Germline regulatory element of Oct-4 specific for the totipotent cycle of embryonal cells

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SUMMARY

The totipotent stem cells of the pregastrulation mouse embryo which give rise to all embryonic somatic tissues and germ cells express Oct-4. The expression is downregulated during gastrulation and is thereafter only maintained in the germline lineage. Oct-4/lacZ transgenes were used to determine how this pattern of expression was achieved, and resulted in the identification of two separate regulatory elements. The distal element drives Oct-4 expression in preimplantation embryos, in migratory and postmigratory primordial germ cells but is inactive in cells of the epiblast. In cell lines this element is specifically active in embryonic stem and embryonic germ cells. The proximal element directs the epiblast-specific expression pattern, including downregulation during gastrulation; in cell lines its activity is restricted to epiblast-derived cells. Thus, Oct-4 expression in the germline is regulated separately from epiblast expression. This provides the first marker for the identification of totipotent cells in the embryo, and suggests that expression of Oct-4 in the totipotent cycle is dependent on a set of factors unique to the germline.

Key words: germline, totipotent cells, gastrulation, germ cells, POU, enhancer

INTRODUCTION

In higher organisms reproduction is achieved through the activity of cells in the germline. One of the important questions in biology has been how the germline is formed, maintained and delivered to the next generation. 110 years ago, August Weismann in his germ plasm theory postulated that the germline is defined by specific substances, which he termed ‘determinants’ (Weismann, 1885). According to his theory, determinants are directly transmitted from one generation to the next in order to propagate the germline. Weismann was referring to genetic material of the nucleoplasm when he wrote of the continuity of germ plasm. However, the investigation of germ plasm concentrated on visible cytoplasmic components which could only be detected in oocytes and primordial germ cells of certain organisms such as insects (reviewed by Beams and Kessel, 1974; Eddy, 1975). In some organisms the germline determinative properties of cytoplasmic components were demonstrated by transplantation experiments (Smith, 1966; Illmensee and Mahowald, 1974). So far, the nature of germline determinants has not been elucidated in any organism.

Despite its fundamental position in the life cycle of organisms, only little is known about the mammalian germline. Mammals lack distinctive visible components in the oocyte that could account for ‘determinants’, and attempts to define mammalian germline determinants have failed (Eddy et al., 1981). Actually, it seems unlikely that mammals contain germline determinants prelocalized in the oocyte, since individual blastomeres of the cleavage stage embryo retain their developmental totipotency (reviewed by Pedersen, 1986). Mammals may have a different mode of establishing the germline than through cytoplasmic determinants. The ‘inside-outside’ hypothesis for early mouse embryo differentiation suggests that different environmental conditions sensed by blastomeres at different positions play a decisive role in maintaining totipotency. According to this hypothesis only inner blastomeres of the preimplantation embryo retain totipotency (Tarkowski and Wroblewska, 1967).

Cells of the mammalian germline are the only ones to be incorporated into the next generation and that have the means to generate new genotypes. The developmental program of the germ cell lineage involves differentiation into two types of highly specialized cells, sperm and oocyte, the fusion of these two and the subsequent regeneration of germ cells. Thus, it represents the only developmental program with a cyclical nature.
Experiments with mouse chimeras have shown that the germ cells are derived from the epiblast (Gardner and Papaioannou, 1975). Although inner cell mass (ICM) cells of blastocysts cannot be distinguished according to their totipotency, only a small subset forms the germ line. Embryos infected with recombinant retroviruses during preimplantation development were analyzed for viral integration at later stages. At least three cells form the germ line and are set aside prior to somatic tissue allocation (Soriano and Jaenisch, 1986).

The murine germline first becomes visible at embryonic day 7, one-third of the way through gestation. Primordial germ cells (PGCs) are the earliest recognizable precursors of gametes and arise outside the gonads. During gastrulation PGCs form a cluster of about 100 cells within the midline extraembryonic mesoderm just posterior to the primitive streak (Ginsburg et al., 1990). Clonal analysis of germ cell progenitors suggests that allocation of PGCs to the germ cell lineage occurs around 7.2 days post coitum d.p.c. (Lawson and Hage, 1994). By as yet unknown mechanisms, PGCs disperse from the base of the allantois (8.0 d.p.c.) and migrate into the hindgut epithelium. Subsequently, PGCs emigrate from the hindgut (9.5 d.p.c.) and move along the dorsal mesentery (10.5 d.p.c.) until they reach the primordia of the gonads (11.5 d.p.c.). PGCs are identified by their large size and abundant levels of the enzyme tissue nonspecific alkaline phosphatase (TAP) (Chiquoine, 1954). However, due to embryonal alkaline phosphatase (EAP) staining in the epiblast, it is difficult to identify germ cells before 8 days of development (Hahnel, 1990).

The Oct-4 gene (also termed Oct-3 or Oct3/4) encodes the only known transcription factor which is likely to be involved in the establishment of the mammalian germline, and which could play a role in early germ cell specification. Oct-4 encodes a maternally expressed POU transcription factor that is present and active in the pregastrulation embryo and in the mammalian germline (reviewed by Schöler, 1991). In the developing mouse embryo Oct-4 is downregulated during the differentiation of the epiblast, eventually becoming confined to the germ cell lineage. Expression in the totipotent cycle suggests that Oct-4 is associated with the undifferentiated state of embryonal stem cells (Fig. 1A). The term totipotent cycle is used to stress that cells in the germline remain totipotent in the sense that they will contribute to a new organism once they go through the cycle.

