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An upstream region of the mouse ZP3 gene directs expression of firefly luciferase specifically to growing oocytes in transgenic mice

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ABSTRACT **The gene encoding the mouse egg primary receptor for sperm, a zona pellucida glycoprotein called ZP3, is expressed exclusively in growing oocytes within ovaries of sexually immature and mature female mice. We have constructed a transgene in which 6.5 kilobases of ZP3 gene 5′-flanking sequence is fused to the coding region of the firefly luciferase gene, and we have generated four independent lines of transgenic mice. In these animals, the transgene is expressed exclusively in ovaries. Furthermore, within ovaries, expression is confined to growing oocytes, and luciferase activity can be detected by assaying individual, isolated oocytes. The pattern of firefly luciferase expression during oocyte growth is similar to that observed in previous studies of ZP3 expression during oogenesis in mice. Observations reported here strongly suggest that cis-acting elements present in the ZP3 gene 5′-flanking region regulate oocyte-specific and, therefore, sex-specific expression of the sperm receptor gene during mouse development. They also suggest that such elements can be used to direct expression of cloned genes specifically to oocytes of transgenic mice and to evaluate the effects of such expression on various aspects of early mammalian development.**

The mouse egg extracellular coat, or zona pellucida, is composed of three glycoproteins—ZP1, ZP2, and ZP3—that are synthesized and secreted continuously by growing oocytes (1, 2). One of the glycoproteins, ZP3, serves as both primary sperm receptor and acrosome reaction-inducer during initial stages of the fertilization process (2–5). The ZP3 gene has been cloned and characterized, and molecular probes were used to examine its expression during mouse development (6). Such studies have revealed that, indeed, the ZP3 gene is expressed only in growing oocytes, in no other cell type, and that ZP3 transcripts accumulate in oocytes to unusually high steady-state levels. Therefore, ZP3 is an example of oocyte-specific and, consequently, sex-specific gene expression in mammals.

Experiments described here were performed to begin to identify cis-acting DNA elements that restrict expression of the ZP3 gene to oocytes. We report that a 6.5-kilobase (kb) fragment of the mouse ZP3 gene 5′-flanking region directs expression of a foreign gene, that encoding firefly luciferase, exclusively to oocytes of transgenic female mice. Furthermore, the developmental time course for appearance of luciferase activity in oocytes of transgenic mice is consistent with the previously determined time course for ZP3 gene expression in oocytes during mouse development. These results strongly suggest that the mouse ZP3 gene 5′-flanking region contains cis-acting elements that are responsible for regulation of oocyte-specific gene expression.

MATERIALS AND METHODS

Construction of Plasmid pZP3/6.5-LUC. The plasmid designated pGEM-G4-HB, encompassing −0.85 to −6.5 kb of ZP3 5′-flanking sequence, was digested with *Hin*dIII and cloned into the *Hin*dIII site of plasmid vector pGEM-7Zf$^+$ (Promega Biotec). The resulting plasmid was digested with *Sal* I and *Bam*HI, restriction fragments were separated on agarose gels, and the largest fragment, containing ≈4.5 kb of ZP3 5′-flanking sequence and the vector, was ligated to a *Sal* I/*Bam*HI fragment isolated from plasmid pGEM4-ZP36.5, representing −0.47 to −2 kb of ZP3 5′-flanking sequence. The resulting plasmid, designated pGEM7-ZP3d0.47, encompassed −0.47 to −6.5 kb of ZP3 5′-flanking sequence. pGEM7-ZP3d0.47 was linearized with *Bam*HI and ligated to the *Bam*HI fragment of pZP30.47-LUC, which contained −0.47 to +0.01 kb of the ZP3 gene linked to a 2572-base-pair (bp) fragment (pJD206) (7) containing the coding region of the firefly luciferase gene and the simian virus 40 splicing and polyadenylylation signals. The resulting plasmid, designated pZP3/6.5-LUC contained 6.5 kb of ZP3 5′-flanking sequence and nucleotides (nt) 1–10 of ZP3 mRNA (noncoding) linked to firefly luciferase (see Fig. 1). This plasmid was digested with *Aat* II and *Bst*XI to excise vector sequence, fractionated on agarose gels, and the ZP3/luciferase fragment was recovered by electroelution. DNA was extracted with phenol/chloroform, precipitated with ethanol, resuspended at 1 μg/ml in injection buffer (5 mM Tris·HCl, pH 7.4/5 mM NaCl/1 mM EDTA), and stored at −20°C.

