins (E2A, HEB, E2-2) were used in a gel mobility shift assay (Fig. 1A). The protein-DNA complex formed using a 32 P-labeled oligonucleotide (30 bp) centered on the Zp2 E-box (−216 bp) incubated with ovarian extract was competed by molar excess amounts of unlabeled oligonucleotide but not if the E-box had been mutated (CACCTG → AGATCT) (Fig. 1A, lanes 1-3). The addition of pre-immune serum or monoclonal antibodies specific for HEB or E2-2 to the ovarian extract had no affect on the mobility of the complex (Fig. 1A, lanes 4-6). However, monoclonal antibodies specific for E2A bHLH transcription factors, resulted in a diminution in the protein-DNA complex and the formation of a more slowly migrating complex (supERSHIFT; Fig. 1A, lane 7). Not all of the protein-DNA complex was shifted which may reflect a lower affinity of the antibodies for mouse compared to the human proteins against which they were raised, although the presence of an additional bHLH complex lacking one of the three known class A proteins could not be excluded.

E2a is a single copy gene that encodes E12 and E47, two transcription factors that arise from differential splicing of 2 exons within E2a and differ only in their bHLH domains (Murre et al., 1989b). Gel mobility shift assays were extended to examine proteins binding to Zp1 (−216 bp), Zp2 (−218 bp) and Zp3 (−181 bp) E-boxes using monoclonal antibodies specific for human E12 and E47. Incubation of equal amounts of mouse ovarian extracts with 32 P-labeled oligonucleotides centered on Zp1, Zp2 and Zp3 E-boxes resulted in protein-DNA complexes that migrated with the same mobility. All three protein-DNA complexes were competed with molar excess amounts of gene-specific unlabeled oligonucleotides but not if they contained a mutated E-box (Fig. 1B, lanes 1-3). The intensity of the protein-DNA complex differed considerably among the three genes decreasing from Zp2, to Zp1 to
FIGα cDNA encodes a bHLH transcription factor

Due to the paucity of biological material, traditional biochemical purificatication methods were not practical for the isolation of the presumptive, tissue-specific class B bHLH transcription factor that heterodimerizes with E12. Instead, a strategy based on the formation of a protein-protein complex by bHLH domains was employed. Following previously described protocols (Blanar and Rutter, 1992; Armand et al., 1994), 32P-labeled recombinant E2A proteins were used to screen an unamplified mouse ovarian cDNA expression library. Twenty-four cDNAs were identified among 4.6 \times 10^6 plaques. Each clone was purified and partially sequenced. All contained a basic helix-loop-helix motif, but when compared to sequences in GenBank, only one was novel. This clone, encoding transcripts present in the ovary and to a much lesser extent in the testis (see below), was designated Factor In the Germline alpha, FIGα.

Since the initial FIGα cDNA was not full-length, 5’ RACE was used to obtain additional sequences that included a methionine in the context of a vertebrate initiator (Kozak, 1991) and an upstream in-frame stop codon. This 68 bp 5’ sequence was combined with the 691 bp initial clone to form a near full length (759 bp) cDNA, designated pFIGα. The longest open reading frame beginning with the initiator methionine was 582 bp and encoded a polypeptide chain of 194 amino acids with a relative molecular mass of 21,910 (Fig. 2A). The deduced amino acid sequence predicted a bHLH domain between residues 59 and 113, but examination of the remaining sequence failed to identify motifs (e.g. acidic-, serine/threonine- or glutamine-rich regions) that have been ascribed to the transactivation domains of transcription factors. There are multiple, potential protein kinase C and casein kinase II phosphorylation sites along the FIGα polypeptide chain. While the activity of some transcription factors is dependent on such post-translational modification, it is unknown if FIGα is modified in vivo. Based on Southern blot analysis of mouse genomic DNA probed with 32P-labeled FIGα cDNA and washed under conditions of high stringency (0.1 \times SSC, 65°C), Figα is most likely a single copy gene (data not shown).