Oct-4 is expressed in several embryonal cell lines, each of which represent cells of distinct developmental stages (Fig. 1A). Cultured cell lines often resemble particular cell types found in part of the embryonic anatomy at a certain developmental stage. However, the correspondence between cell lines and in vivo cell types is never complete. Rather it is based on the original derivation of the cell line and the overlap in the expression and regulation of as many marker genes as are available. Embryonic stem (ES), embryonal carcinoma (EC) and embryonic germ (EG) cells used in this report resemble cells found in the ICM of blastocysts, epiblast cells and primordial germ cells, respectively (reviewed by Robertson, 1987 and Hogan et al., 1994). ES cells are derived from blastocysts, can be grown in culture for many generations and contribute and embryonic germ (EG) cells used in this report resemble cells found in the ICM of blastocysts, epiblast cells and primordial germ cells, respectively (reviewed by Robertson, 1987 and Hogan et al., 1994). ES cells are derived from blastocysts, can be grown in culture for many generations and contribute and embryonic germ (EG) cells used in this report resemble cells found in the ICM of blastocysts, epiblast cells and primordial germ cells, respectively (reviewed by Robertson, 1987 and Hogan et al., 1994). ES cells are derived from blastocysts, can be grown in culture for many generations and contribute and embryonic germ (EG) cells used in this report resemble cells found in the ICM of blastocysts, epiblast cells and primordial germ cells, respectively (reviewed by Robertson, 1987 and Hogan et al., 1994). ES cells are derived from blastocysts, can be grown in culture for many generations and contribute.
GOF-6 was prepared by cloning the lacZ-containing HindIII fragment of Oct-4 (see above) into the HindIII site of the 5 kb BamHI fragment of genomic Oct-4 that has been subcloned in pBluescript KS (T7; 5’s to 3’s; T3). GOF-5 was derived from GOF-6 by deleting the 1.08 kb upstream SalI/AatII fragment by partial digestion, followed by religation in the presence of a linker with SalI/AatII overlap. The linker used for this purpose consisted of: 5’-TCGACGGACACTT-GACGT-3’ and 5’-CAGGTGTCCGC-3’. GOF-9 and GOF-13 were derived from GOF-12 and GOF-18, respectively, by taking the 5’side SalI/AatII fragments of the latter constructs to replace the corresponding 5’side SalI/AatII fragment in GOF-6. In this exchange cloning process, the DNAs had to be partially digested with AatII after a complete digestion with SalI.

GOF-18ΔPP, GOF-18ΔPE, GOF-18ΔDE

GOF-18 was further modified by introducing deletions in the promoter (ΔPP), in the proximal (ΔPE) and in the distal enhancer (ΔDE), respectively. To prepare GOF-18ΔPP and GOF-18ΔPE deletions were first introduced into the lacZ-containing HindIII fragment of Oct-4 that had been cloned as above into pBluescript KS. For ΔPP the above plasmid, a 236 bp BstEI/AvrII fragment was deleted, followed by filling-in with Klenow enzyme and religation. For ΔPE the plasmid was digested completely with BstEI and partially with BamHI to delete a 986 bp BamHI/BstII fragment. The appropriate fragment was isolated from an agarose gel after size fractionation, treated with Klenow enzyme, and then religated. Finally, GOF-18ΔPP and GOF-18ΔPE were constructed by replacing the 2.07 kb HindIII fragment in GOF-18 with the lacZ-containing HindIII fragment modified as above. GOF-18ΔDE was prepared from GOF-18 by partial digestion with BamHI to delete the 3.29 kb fragment.

Cloning of BamHI genomic Oct-4 fragments in front of TKlacZ

Upstream and downstream BamHI genomic Oct-4 fragments were isolated and cloned individually in both orientations into a unique BamHI site of TKlacZ (Schöler et al., 1989b). The orientation of the insert was determined by sequence analysis using a primer specific for the TK promoter.

Generation of transgenic animals and staining for β-galactosidase activity

All standard mouse techniques according to Wassarman and DePamphilis (1993) were used. The Oct-4lacZ constructs for generating transgenic animals were isolated as linear NotI/Sall vector-free fragments by fractionating the restriction enzyme-digested plasmid DNA on an agarose gel. After electrophoresis, the DNA solution was extracted three times with phenol, followed by extensive dialysis in the injection medium (10 mM Tris, pH 7.4, 0.2 mM EDTA). The DNA solution (1-2 ng/μl) was microinjected into the pronuclei of C57BL/6J x SJL/JF1 oocytes fertilized with sperm from BDF1 as described.

Transient transgenic embryos

Microinjected oocytes were allowed to develop in vitro. Embryos were cultured for 3 days in microdrops of CZB medium under oil at 37°C in a 5% CO2 incubator, and then stained for β-galactosidase activity, as described. Embryos resulting from such experiments usually consist of a mixed population representing various developmental stages of preimplantation due to temporal randomness of developmental arrest after the microinjection procedure.

Stable transgenic animals

Embryos derived from stable transgenic lines were dissected out at different days post-coitum, fixed and stained for β-galactosidase activity at 30°C. For clearing, the embryos were post-fixed in 4% paraformaldehyde, sequentially dehydrated in 25, 50, 70, 80 and 100% ethanol and then cleared with a solution consisting of 2 parts benzyl benzoate and 1 part benzyl alcohol (Merck), before being photographed.

Cell lines, transient transfection experiments and band shift assays

P19 and Rac65 cells were grown as described (Sylvester and Schöler, 1994). MBL-1 cells were maintained in the presence of murine leukaemia inhibitory factor (LIF; ESGRO™; 10³ units/ml) as described (Williams et al., 1988). The EG cells were derivatives of PGCs of different developmental stages, namely from 8.0, 8.5 or 12.0 day embryos. Isolation and culturing of EG cells on embryonic fibroblasts was done as described in Labosky et al. (1994b). To remove the feeder cells from the EG cell cultures for transfection the cells were trypsinized and replated on uncoated dishes. The cells were incubated for 20 minutes and then the supernatant was replated onto plates precoated with a 0.1% gelatin solution. This procedure removes about 90% of the feeder cells. Five hours after plating the EG cells were transfected by the calcium phosphate coprecipitation technique as described (Schöler et al., 1991). The EG cells were cultured during the transfection procedure with 2×10³ units/ml LIF. To harvest EG cells, the dishes were washed twice with cold PBS. Then 1 ml of cold PBS was added per each 6 cm dish. The dishes were carefully vortexed at low setting for 2 minutes. By this procedure, most of the EG cells detached from the dishes, whereas the feeder cells remained attached. Culturing of HeLa and 3T3 cells and transfection were performed as described (Schöler et al., 1991).