Transfer of DNA into Fertilized Eggs. The purified DNA fragment described above (ZP3/luciferase) was microinjected into the male pronucleus of fertilized mouse [(C57BL/6J × DBA/2)F_2; The Jackson Laboratory] eggs. Microinjected eggs were then transferred into oviducts of CD-1 (Charles River Breeding Laboratories) foster mothers according to published procedures (8).

Characterization of Transgenic Mice. Identification of transgenic founders was carried out by polymerase chain reaction (PCR) analysis. A small piece of mouse tail was excised and digested in 400 μl of a buffer containing 50 mM Tris·HCl (pH 8.3), 100 mM NaCl, 5 mM EDTA, 1% SDS, and 300 μg of proteinase K for 5 hr at 55°C. Samples were extracted with phenol/chloroform, and nucleic acid was precipitated with ethanol and resuspended in 500 μl of water. Two oligonucleotide primers each were used for PCR analysis of the firefly luciferase gene (5′-CCGGGCGCGGTCGGTAAAG-3′ and 5′-CGGCGGCGGGAAGTTCACCGGCG-3′) and, as an internal control, the endogenous ZP3 gene (5′-CAGCTCTACATCACCTGCCA-3′ and 5′-CACTGGGAAGAGACACTCAG-3′). These primers amplify 359- and 511-bp segments of the luciferase and ZP3 genes, respectively. Two microliters of tail DNA was added to 18 μl of PCR mixture [50 mM KCl/10 mM Tris·HCl, pH 8.4/1.5 mM $MgCl_2$/gelatin (20 μg/ml)/0.2 mM NTPs (Pharmacia)/0.6 unit of *Thermus aquaticus* DNA polymerase (*Taq* polymerase) (Perkin–Elmer/Cetus)/200 ng of each oligonucleotide primer]. Samples were overlaid with 35 μl of mineral oil and reactions were run in a thermal cycler (M-J Research). PCR

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Abbreviations: nt, nucleotide(s); PCR, polymerase chain reaction; LU, light unit(s); ZP, zona pellucida.

conditions used were initial denaturation, 2 min at 94°C and amplification, 30 cycles of 90 sec at 55°C, 1 min at 72°C, and 1 min at 94°C. Upon completion of the reaction, a 10-μl sample was subjected to electrophoresis on 2% agarose gels and DNA bands were visualized under UV light. Positive founders were mated to generate transgenic pedigrees.

Luciferase Assays in Tissues and Oocytes. Organs were excised from mice and either homogenized or sonicated in a buffer containing 25 mM glycylglycine (pH 7.8), 15 mM magnesium sulfate, 1 mM dithiothreitol, and 1% Triton X-100. Extracts were centrifuged for 10 min at 13,000 × *g* and supernatants were stored at −70°C. Growing and fully grown oocytes (and follicles) were isolated essentially as described (9) and stored at −80°C in 50 μl of homogenization buffer. Fifty microliters of tissue and cell extracts was added to 300 μl of luciferase reaction buffer containing 25 mM glycylglycine (pH 7.8), 15 mM magnesium sulfate, 5 mM ATP (pH 7), 100 μg of bovine serum albumin per ml, and 1 mM dithiothreitol and was assayed in the presence of 1 mM luciferin (Analytical Luminescence). Light emissions were integrated for the initial 10 sec of emission at 25°C in a luminometer (Monolight 2001, Analytical Luminescence). Data were standardized by using aliquots of purified firefly luciferase (Sigma) and expressed as light units (LU), with ≈2278 LU equivalent to 1 pg of luciferase.