The deduced FIGα protein sequence contains the consensus amino acids that define the basic helix-loop-helix domain (Fig. 2B). Many bHLH transcription factors fall into small families that share considerable homology within the bHLH region (e.g., myoD, myogenin, myf5, MRF4 (Lassar and Munsterberg, 1994); Hxt, Hed (Olson et al., 1996); scleraxis, paraxis, parvis).
recombinant FIGα antisera raised against a FIGα membrane. Each primary antibody was diluted 1:500 and detected by 12% polyacrylamide gel prior to transfer to a nitrocellulose membrane. Each primary antibody was diluted 1:500 and detected by ECL. (B) Gel mobility shift assay using recombinant E12 and myoD with a 32P-labeled oligonucleotides (24 bp) containing an E-box upstream of the creatine kinase gene that binds myoD/E12 complexes (Shirakawa and Paterson, 1995): no antisera (lane 1); 1 μl of anti-myoD antiserum (lane 2) or 1.5 μl of anti-FIGα peptide antiserum (lane 3). The arrow at the right indicates the myoD/E12-DNA complex that is shifted by antisera against myoD (lane 2) but not by antisera against FIGα (lane 3).

Fig. 3. Specificity of anti-FIGα antisera. (A) Western blot of purified recombinant FIGα (lanes 1,3) and E12 (lanes 2,4) probed with the antisera raised against a FIGα peptide (residues 156-170; lanes 1,2) or a monoclonal antibody specific to E12 (lanes 3,4). Proteins (0.5 μg) were separated by SDS-PAGE under reducing condition on a 12% polyacrylamide gel prior to transfer to a nitrocellulose membrane. Each primary antibody was diluted 1:500 and detected by ECL. (B) Gel mobility shift assay using recombinant E12 and myoD with a 32P-labeled oligonucleotides (24 bp) containing an E-box upstream of the creatine kinase gene that binds myoD/E12 complexes (Shirakawa and Paterson, 1995): no antisera (lane 1); 1 μl of anti-myoD antiserum (lane 2) or 1.5 μl of anti-FIGα peptide antiserum (lane 3). The arrow at the right indicates the myoD/E12-DNA complex that is shifted by antisera against myoD (lane 2) but not by antisera against FIGα (lane 3).

( Cross et al., 1995). However, aside from the conserved consensus residues, FIGα is distinct from these families (Fig. 2B). Whether there are other bHLH proteins that are more closely related to FIGα and whether or not their expression is also restricted to germ cells, remains to be ascertained.

To confirm that the bHLH protein encoded by FIGα cDNA participates in the protein complex that binds to the E-box upstream of mouse Zp1, Zp2, and Zp3, a synthetic peptide based on the cDNA sequence (amino acids 156-170) was synthesized and coupled to KLH for immunization of mice (Fig. 2B). The resultant antisera detected recombinant FIGα but not E12 on western blots (Fig. 3A) and had no affect on the MyoD/E12 complex in a gel mobility shift assay (Fig. 3B). The anti-FIGα antisera was used in a gel mobility shift assay with oligonucleotides centered on the E-box from Zp1, Zp2, and Zp3 promoters (Fig. 4). The protein-DNA complexes formed with the oligonucleotides from each of the three genes (Fig. 4A-C, lane 1) were retarded by the anti-FIGα antiserum (Fig. 4A-C, lane 3) but not by pre-immune serum (Fig. 4A-C, lane 2). Similar results were obtained with antisera from three different animals (data not shown) and demonstrate that pFIGα encodes native FIGα protein. The completeness of the retardation reflects the high affinity of the antisera for mouse FIGα and the absence of other supershifted bands indicates that FIGα homodimers (42×103 M), which if bound to the labeled oligonucleotide would have a different mobility than E12/FIGα heterodimers (86×103 M), are not present in the ovarian extracts.

Germ cell-specific expression of FIGα

RT-PCR analyses, using primers specific for FIGα and HPRT (positive control) transcripts, were performed on total RNA from 13 different tissues (Fig. 5A). HPRT, as expected, was ubiquitous among mouse tissues, but FIGα transcripts were detected only in the ovary and testis (Fig. 5A, lanes 8,11). Northern blot analysis revealed a single, prominent transcript of approximately 0.9 kb in ovarian RNA and a similar but much fainter transcript in testis RNA (Fig. 5B). The size of the mRNA was in agreement with the predicted transcript length (759 bp cDNA plus 150-200 nt polyA tail). Densitometric analysis of the autoradiograph indicated that the abundance of FIGα in the ovary was at least ten-fold greater than in the testis. The relative amounts of FIGα transcripts in the ovary and testis corresponded to the amounts of FIGα protein-DNA complex detected on gel mobility shift assays using extracts from the two tissues (Millar et al., 1991).