For transfections 10 μg of doubly CsCl-purified DNA was used for...
1.5-1.8×10^5 cells in 6 cm dishes (Sylvester and Schöler, 1994). RSV-
luciferase DNA (1 μg/dish) was included in each transfection as an
internal standard. Relative β-galactosidase activity was calculated by
normalizing the β-galactosidase activity to that of luciferase, both
assayed as described elsewhere (De Wet et al., 1987; Wassarman and
DePamphilis, 1993). In some experiments, the results obtained from
different cell lines were normalized further using the values for TK-
lacZ in each cell line. The band shift assay and incubation with Oct-
4-specific antibodies were described elsewhere (Palmieri et al., 1994).

RESULTS

Oct-4/lacZ transgenics reproduce the endogenous
pattern of the Oct-4 gene

The minimal genomic fragment that faithfully reproduces the
endogenous expression pattern of Oct-4 during mouse embry-
genesis was determined using reporter transgenes. Initial tests
showed that fusion proteins of Oct-4 and lacZ strongly reduce
β-galactosidase activity, even if only small regions of the
amino terminus of Oct-4 were present (data not shown). In
addition, approximately 5 times higher levels of β-galactosi-
dase activity were obtained in tissue culture experiments when
the SV40 polyadenylation signal was used rather than the Oct-
4 signal (data not shown). Therefore, the lacZ gene was
inserted immediately adjacent to the start codon of Oct-4 and
terminated by the SV40 polyadenylation signal (Fig. 1B).

Three fragments of different size, each harboring the lacZ
insertion, were microinjected into the pronuclei of fertilized
oocytes and stable transgenic mouse lines were generated. The
constructs are named GOF-32, GOF-18 and GOF-12, the
numbers referring to the size of the respective Genomic Oct-4
Fragment in kb (Fig. 1B). Analysis of the transgenic lines was
initially restricted to four stages: early blastocyst, day 6.5
epiblast, and day 8.5 and day 11.5 embryos. GOF-32 and GOF-

Fig. 2. Oct-4/lacZ transgene expression during early mouse development. Expression pattern of GOF-18 during pre- and early postimplantation
development in stable transgenic animals. Mouse lines were generated which contained the GOF-18 construct. Embryos at 3.5 days through 7.5
days post-coitum (d.p.c.), were stained for β-galactosidase activity: (A) 3.5 d.p.c., blastocyst; (B) 5.5 d.p.c., pre-streak; (C) 6.0 d.p.c., prestreak;
(D) 7 d.p.c., early streak; (E-H) 8.0 d.p.c. to 8.5 d.p.c., head-fold. (H) embryo proper, early head-fold, was prepared to show neural fold and
underlying mesoderm. Staging was according to Kaufman (1993) and Downs and Davies (1993). All stages shown were derived from one
transgenic line. The pattern of staining is identical to GOF-32 and to three different GOF-18 lines at all stages tested. HF, head-fold; AL,
allantois. The embryos shown in C-G were cleared after staining for β-galactosidase.
reproduced the endogenous pattern of Oct-4 (data not shown). Two independent GOF-32 transgenic lines and three independent GOF-18 lines were analyzed, each of which reproduced the Oct-4 pattern. In the two GOF-12 lines analyzed, the β-galactosidase signals at the epiblast stage appeared to be weaker. In addition, GOF-12 showed ectopic expression of β-galactosidase at later stages.

To obtain sufficient levels of β-galactosidase activity and to minimize ectopic expression, GOF-18 was used as the reference fragment in our further analyses. The initial analysis, which was restricted to four stages, was then extended in a detailed analysis of the GOF-18 transgene expression pattern. β-Galactosidase activity is found in oocytes and morulae (not shown) and is restricted to the ICM of the expanding blastocyst (Fig. 2A). Staining of the blastocysts of different transgenic lines often showed heterogeneity of staining in the ICM. The reason for this heterogeneity is not clear. In early postimplantation embryos, transgene expression could easily be detected in the epiblast with the highest levels at day 6.5-7 (Fig. 2B-D). No expression was found in hypoblast, trophoblast, allantois and other extraembryonic tissues. Transgene expression was rapidly downregulated during gastrulation, from anterior to posterior (Fig. 2E-H). Downregulation of the transgene was similar to the endogenous Oct-4 RNA downregulation, indicating that the stability of β-galactosidase during these stages is not affecting the expression pattern (Rosner et al., 1990; Schöler et al., 1990a).

Transgene expression was not detected after day 9 in uncleared embryos (data not shown). A weak transgenic signal was found in cleared 9 day embryos close to the posterior neuropore and in the hindgut area where the PGCs are located (Fig. 3A, arrowhead). Those cells that were blue in the bright-field image became red in the dark-field image of the same embryo (Fig. 3B). The increased sensitivity of dark-
The Oct-4 gene contains a transcriptional enhancer specifically active in MBL-1 ES cells. (A) Physical map of Oct-4 showing BamHI restriction fragments in the 18 kb (GOF-18) clone. Numbers in the boxes indicate the respective BamHI fragments cloned upstream of the TK minimal promoter. The size of the BamHI fragments 1-5 in kilo basepairs are: 4.18, 3.29, 1.39, 1.11, 3.01, respectively. Fragment 2 (hatched) contains the DE. (B) Enhancer mapping in MBL-1 cells. Abscissa indicates the respective BamHI restriction fragments in the 18 kb (GOF-18) clone. Numbers in the boxes indicate the respective BamHI fragments 2 (hatched) and 3 (white) in the TK promoter. RSV-luciferase was cotransfected as an internal standard to normalize -Galactosidase activity was almost at background levels in transgenic signal was localized to an increased number of +) and (−) represent the orientation of both fragments relative to the TK promoter. RSV-luciferase was cotransfected as an internal standard. Relative -galactosidase activity was calculated by dividing by the activity of TK-lacZ which was set at 1.