***In Situ* Hybridization.** *In situ* hybridizations were performed essentially as described (10). Briefly, ovarian paraffin sections (≈4 μm) were dewaxed, hydrated, and immersed in Pronase (20 μg/ml) for 5 min. Samples were acetylated in 0.1 M triethanolamine, pH 8/0.25% acetic anhydride for 10 min, dehydrated, and hybridized immediately. Radiolabeled (^{35}S) sense and antisense luciferase RNA probes were prepared by using plasmid pGEM3-Dluc [contains a 398-bp insert (positions 710–1107) of the luciferase coding region] and SP6 or T7 RNA polymerase, respectively. Probes were added to hybridization solution [0.48 M NaCl/8 mM Tris·HCl, pH 7.5/1.6 mM EDTA/0.8× Denhardt's solution (1× Denhardt's solution = 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone)/yeast tRNA (0.4 mg/ml)/poly(A) (100 μg/ml)/20% dextran sulfate] at a final concentration of 0.2 $\mu g \cdot ml^{-1} \cdot kb^{-1}$. Hybridizations were carried out at 42°C for 3 hr. Slides were washed twice for 15 min each at 50°C in 50% formamide/2× SSC/0.1% 2-mercaptoethanol and once for 30 min at 37°C in RNase A (20 μg/ml)/0.5 M NaCl/10 mM Tris·HCl, pH 8. Finally, slides were washed twice for 15 min each at 50°C in 50% formamide/2× SSC/0.1% 2-mercaptoethanol, followed by 0.1× SSC/1% 2-mercaptoethanol. Slides were then dehydrated, air dried, and subjected to autoradiography with Kodak NTB2 emulsion.

RESULTS

Generation of Transgenic Animals and Pedigrees. A total of 328 fertilized mouse eggs were microinjected with the ZP3/luciferase transgene and were transplanted into oviducts of pseudopregnant CD-1 mice. As a result, a total of 51 mice were born. PCR analysis of tail DNA was used to assess incorporation of the ZP3/luciferase transgene into the mouse genome. Four of the 51 newborn animals exhibited a DNA band on agarose gels that corresponded to amplification of a 359-bp segment of the luciferase gene (Fig. 1). In addition to this 359-bp band, these samples also contained a DNA band that corresponded to amplification of a 511-bp segment of the endogenous ZP3 gene ("internal control"; see *Materials and Methods*). The relative degrees of amplification of the 359-bp segment correlated directly with transgene copy number, with transgenic founder animals 51 and 2 bearing the highest and lowest number of copies, respectively (data not shown). It should be noted that analysis of the progeny of founder 42 (Fig. 1; designated as transgenic line 42p), as well as progeny