The FIGα transcripts were further localized within the ovary by in situ hybridization. Ovaries from 12- to 14-day old mice were fixed, sectioned and hybridized with 33P-labeled sense- and anti-sense FIGα probes (Fig. 6). FIGα transcripts were most notably localized in the resting oocytes of primordial follicles where they are seen as bright spots concentrated at both the periphery of the ovary and in the hilar region (arrow, Fig. 6A). FIGα transcripts were also visible in growing oocytes (Fig. 6A,B,E,F) but, the intensity of the signal in the surrounding follicle cells was no greater than background. The more intense signals in resting oocytes suggested a higher concentration of FIGα transcripts in oocytes prior to the onset of growth. This early and oocyte-specific presence of FIGα transcripts corresponds with the tissue localization of the FIGα protein-DNA complex determined by gel mobility shift assays (Millar et al., 1991) and is consistent with a hierarchical relationship in which FIGα plays an early role in modulating the oocyte-specific expression of the zona pellucida genes.

Transactivation of zona gene promoters by E12 and FIGα

To examine the activation potential of FIGα on mouse Zp1, Zp2 and Zp3 promoters, transient transactivation studies were

Fig. 4. The protein encoded by FIGα cDNA binds to Zp1, Zp2 and Zp3 promoters in vitro. (A) Gel mobility shift assay with ovarian extracts (7-day old mice) and 32P-labeled oligonucleotide (30 bp) centered on the Zp1 E-box with: no antisera (lane1); pre-immune mouse serum (lane 2); or anti-FIGα antiserum (lane 3). (B) Same as A except a 32P-labeled oligonucleotide (30 bp) centered on the Zp2 E-box was used. (C) Same as A except a 32P-labeled oligonucleotide (30 bp) centered on the Zp3 E-box was used. X-ray film for B (Zp2) was exposed for 8 hours and for A (Zp1) and C (Zp3) for 2 days. The arrow at the right indicates the FIGα protein-DNA complex; the star denotes the supershift band.
performed in mouse 10T\textsubscript{1} embryonic fibroblast cells. Full length mouse E12 and FIG\textgreek{a} cDNA were subcloned into mammalian expression vectors under control of cytomegaloviral promoters. Luciferase reporter plasmids containing Zp1, Zp2 or Zp3 promoters (1.5-1.6 kb) were co-transfected with equal amounts of E12 and FIG\textgreek{a} expression plasmids, either individually or together. A \(\beta\)-galactosidase expression vector with an early SV-40 promoter was used as an internal control for normalization of transfection efficiency. Luciferase activity from transfections with the reporter plasmid alone was set at a value of 1.

 Cotransfections of the luciferase reporter plasmids with either E12 or FIG\textgreek{a} caused relatively small increases in luciferase activity. However, the presence of both E12 and FIG\textgreek{a} resulted in substantial transactivation of 10.1±1.8, 19.5±3.5 and 9.0±3.8 fold (s.e.m.) for the Zp1, Zp2 and Zp3 promoters, respectively (Fig. 7). The degree of transactivation of individual zona gene promoters varied, with Zp2 transactivation approximately twice that of either Zp1 or Zp3. These results indicated that FIG\textgreek{a} was biologically active in transactivating reporter genes downstream of Zp1, Zp2 and Zp3 promoters. When these studies were repeated using identical zona promoter-luciferase reporter constructs except for 6 bp mutations of their E-box (CANNTG → AGATCT) at −218 bp (Zp1), −216 bp (Zp2) and −181 bp (Zp3), transactivation was substantially reduced with the Zp1 or Zp2 promoters but persisted with the Zp3 promoter (Fig. 7).