BamHI fragments

A

Oct-4

1

2 3

4 5

GOF-18

MBL-1 ES

2(+)

2(−)

3(+)

3(−)

TK

fragment

C

3(+) 3(−) TK

Molecules which were derived from P19 cells but lack the ability to differentiate in response to retinoic acid (Jones-Villeneuve et al. 1983). B and C show enhancer activity of fragments 2 and 3 in MBL-1 ES and P19 EC cells, respectively. Consistent with their role as enhancers both activate transcription in an orientation-independent manner. (+) and (−) represent the orientation of both fragments relative to the TK promoter. RSV-luciferase was cotransfected as an internal standard. Relative -galactosidase activity was calculated by dividing by the activity of TK-lacZ which was set at 1.

BamHI fragments

A

Oct-4

1

2 3

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GOF-18

MBL-1 ES

2(+)

2(−)

3(+) 3(−)

TK

fragment

C

3(+) 3(−) TK

P19 EC

2(+)

2(−)

3(+)

3(−)

TK

fragment

Fig. 4. The Oct-4 gene contains a transcriptional enhancer specifically active in MBL-1 ES cells. (A) Physical map of Oct-4 showing BamHI restriction fragments in the 18 kb (GOF-18) clone. Numbers in the boxes indicate the respective BamHI fragments cloned upstream of the TK minimal promoter. The size of the BamHI fragments 1-5 in kilo basepairs are: 4.18, 3.29, 1.39, 1.11, 3.01, respectively. Fragment 2 (hatched) contains the DE. (B) Enhancer mapping in MBL-1 cells. Abscissa indicates the BamHI fragments described in A. The fragments were cloned upstream of TK-lacZ. TK is TK-lacZ in which the HSV TK minimal promoter lacking upstream control sequences, is linked to lacZ (Schöler et al. 1989a). (C) Cell-type specificity of fragment 2. Activity of the fragment 2-TK plasmid in comparison to TK-lacZ was tested in several cell lines in transient transfection assays as indicated in the figure. Rac65 cells are derived from P19 cells but lack the ability to differentiate in response to retinoic acid (Jones-Villeneuve et al. 1983). B and C are transient transfection assays. RSV-luciferase was cotransfected as an internal standard to normalize -galactosidase activities. Relative -galactosidase activity was calculated by dividing by the activity of TK-lacZ which was set at 1. In C the increase of activity in each cell line was compared to the activity of TK.

Detected in the head folds. In addition, more positive spots in the hindgut area were visible (Fig. 3B, white arrowhead). The dark-field image of a stage-matched, non-transgenic embryo was an opaque white shadow (Fig. 3D), demonstrating that the red color in the transgene analysis resulted from -galactosidase.

- Galactosidase activity was almost at background levels in ectodermal and mesodermal derivatives after embryonic day 9 (Fig. 3E-G, dark-field images not shown). At day 9.25, the transgenic signal was localized to an increased number of -galactosidase-positive cells in the hindgut area (Fig. 3E, arrowhead). At subsequent stages the positive cells increased in number, were located at a more anterior position in the embryo and had a stronger -galactosidase signal (Fig. 3E,F). In 10.5-day embryos some of the positive cells appeared to have moved towards the genital ridges that are being formed (Fig. 3G, H). All positive cells were located in the genital ridges by day 11.5 (data not shown, see also Fig. 9E). The developing gonads of 12.5 d.p.c. female and male mice were distinguishable by the pattern of positive cells. The spotty...
An upstream enhancer specifically active in embryonic stem cells

The activity of GOF-18 subfragments was tested in embryonic stem cell lines to localize regulatory sequences within the 18 kb genomic fragment. Five BamHI fragments from either upstream or downstream of the coding region of Oct-4 were individually inserted upstream of TK-lacZ (Fig. 4A). Only fragment 2 increased transcription from the TK minimal promoter in MBL-1 ES cells (approx. 25 fold; Fig. 4B). The transcriptional enhancing activity of fragment 2 was also observed in E14 ES cells (not shown), indicating that it contains transcription activation sequences active in ES cells. Fragment 2 enhancer activity was then compared in various cell lines to assess possible cell-type specificity (Fig. 4C). Fragment 2 was inactive in terminally differentiated cell types such as NIH3T3 or HeLa. Interestingly, it was also inactive in EC cells such as P19 and Rac65, which are both known to express Oct-4 at high levels. Thus, fragment 2 of the Oct-4 gene contains an element which, in its activity, appears to be restricted to ES cells.

Reciprocal activity of two upstream enhancers in MBL-1 ES cells and P19 EC cells

The same five BamHI fragment reporters were tested in P19 EC cells. Only fragment 3, which did not stimulate transcription in ES cells (Fig. 4B), increased transcription from the TK minimal promoter (data not shown). Next, we compared the cell-type specificity of fragments 2 and 3 in both orientations in MBL-1 ES and P19 EC cells (Fig. 5). A strikingly reciprocal activity profile was observed, fragment 2 showing activity only in ES cells while fragment 3 activates transcription more strongly in EC cells. In ES cells fragment 3 stimulated TK very weakly, but reproducibly, ranging from 1.5 to 2 fold (Fig. 5B, compare 3(+), 3(−) and TK). In contrast, fragment 2 always reduced the levels of TK activity in P19 EC cells by 1.5 to 2.5 fold (Fig. 5C, compare 2(+), 2(−) and TK). The cell-type-specific manner with which fragments 2 and 3 activate transcription is consistent with stage-specific roles for both during embryogenesis. Since both fragments show similar activation in either orientation, they are considered to be enhancers, fragment 2 will be referred to as the distal enhancer (DE), fragment 3 as the proximal enhancer (PE). An enhancer which is active in P19 EC cells and is located in fragment 3 has previously been described (Okazawa et al., 1991).