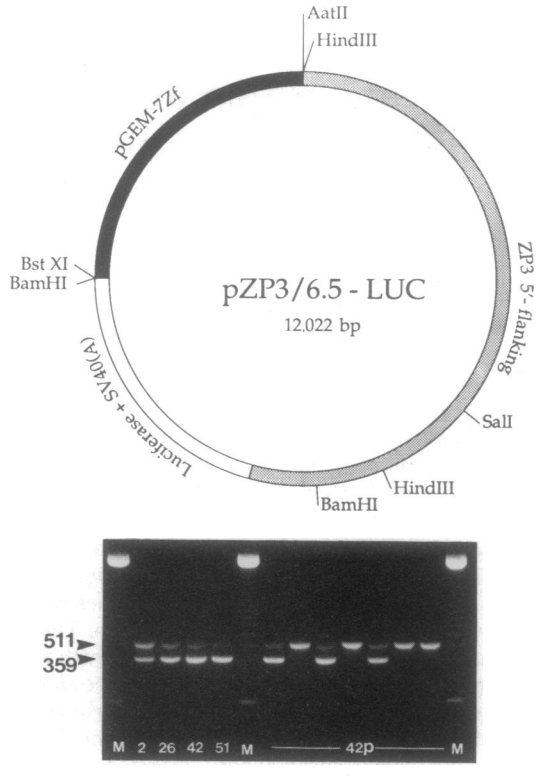

FIG. 1. Schematic diagram of plasmid pZP3/6.5-LUC and PCR analysis of transgenic mice harboring ZP3/luciferase. (*Upper*) Schematic diagram of plasmid pZP3/6.5-LUC. The 6.5-kb ZP3 gene 5′-flanking region is indicated by the stippled region; the firefly luciferase sequences, simian virus 40 (SV40) polyadenylylation signal, and SV40 splicing site are indicated by the open region; the pGEM-7Zf vector backbone is indicated by the solid region. Restriction enzymes and restriction sites used in constructing the plasmid and in isolation of the transgene ZP3/luciferase are indicated. (*Lower*) PCR analysis of tail DNA. PCR analysis was performed on tail DNA from founder mice carrying the transgene (lines 2, 26, 42, and 51) and from seven progeny of transgenic line 42p. Shown is an agarose gel on which amplified DNA was fractionated. The 511-bp amplified DNA fragment represents the endogenous ZP3 gene, whereas the 359-bp amplified DNA fragment represents the ZP3/luciferase transgene. Lanes M contained DNA fragments of a 123-bp DNA ladder (BRL).

from each of the other three transgenic lines (2p, 26p, and 51p), indicated that the pattern of amplification was reproducible. Transgenic lines, or pedigrees, were established from all four positive animals and >300 progeny were analyzed. Founder animals 2, 42, and 51 distributed the transgene to 60%, 44%, and 42% of their progeny, respectively. Founder animal 26 distributed the transgene to only 14% of its progeny, suggesting mosaicism in its germ cells. The mosaicism was corrected by mating some male progeny to nontransgenic females.

Analysis of Luciferase Activity in Mouse Tissues. Various tissues were excised from progeny of the four transgenic lines, as well as from nontransgenic animals of the same ages, and were assayed for luciferase activity (see *Materials and Methods*). As shown in Table 1, all four transgenic lines exhibited very high levels of luciferase activity in ovaries ($2.15–5.20 \times 10^5$ LU; 95–228 pg of luciferase) excised from 15-day-old animals. On the other hand, all other tissues exhibited only background levels of luciferase activity (50–92 LU), compared with tissues excised from nontransgenic animals (50–75 LU) of the same age. On average, ovaries excised from 15-day-old transgenic animals exhibited ≈6400 times more luciferase activity (3.98×10^5 LU; 175 pg of luciferase) than other tissues (62 LU). It should be noted that

Table 1. Luciferase activity in transgenic mouse tissues

Tissue	Mean luciferase activity, LU (range; no. of lines)
Adrenal	56 (53–61; 3)
Brain	92 (51–169; 4)
Epididymis	66 (40–103; 4)
Gut	53 (51–54; 4)
Heart	56 (47–75; 4)
Kidney	51 (48–52; 4)
Liver	65 (48–85; 4)
Lung	54 (47–59; 4)
Ovary	3.98×10^5 ($2.15–5.20 \times 10^5$; 4)
Spleen	50 (46–58; 4)
Testis	89 (61–141; 4)
Thymus	54 (—; 1)

Aliquots of transgenic mouse tissue homogenates were assayed for luciferase activity. With the exception of adrenal and thymus, tissues were examined for progeny from all four transgenic lines (2p, 26p, 42p, and 51p). The data were corrected for background (homogenization buffer only) values (mean, 110 LU) and for small variations from one experiment to the next by construction of a standard curve for each experiment by using different concentrations of purified luciferase (1 pg of luciferase equals ≈2278 LU). The mean values reported are for total tissue homogenates (e.g., 3.98×10^5 LU per ovary vs. 89 LU per testis) averaged for all transgenic lines tested. Known amounts of purified luciferase were added to control tissue samples to determine percentage recovery (see *Results*). The data for ovaries were obtained with transgenic animals 14–16 days old.

recovery of luciferase activity in control samples was estimated to be 75–100% for all tissues examined, with the exception of gut (30%); these values are consistent with previous reports (11). These results strongly suggest that expression of the reporter gene luciferase is directed specifically to the ovary in all four lines of transgenic animals described here.