Thus, the ability of FIG\textgreek{a} to transactivate depended on an intact E-box located −200 bp upstream of the transcription start site of the Zp1 and Zp2 genes. However, the integrity of the mouse Zp3 E-box (−181 bp), originally characterized by microinjection of reporter constructs into growing oocytes (Millar et al., 1991), was not critical in 10T\textsubscript{1} fibroblast cells and indicates that the E12/FIG\textgreek{a} complex transactivated the reporter construct using a different E-box in fibroblasts. Nevertheless, it remains most likely that the E-box at −181 bp, shown to be essential for reporter gene expression in oocytes (the only cell in which zona genes are normally expressed), is utilized in vivo. To examine whether E12 and FIG\textgreek{a} were sufficient for endogenous zona gene activation, 10T\textsubscript{1} fibroblast cells were transfected with E12 and FIG\textgreek{a} expression plasmids (individually and together). Total RNA was isolated and analyzed for the presence of ZP2 transcripts by RT-PCR. No ZP2 transcripts were detected in the transfected cells (data not shown), indicating that although E12 and FIG\textgreek{a} may be necessary for zona gene expression, they are not sufficient and additional factors or modifications of pre-existing factors, are needed for the activation of the zona genes in vivo.

**DISCUSSION**

The assembly of the zona pellucida requires the coordinate expression of three single-copy genes, Zp1, Zp2 and Zp3, located on disparate chromosomes in the mouse genome. Expression of the zona genes is restricted to oocytes where their protein products are posttranslationally modified and secreted to form an extracellular matrix. This matrix is critical for in vivo fertilization and pre-implantation development. Mice with a single genetically disrupted zona gene lack a zona pellucida matrix and are infertile (Liu et al., 1996; Rankin et al., 1996). We have presented data that is consistent with a model in which a tissue-specific basic helix-loop-helix (bHLH) transcription factor, FIG\textgreek{a}, forms a heterodimer with E12, an ubiquitous bHLH protein, and binds to a conserved E-box (CANNTG) upstream of the transcription start site of the three mouse zona genes. While FIG\textgreek{a} is not sufficient to activate endogenous zona gene expression in 10T\textsubscript{1} embryonic fibroblasts, our model suggests that it plays an important role, presumably in conjunction with other factors, in coordinating the oocyte-specific expression of mouse Zp1, Zp2 and Zp3. E-boxes with similar biological activity in vitro are comparably located in the promoters of the three human zona genes, raising the possibility that the role of FIG\textgreek{a} in coordinating the expression of the zona pellucida genes may be conserved among mammals (Millar et al., 1991; Liang and Dean, 1993; Epifano et al., 1995a).

The observed intensity of the FIG\textgreek{a}/E12 complex binding to the E-box in the three zona promoters varies as does the
observed transactivation of reporter gene constructs in heterologous 10T½ cells. The 1.5 kb Zp2 promoter which has 20-fold transactivation, also has the strongest signal in gel mobility shift assays whereas the Zp3 promoter with 9-fold transactivation has the weakest. Transactivation of the Zp1 promoter and FIGα/E12 binding to its E-box lie between these levels, albeit closer to that of Zp3 than Zp2. Each zona promoter has multiple (7-14) E-boxes, but the transactivation of the two Zp1, Zp2 promoters is dependent on the canonical CANNTG E-box sequence at −218 bp and −216 bp, respectively, relative to the transcription start sites. Transactivation in 10T½ cells with the Zp3 promoter persists even if the E-box located at −181 bp is mutated. This observation is in contrast to the required integrity of this E-box for reporter gene activity of a shorter Zp3 promoter (0.5 kb) microinjected into the nucleus of growing mouse oocytes (Millar et al., 1991). When the shorter promoter construct was co-transfected into 10T½ embryonic fibroblast cells with E12, transactivation comparable to that observed with the 1.6 kb promoter (9-fold) was observed and also was not dependent on the integrity of the E-box at −181 bp (data not shown). In the shorter Zp3 promoter, there are only two additional E-boxes, located −146 bp and −55 bp to the transcription start site of Zp3. It is possible that either one of these E-boxes serves as a binding site for FIGα/E12 in the heterologous 10T½ embryonic fibroblast cells whereas the E-box at −181 is of critical importance in oocytes, the tissue in which the endogenous zona genes are active.