The intact Oct-4 gene reporter (GOF-18) was expressed both in MBL-1 ES and P19 EC cells. The contribution of each enhancer to Oct-4 gene activity was investigated by introduc-
ing several deletions into the GOF-18 construct (Fig. 6). In transient transfection experiments, deletion of the downstream BamHI fragments 4 and 5 had no effect on gene activity in either ES or EC cells (Fig. 6, compare GOF-18 and GOF-13). GOF-18 was then compared with constructs containing progressive deletions of upstream sequences. Reporter expression in MBL-1 ES cells remained unaffected by deletion of the upstream BamHI fragment 1 (Fig. 6B; compare GOF-13 and GOF-9). However, further deletion of the 2.7 kb BamHI-HindIII fragment resulted in a 10-fold decline in activity, indicating that the DE in this fragment was required for expression of Oct-4 in ES cells (compare GOF-9 and GOF-6). In contrast, a two-step decrease in activity was observed in P19 EC cells indicating that two additional elements, located up- and downstream of the DE, are required for expression in EC cells (Fig. 6C; compare GOF-13 and GOF-9, and GOF-6 and GOF-5). The upstream EC cell-specific element (fragment 1) was not investigated further in this study. The downstream EC cell-specific activity is most likely due to the PE.

The contribution of three regulatory regions, namely the promoter, the PE and the DE, to GOF-18 activity was tested in transient transfection experiments by excising them from the GOF-18 construct (Fig. 6, right side). Deletion of about 230 bp of the promoter region abolished Oct-4 expression in both MBL-1 ES and in P19 EC cells (GOF-18ΔPP in Fig. 6B and C). Thus, the presence of additional promoter sequences within the GOF-18 fragment is unlikely. Deletion of the PE only reduces the gene activity in ES cells by about 25% (Fig. 6B, compare GOF-18 and GOF-18ΔPE). In contrast, deletion of the DE severely decreases GOF-18 reporter function in ES cells, indicating that the DE is the major transcriptional enhancer driving Oct-4 expression in ES cells (Fig. 6B, compare GOF-18 and GOF-18ΔDE). Deletion of the DE only marginally decreased reporter activity in P19 EC cells (Fig. 6C; compare GOF-18 and GOF-18ΔDE). Deletion of the PE resulted in a 2.5- to 3-fold reduction of GOF-18 activity, consistent with the idea that the PE is important for Oct-4 activity in P19 EC cells (Fig. 6C; compare GOF-18 and GOF-18ΔPE). However, DE still activates transcription to a considerable extent, suggesting that GOF-18 contains additional elements that stimulate expression in P19 EC cells. A likely location for such an element is fragment 1, whose deletion caused the first step-decrease described above (Fig. 6C; compare GOF-13 and GOF-9).

In summary, in ES or EC cells the same fragments increase TK and Oct-4 promoter activity. Analyses of the external and the internal deletions, as well as the analyses of the different Oct-4 fragments combined with a heterologous promoter provide mutually supportive results. Thus, the DE is ES cell-specific, whereas the PE is P19 EC cell-specific.

The distal enhancer is active in cells of the germ cell lineage

ES cells resemble cells of the ICM of blastocysts, whereas P19 EC cells have a number of features in common with cells of the epiblast (Fig. 1A; Robertson, 1987). Characterization of ES- and EC-cell-specific enhancers within the sequences sufficient for reproducing both the germline and epiblast expression patterns suggested that the DE and PE may act as stage-specific enhancers, required for preimplantation and postimplantation expression of Oct-4, respectively. A second possibility is that the DE functions as a lineage-specific enhancer, which is active in cells of a particular lineage throughout development.

It is not feasible to isolate sufficient numbers of PGCs to do transient transfection experiments. Therefore, expression of the Oct-4 gene was studied in EG cell lines which were established from PGCs at various times during development, namely days 8.0, 8.5 and 12.5 (Labosky et al., 1994a, b). The presence of Oct-4 protein in EG cells was shown in band shift assays (Fig. 7A). The binding pattern of Oct-1, Oct-4 and Oct-6 to the octamer motif has been described previously for ES and EC cells and is shown in lanes 1 to 3 (Schöler et al., 1989a; Suzuki et al., 1990; reviewed by Schöler, 1991). The binding profile with all three EG cell extracts was almost identical to that of ES and EC cells, indicating that all cell lines used in this study contain similar amounts of Oct-4. The profile differs only with respect to Oct-6 where a stronger band is obtained with the EG extracts (compare lanes 1-3 with 4-6). The presence of Oct-4 in the EG cell lines is confirmed by Oct-4-specific antibodies (compare lanes 4-6 and 8-10). The Oct-4 complex is specifically abolished, whereas Oct-1 and Oct-6 remain unaffected. The fibroblasts on which the EG cells are grown are included as a control (lane 7). These cells do not express Oct-4 and Oct-6 but do express Oct-1, which is found in all cell lines tested (Schöler, 1991 for review).