Analysis of Luciferase Activity in Mouse Ovaries. During the first 21 days or so after the birth of female mice (sexually immature animals), an unusually large number of oocytes begin to grow and, concomitantly, to lay down a zona pellucida (12, 13). The diameter of growing oocytes isolated from excised ovaries is directly related to the age of the donor mice during days 3–21 postpartum (14). The ZP3 gene is expressed in oocytes at very high levels during their growth phase, with ZP3 mRNA reaching a steady-state level of nearly 250,000 copies in each fully grown oocyte (6, 15). Accordingly, we examined the levels of luciferase activity in ovaries excised from transgenic animals of different ages.

Overall, ovarian luciferase activity increased substantially in transgenic mice harboring ZP3/luciferase during the first 21 days or so postpartum (Fig. 2). During this period, growing oocytes increase from about 15 to 80 μm in diameter (12, 14). As expected, the four transgenic lines exhibited quantitative differences (as much as 5-fold) in levels of ovarian luciferase activity. This could be due to one or more of a number of factors that can affect expression of transgenes, including differences in copy number, integration sites, and/or methylation (16). However, in this context, it should be noted that the lowest levels of luciferase activity were found in line 42p, which, together with line 51p, harbors the highest number of copies of the transgene. Ovarian luciferase activity decreased significantly in older animals (Fig. 2), apparently reflecting the much smaller number of growing oocytes in sexually mature (>21 days old), compared with immature animals (see below). These results are entirely consistent with the pattern of ZP3 expression observed during mouse development (15, 17).

Analysis of Luciferase Activity in Isolated Mouse Oocytes and Follicles. To determine the site of luciferase activity within ovaries of transgenic mice harboring ZP3/luciferase, individual oocytes and oocyte-free ("empty") follicles were isolated and assayed for luciferase activity (see *Materials and Methods*). Empty follicles isolated from all four transgenic lines exhibited only background levels of luciferase activity (mean, 99 ± 42 LU; range, 45–187 LU), where background was taken as luciferase activity in individual oocytes isolated from nontransgenic mice (mean, 99 ± 17 LU; range, 83–116 LU), regardless of the age of the donor mice (14–40 days postpartum). On the other hand, individual oocytes isolated from transgenic mice exhibited luciferase activity significantly above background levels (830 ± 190 to 5102 ± 160 LU; 0.36–2.2 pg of luciferase, respectively) and, like ovarian extracts (Figs. 2 and 3), the amount of activity per oocyte increased with the age of the donor mice (i.e., with increasing oocyte diameter) during the first 21 days or so postpartum (Fig. 3). These results strongly suggest that luciferase activity is localized to growing oocytes, not to follicle cells, within the ovaries of transgenic mice and they account for the increasing