The appearance of the extracellular zona pellucida matrix is a biochemical marker of oocyte growth within the ovary and transcription factors which regulate zona gene expression could serve as earlier markers of which oocytes will grow and which will remain quiescent. FIGα mRNA is most concentrated in resting oocytes and although its concentration decreases as oocytes grow, it persists in mid-sized oocytes (Fig. 6). In contrast, individual zona transcripts are either absent or present in low copy number in resting oocytes but then become increasing abundant as oocytes grow to mid-size (Epifano et al., 1995b). This temporal-spatial correlation is consistent with FIGα having an upstream hierarchical role in the oocyte-specific expression of the zona pellucida genes. However, the presence of FIGα in all resting oocytes, indicates that while it may be an enabling factor, it is not the sole arbiter of which oocytes will grow and express zona genes.

Several lines of evidence suggest that additional transcription factors (Molkentin and Olson, 1996) or tissue-specific modifications of pre-existing factors (Karlin, 1994; Sloan et al., 1996) are required for the activation of the zona pellucida genes. First, the observed 1:4:4 stoichiometry of ZP1, ZP2 and ZP3 transcripts, respectively, present throughout oogenesis (Epifano et al., 1995b) is not paralleled by the intensity of FIGα/E12 binding to individual zona promoters nor to the relative ability of the Zp1, Zp2, and Zp3 promoters to be transactivated by the two transcription factors in heterologous cells. Second, although FIGα mRNA and FIGα/E12 complexes are detected in the testis (albeit in small amounts), no zona gene expression has been observed in the male gonad (Ringette et al., 1986; Liang et al., 1990; Epifano et al., 1995b). Third, co-transfection of E12 and FIGα expression plasmids into 10T½ fibroblast cells does not result in the expression of endogenous Zp2. If additional transcription factors are needed, they need not be tissue specific, but could be expressed in oocytes (e.g. GATA binding proteins in vertebrates, Schickler et al., 1992; Partington et al., 1997) as well as in other tissues as reported for dMyc and dMax in Drosophila melanogaster (Gallant et al., 1996). If some of these proteins form a FIGα-like subfamily of bHLH proteins with conserved domains, it may be possible to use this conservation to identify other transcription factors in this presumptive cascade. Having FIGα as an upstream molecular

![Fig. 6.](image-url) Detection of FIGα by in situ hybridization of mouse ovaries. 32P-labeled FIGα probes were hybridized to formaldehyde-fixed, paraffin embedded ovarian sections from 12-day old mice. Washed sections were exposed for 21 days prior to photography. Dark-field (A,B,C,D) and bright-field (B,D,F) photomicrographs were obtained for sections hybridized with anti-sense (A,B,E,F) and sense (C,D) probes. FIGα transcripts were detected in oocytes within growing follicles throughout the ovary but were particularly concentrated in the small primordial oocytes in the periphery and in the hilar region (arrow). Grain density over the granulosa cells surrounding oocytes, was no greater than background. Scale bar, 100 μm (A,B,C,D); 50 μm (E,F).
able to substitute for E12/E47 and the derivation of mice mutant at these loci (Zhuang et al., 1996) will allow this hypothesis to be tested in oogenesis. Alternatively, because of the critical importance of the zona pellucida to fertilization and early embryonic survival, it may be that additional, redundant strategies have evolved to ensure the integrity of the pathways that lead to the coordinate, oocyte-specific activation of the zona pellucida genes.

We appreciate the gifts of cDNAs encoding E12 and E47 from Dr C. Murre; the FLAG-fusion protein expression plasmid pAR(ΔRI)59/60 from Dr M. Blanar; and the recombinant myoD/E12 proteins, creatine kinase E-box oligonucleotides and the myoD-specific antisera from Dr B. Paterson. We are grateful to Drs P. Castle and J. Berzofsky for consultations on peptide selection and immunization procedures and very much appreciate the critical reading of the manuscripts by Dr B. Paterson.

REFERENCES


(Accepted 23 September 1997)