The set of constructs described in Fig. 5 was used to investigate the contribution of the DE and the PE to Oct-4 gene activity in each EG cell line (Fig. 7B-D, left side). Fragment 3 was inactive in this analysis, whereas fragment 2 activated transcription in all three EG cell lines. Therefore, as in ES cells, the DE but not the PE was active in EG cells. However, the levels of activation by the DE were about 5-fold lower in EG cells than in ES cells (compare Figs 5 and 7). The contribution of both enhancers to Oct-4 gene activity was further investigated by comparing GOF-18 with its deletion variants lacking the PE or the DE (Fig. 7B-D, right side). The activities of GOF-18 and GOF-18ΔPE were similar, indicating that the PE is dispensable for Oct-4 gene activity in EG cells. In contrast, deletion of the DE resulted in a 2.5- to 5-fold decrease of reporter activity. Thus, both sets of assays indicate that in EG cells, the DE is required for Oct-4 gene activity and that the PE is dispensable. These results are consistent with the idea that the DE acts as a lineage-specific rather than a stage-specific enhancer.

Preimplantation Oct-4 expression is DE dependent

Considering the cell-type specificities observed for the PE and the DE in transfection studies of ES, EC and EG cells, together with the embryonic origins of these lines it is possible that the DE is responsible for Oct-4 expression in blastocysts and the PE for expression in the epiblast. In order to verify this point, the contribution of both enhancers was directly tested during mouse development. lacZ expression from the transgenes containing the DE and the PE was clearly detectable in preimplantation embryos (Fig. 2A). However, at this stage of development the β-galactosidase activity in embryos of these transgenic lines was low irrespective of the content of the genomic sequences flanking the DE and the promoter of Oct-4.

To compare the activities of the Oct-4 transgenes in larger numbers of preimplantation embryos, an Oct-4/lacZ transient
transgenic assay was performed in at least 100 embryos per construct. For this the reporter constructs were microinjected into the pronuclei of fertilized oocytes and the embryos were cultured until most reached the blastocyst stage (Fig. 8B). Five β-galactosidase-positive preimplantation embryos, representative of the results with any of the functional constructs, are shown (Fig. 8C). Constructs lacking the DE but containing the PE exhibited no staining of morulae or blastocysts in this transient transgenic assay (GOF-6 in Fig. 8B, other constructs not shown), consistent with an essential role for the DE in the expression of Oct-4 in preimplantation embryos. When the DE

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**Fig. 7.** Enhancer function in embryonic germ cell lines. (A) Presence of Oct-1, Oct-4 and Oct-6 proteins in various embryonal cell extracts and fibroblasts (lane 7). Band-shift analysis of ES (lane 1), EC (lanes 2 and 3) and EG (lanes 4-6 and 8-10) extracts. Whole cell extracts (10 μg) were analyzed with a radiolabeled oligonucleotide probe containing a canonical octamer motif (Schöler et al. 1989b). In lanes 8-10, EG cell extracts were incubated with Oct-4-specific antibodies (Palmieri et al. 1994). (B-D) Embryonic germ cell lines, EG 8.0, EG 8.5 and EG 12, were transiently transfected with TK reporters containing BamHI restriction fragments 2 (hatched) and 3 (white) in both orientations (+ and −) in front of TK. The activity of the TK promoter is in black. (Right panels) The same embryonic germ cell lines transfected with either GOF-18, GOF-18ΔPE or GOF-18ΔDE. RSV-luciferase DNA was included in each transfection as an internal standard. Relative β-galactosidase activity was calculated with the activity of TK set to 1 (left panels) or the activity of GOF-18 set to 100 (right panels).

**Fig. 8.** Oct-4 expression in preimplantation embryos requires the distal enhancer. (A) Deletion constructs used in the transient transgenic analyses to define the contribution of the distal and proximal enhancer for Oct-4 gene activity in preimplantation embryos. Constructs are the same as in Fig. 3. (B) Quantitative analysis of the transcriptional enhancer activity of Oct-4 flanking sequences in transient transgenic preimplantation embryos. Embryos were injected with the constructs outlined in A. At least 100 blastocysts or morulae were investigated per construct. The percentage of lacZ-positive embryos is plotted for each construct. C shows a collection of four lacZ-positive blastocysts and one morula (upper left), representatives of positive embryos.
was present, strong Oct-4 transcription was observed (GOF-9, -13, -18). Deletion of the PE in constructs containing DE (GOF-18ΔPE) did not influence lacZ expression. Thus the PE is not required for Oct-4 expression in preimplantation embryos. In contrast to a previous report (Okazawa et al., 1991), genomic fragments containing only the PE were inactive in the blastocyst, even though several different PE-containing constructs were tested in at least 300 embryos. The transient transgenic results agreed with those obtained using ES cells (Fig. 6). Therefore, expression of Oct-4 in preimplantation embryos and ES cells is dependent on the DE but not on the PE.

**Proximal enhancer is required for epiblast expression**

The PE is active in EC cell lines which are derived from epiblast cells (Fig. 5), suggesting that it serves as an epiblast-specific enhancer. To test this idea directly, several stable GOF-6 transgenic lines were established and four were analyzed (data not shown). In postimplantation embryos the expression pattern was similar to that of GOF-18. The GOF-6 line showing the strongest expression was indistinguishable from GOF-18 during early postimplantation development up to about day 8.5 (Fig. 2B-H for GOF-18), suggesting that GOF-6 contains most of the elements required for normal expression in the epiblast and for downregulation of the transgene during gastrulation. However, in contrast to GOF-18 transgenic embryos, the levels of β-galactosidase activity in GOF-6 transgenics varied. In several GOF-6 lines the levels were lower than in GOF-18, and the transgenic signal was not detectable by day 8. Importantly, none of the transgenic lines transgenic for GOF-6 stained the PGCs after day 8.5, indicating that elements required for expression in PGCs are missing from this construct. These results also demonstrate that the DE was not necessary for Oct-4 expression in the epiblast. Transgenic lines carrying GOF-18ΔPE or GOF-5 did not show expression in the epiblast, indicating that the PE is required for in vivo Oct-4 expression in the epiblast (for GOF-18ΔPE see Fig. 9A, GOF-5 not shown). Therefore, the PE is a stage-specific enhancer active in the epiblast of mouse embryos. These data emphasize the cell-type similarity of P19 EC and epiblast cells.