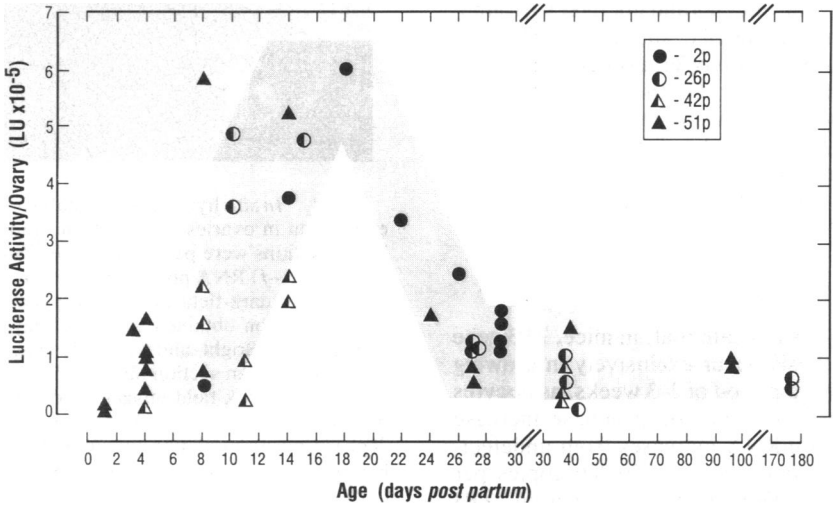

FIG. 2. Analysis of luciferase activity in ovaries excised from transgenic female mice of different ages. Ovaries were excised from progeny of transgenic lines 2p, 26p, 42p, and 51p at various times postpartum and were assayed for luciferase activity. Shown are luciferase activities for individual ovaries (LU × 10^{-5}) plotted as a function of age of the mice (1–177 days postpartum). The data were corrected as described in the legend to Table 1. Overall, 33 transgenic mice were used to collect these data and, in some cases, both ovaries of individual mice were assayed. Shading is used to indicate the general pattern of the data.

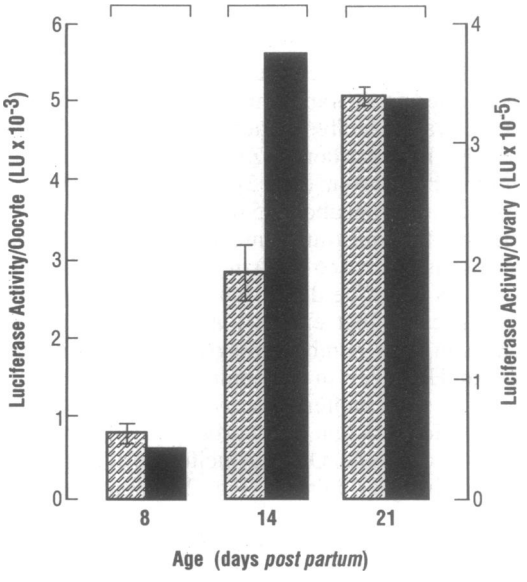

FIG. 3. Analysis of luciferase activity in individual oocytes isolated from ovaries excised from transgenic female mice of different ages. Individual growing oocytes were isolated and assayed for luciferase activity. Progeny from transgenic line 2p were used for all measurements reported and at least 15 oocytes were assayed individually for each of the three age groups—8, 14, and 21 days postpartum. Shown is the average luciferase activity for individual oocytes (LU × 10^{-3}; hatched bar) and for the entire contralateral ovary (LU × 10^{-5}; solid bar) excised from the same mouse from which oocytes were recovered. The data were corrected as described in the legend to Table 1 and the standard deviation of the mean is indicated for oocyte measurements.

levels of luciferase activity found in ovaries as transgenic animals increase in age during the first 21 days postpartum.

***In Situ* Hybridization Analysis of Luciferase Expression.** Results described above strongly suggest that the 6.5-kb upstream region of the ZP3 gene directs expression of the luciferase gene specifically to growing oocytes, not to follicle cells. To provide additional support for this conclusion, *in situ* hybridization analyses were carried out with ^{35}S-labeled RNA probes directed against luciferase mRNA and ovarian sections from transgenic mice harboring ZP3/luciferase (see *Materials and Methods*). As shown in Fig. 4, silver grains were concentrated in growing oocytes and not over surrounding follicle cells when a radiolabeled antisense probe was used. In general, the number of silver grains per oocyte was directly related to oocyte diameter. On the other hand, only background levels of silver grains were observed on comparable ovarian sections when a radiolabeled sense probe was used (Fig. 4). These *in situ* results are entirely consistent with the ovarian and isolated oocyte luciferase activity measurements described above.

DISCUSSION

Results of previous analyses indicate that, in mice, ZP3 gene expression and ZP3 synthesis occur exclusively in growing oocytes (15, 17–21). During a period of 2–3 weeks, as oocytes arrested in dictyate of the first meiotic prophase increase ≈300-fold in volume, ZP3 mRNA increases from undetectable levels in nongrowing oocytes to ≈250,000 copies per fully grown oocyte (6, 15). During this same period, ZP3 synthesis increases in growing oocytes from undetectable levels to as much as 2–3% of total protein synthesis (1, 22). ZP3 gene expression and ZP3 synthesis cease when fully grown oocytes resume meiotic progression, are ovulated, and become unfertilized eggs. Thus, ZP3 is currently the only well-documented example of oocyte-specific gene expression in mammals.

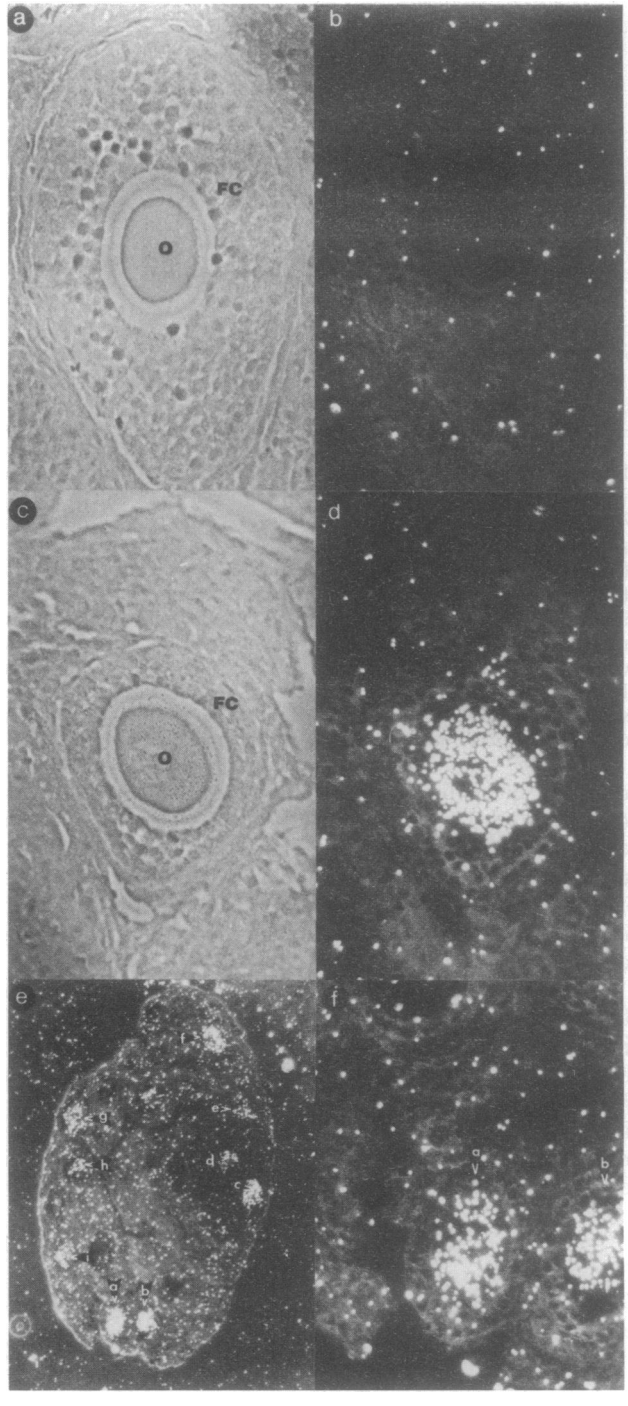

FIG. 4. *In situ* hybridization analysis of the site of luciferase gene expression in ovaries excised from transgenic female mice. *In situ* hybridizations were performed with ^{35}S-labeled sense (*a* and *b*) and antisense (*c–f*) RNA probes specific for luciferase mRNA. (*a* and *b*) Bright- and dark-field images, respectively, of a follicle within an ovarian section obtained from a 27-day-old mouse, transgenic line 51p. (*c* and *d*) Bright- and dark-field images, respectively, of a follicle within an ovarian section obtained from the same mouse as in *a* and *b*. (*e* and *f*) Dark-field images at low and high magnification of an ovarian section obtained from a 13-day-old mouse, transgenic line 26p. The positions of nine oocytes (labeled a–i) are indicated in *e*, and two of these oocytes (a and b) are shown at higher magnification in *f*. FC, follicle cells; O, oocyte. (*a–d* and *f*, ×160; *e*, ×60.)