**The distal enhancer functions in the germ cell lineage**

ES and EG cell lines are thought to resemble cells of the germline lineage. The cell-type specific function of the DE in these cell lines suggested that the DE is a germline-specific enhancer. This idea was investigated directly during the embryogenesis of transgenic mouse lines. Deletion of the epiblast-specific PE allowed germline expression to be separated from epiblast expression. GOF-18ΔPE expression could not be detected in the day 6.5 epiblast even in the sensitive dark-field image (Fig. 9A, dark-field image not shown).

In situ hybridization experiments have shown that endogenous Oct-4 RNA expression is not restricted to the germ cell lineage prior to day 8.75-9 (Schöler et al., 1990a). Allocation of cells to the germ cell lineage occurs at about embryonic day 7.2 (Lawson and Hage, 1994). Analysis of Oct-4 expression in 7.2 d.p.c. germ cells, either by in situ hybridization or by the GOF-18 transgene, was previously not reliable due to the strong signal from the epiblast. Ablation of epiblast expression by deletion of the PE allows us to determine if the DE can confer expression soon after the germ cell allocation. The GOF-18ΔPE transgene activity was carefully analyzed in 7.5-8.25 d.p.c. embryos. No activity was found in the dark-field image (data not shown). However, a small cluster of 35-40 red cells was detected in the dark-field image at the posterior end of the embryo (Fig. 9B).

At later stages (9.25-12.5 d.p.c.) transgene activity of GOF-18ΔPE could easily be demonstrated exclusively in the migrating and non-migrating germ cells (Fig. 9C-E). Staining at 9.25 d.p.c. (Fig. 9C) showed that only germ cells were stained. In contrast, GOF-18 transgenes showed staining in both epiblast-derived tissues and germ cells at this time (Fig. 3A-C). After downregulation of PE in the epiblast around 9.25 d.p.c., staining of GOF-18ΔPE lines is indistinguishable from that observed in lines bearing the complete GOF-18 transgene (compare Fig. 9E with Fig. 3C). Clearly germ cell expression could occur in the absence of the PE. The results are consistent with the idea that Oct-4 germ cell expression is DE-dependent.

The epiblast stage may represent the only pause in DEdriven Oct-4 expression. High resolution analyses will have to be undertaken to determine if Oct-4 expression commences at or before allocation of the germ cell lineage, or if expression is continuous throughout the 5.5 d.p.c. to 7.5 d.p.c. window.

**DISCUSSION**

In this study we showed that a proximal and a distal enhancer, PE and DE, respectively, activate Oct-4 expression in pluripotent and totipotent cells of the developing mouse embryo, respectively. The PE is specifically active in P19 EC cells and stimulates transgenic expression in the epiblast of mouse embryos. The DE, in contrast to PE, is active in embryonic stem and embryonic germ cell lines; in the mouse the DE specifically activates Oct-4 expression in the germline. We conclude that the PE is stage- and tissue-specific and that the DE is lineage-specific. Subsequently, the PE and the DE will be referred to as epiblast and germline enhancers, respectively. Our results show that both the epiblast enhancer and the germline enhancer are required to obtain the complete expression pattern of Oct-4.

**Enhancer switch during implantation**

The ICM of the preimplantation embryo expresses Oct-4. Expression was mediated by the germline enhancer and did not require the epiblast enhancer (Fig. 8). Shortly after implantation GOF-18 and GOF-6 expression are observed in the epiblast (Fig. 2B-D; data not shown). Upon deletion of the epiblast enhancer no expression was observed at this stage (Fig. 9A), indicating that early postimplantation expression is driven by the epiblast enhancer. Perhaps a very small population of cells still uses the germline enhancer at this stage but we cannot detect it (see below). Thus, Oct-4 expression is driven by the germline enhancer and the epiblast enhancer, before and after implantation of the blastocyst, respectively. Perhaps the interaction with the uterine wall or the ‘estrogenic surge’ at about 4.5 d.p.c., just before the time for implantation, triggers a switch in enhancer usage.
Downregulation in the epiblast

Oct-4 expression in the epiblast is turned off during gastrulation (Fig. 2). During the RA-mediated differentiation of P19 EC cells, the activity of the epiblast enhancer also decreases, eventually becoming inactive in all differentiated cells (data not shown; Okazawa et al., 1991). Downregulation of the epiblast enhancer by RA in EC cells may represent a close parallel to the downregulation in the epiblast in vivo. RA is a substance known to be present in Hensen’s node, a key organizer of gastrulation (Hogan, 1992). It also alters the Hox gene expression pattern in the gastrulating embryo and in EC cells (reviewed by Boncinelli, 1991).

It has not yet been shown that the epiblast enhancer alone is sufficient for directing the Oct-4 epiblast expression in vivo, including its downregulation. Therefore, it is possible that downregulation may alternatively depend on the activity of other elements in the Oct-4 gene. One such element which may take part in downregulation is the Oct-4 promoter (PP, see Fig. 6; Pikarsky et al., 1994; Schoorlemmer et al., 1994; Sylvester and Schöler, 1994; Ben-Shushan et al., 1995). The Oct-4 PP lacks a canonical TATA box, and a GC-rich box is the only element so far known to be required for Oct-4 gene expression.

The GC-rich box is a high-affinity Sp1 site, and a point mutation that abolishes Sp1 binding in band shift assays decreases gene activity more than 25-fold in different ES and EC cell lines when introduced into GOF-18 (Minucci et al., 1996). Based on cotransfection experiments it has been suggested that nuclear receptors interfere with binding of a cellular factor to the GC-rich box. This could occur by binding of nuclear receptors to a hormone responsive element that overlaps the Sp1 site. RARα and RARγ are expressed in P19 EC cells, whereas RARβ and the orphan nuclear receptors COUP-TF1, ARP-1 and EAR-1 are upregulated by RA during differentiation (Kruyt, 1991; Jonk, 1994). Any of these transcription factors could possibly mediate the downregulation of epiblast-specific Oct-4 expression.