Results reported here on the ZP3/luciferase transgene provide additional support for oocyte-specific expression of

the ZP3 gene and complement previous reports of germ cell-specific expression of transgenes in males (23–25). We have found that an upstream region of the mouse ZP3 gene directs expression of a reporter gene (that encoding firefly luciferase) specifically to growing oocytes of transgenic mice harboring the ZP3/luciferase construct. Overall, these findings are consistent with the generally held belief that cis-acting sequences, together with DNA-binding proteins, regulate tissue-specific expression of eukaryotic genes (26–29). Such a situation applies to unicellular as well as multicellular organisms. Various experimental approaches, including the use of transgenic animals (16, 30, 31), strongly support such a conclusion.

The ZP3 gene 5′-flanking region contains a potential TATA box located 29 nt upstream from the transcription start site, but no other canonical upstream promoter elements, such as a CCAAT box, are present (6, 32–34). However, the region does contain a 54-nt consensus sequence, present as six tandem repeats between −826 and −508 nt; four tandem repeats of the same sequence are present in the seventh intron (6, 32, 33). This may be relevant since tandem repeats have been reported to function in regulation of gene expression in a variety of systems (26, 35–37). In addition, the sequence TCACGT, located 183 nt 5′ of the ZP3 mRNA start site, is identical to the conserved "chorion box" sequence found in the 5′-flanking region of moth and fly chorion (eggshell) genes (38–40). The chorion box is considered to be an essential regulatory element for expression of chorion genes in the follicular epithelium during oogenesis in moths and flies. It is interesting to note that both the 54-nt consensus sequence and the TCACGT sequence are also present in the hamster ZP3 gene 5′-flanking region (45). It remains to be determined, by using other ZP3/luciferase constructs in transgenic mice, whether or not the 54-nt repeated sequences, TCACGT sequence, and/or other 5′-flanking sequences are involved in regulation of expression of the ZP3 gene.

It is estimated that each fully grown oocyte isolated from the transgenic mice (21 days old) described here contains ≈2.2 pg of firefly luciferase (Fig. 3). In this context, it has been estimated that each fully grown mouse oocyte contains >100 pg of actin, >250 pg of tubulin, and >200 pg of lactate dehydrogenase (22). Furthermore, during its growth phase, each mouse oocyte must synthesize >1 ng of ZP3 for assembly into the zona pellucida (1, 22). Although firefly luciferase was present in all growing oocytes isolated from the transgenic mice, when compared with the native proteins described above, the foreign protein, luciferase, accumulated to rather low steady-state levels during oocyte growth. It remains to be determined whether or not these low levels of luciferase per oocyte reflect relatively low rates of transcription and/or translation and/or relatively high rates of luciferase degradation during oocyte growth. In this context, for example, it has been reported that the presence of intervening sequences (absent from ZP3/luciferase transgene) improves the transcriptional efficiency of other transgenes by as much as 10- to 100-fold (41). Furthermore, it is possible that, as in the case of other eukaryotic genes (42–44), additional regulatory elements may be required for high level, copy-dependent expression of the ZP3/luciferase transgene.

In summary, we have presented evidence that oocyte-specific expression of the ZP3 gene, which encodes the mouse sperm receptor, is regulated by cis-acting DNA elements located within a 6.5-kb segment of the ZP3 gene 5′-flanking region. These studies also indicate that now it should be possible to direct expression of cloned genes specifically to oocytes of transgenic mice and to evaluate effects of such expression on both follicular development and early embryogenesis.

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