Recent observations might argue against a direct role of nuclear receptors in the downregulation of Oct-4 (Minucci et al., 1996). In undifferentiated EC and ES cells, strong in vivo footprints were detected as revealed by protection and hypermethylation of specific sites in the PP, PE and DE. The footprint was promptly lost upon RA treatment in ES cells and in P19 EC cells, in parallel with the sharply reduced Oct-4 mRNA levels. Thus, the occupancy of regulatory elements is coupled with Oct-4 expression, and RA treatment causes coordinated factor displacement, leading to extinction of gene activity. However, RA treatment did not generate new footprints in the regions tested and thus in these experiments in vivo binding of nuclear factors to any of these sites could not be demonstrated.

Oct-4 expression in gastrulation

The GOF-18 transgenic lines recapitulated all of the previously reported aspects of the Oct-4 expression patterns (Rosner et al., 1990; Schöler et al., 1990a,b; Yeom et al., 1991, and our unpublished data). In situ hybridization analyses of day-8.0 embryos had shown that ectoderm expressed Oct-4 at higher levels than the underlying mesoderm (Rosner et al., 1990; Schöler et al., 1990a). However, in those studies it was unclear if the signal detected in dark-field images of early somites was above the background level. Analysis of the GOF-18 lines in the present study revealed β-galactosidase expression in the paraxial mesoderm during the initial phase of somitogenesis up to day 9. After day 9 no expression was detected in mesodermal or ectodermal derivatives. Primordial germ cells were the only cells in the embryo which expressed the transgene at this time. Thus, it is likely that Oct-4 is expressed in mesoderm for 1.5 days after the onset of gastrulation.

Expression in the early germ cell lineage

PGCs of early embryos (7 d.p.c.) are identified by their large size and abundant levels of TNAP (Chiquoine, 1954). The conclusion that the AP-positive cells are the germ cells is supported by the AP phenotype of sterile mouse mutants. In two examples, white spotting (W) and Steel (SI), the number of AP-positive cells is greatly reduced and no or only a few PGCs are found in the early gonads (Mintz and Russell, 1957; McCoshen and McCallion, 1975). The idea that all cells expressing AP during the establishment of the germ cell lineage represent PGCs has been challenged recently for the initial population of germ cells (Lawson and Hage, 1994). They suggested in their cell lineage analyses that primordial germ cell precursors are located in the epiblast, proximal to the extraembryonic ectoderm, in both pregastrulation and early-streak stage embryos. Based on their calculations a founding population of about 45 primordial germ cells is allocated at the midstreak stage at 7.2 d.p.c. The AP-positive cluster at that stage contains about 100 cells (Ginsburg et al., 1990). Thus, it is possible that not all cells of the AP-positive cluster become primordial germ cells. In day-7.5 embryos from the GOF-18ΔPE lines about 35-40 β-galactosidase-positive cells were detected at the posterior end of the embryo in a position expected for primordial germ cells. Further experiments will be needed to clarify if all germ cells express β-galactosidase.

The original in situ hybridization analyses of Oct-4 expression did not allow clear resolution of the pattern of Oct-4 expression during migration of the primordial germ cells (Schöler et al., 1990a). In contrast, analysis of the GOF-18 transgenic lines provided a detailed picture of migratory and postmigratory primordial germ cells in the developing embryo. The ease with which these cells can be visualized (Figs 3 and 9) suggests that GOF-18ΔPE transgenic lines will be very useful in analyzing mouse mutations that affect germ cell migration and proliferation. Moreover, the in situ analysis did not show when the Oct-4 gene is downregulated during spermatogenesis. This is currently investigated in a detailed transgenic analysis.

So far, the only difference between ES and EG cells has been found in the methylation imprint of the insulin-like growth factor 2 receptor gene (Labosky et al., 1994b). The binding profile of Oct-1, Oct-4 and Oct-6 indicate that EG cells contain a higher Oct-6 binding activity than ES and EC cells which might reflect a functional difference of ES and EG cells (Fig. 7A). A further analysis should show if higher levels of Oct-6 are also present in PGCs.

The gap in germline enhancer function

Enhancers can act as functional entities that generate subsets of a total expression pattern. The Oct-4 gene contains two enhancers which are exclusively active either in the totipotent cycle or in the epiblast. It remains unclear how the two
enhancers are regulated if their respective sets of activating factors overlap. The epiblast enhancer is strongly active in the total epiblast even in the absence of the germline enhancer. Upon deletion of the epiblast enhancer, Oct-4 transgenes still functioned in preimplantation embryos. However, activity of the germline enhancer could not be detected in any part of the epiblast in the pregastrulation embryo although precursors of the primordial germ cells are supposed to be located in its proximal region (Lawson and Hage, 1994). Perhaps the activity of the germline enhancer between implantation and day 7.5 is very low or present in very few cells and therefore escaped detection with the present assay. Alternatively, inactivity of the germline enhancer in the epiblast is a consequence of regulatory inter- actions between the two enhancers. One possible reason for this lack of activity may be that the germline enhancer requires additional elements outside the GOF-18 fragment. Another possibility is that in the epiblast, but not in totipotent cells, repressors bind to a silencer which regulate the germline enhancer. A third possibility is that the germline enhancer is not functional when the epiblast enhancer is active. This situation might occur if the transcription factors binding to the promoter could interact with those binding to the epiblast enhancer and not to those binding to the germline enhancer. Finally, it is also possible that the germline enhancer is in fact composed of two enhancer elements, one for the preimplantation embryo and the other for germ cells.

Enhancer function in the totipotent cycle

There has been a long search for a marker for the mammalian germline. TNAP has been a possible candidate, but the analysis of the TNAP enzyme activity was confounded by that of EAP at early stages and thus was only useful as a germ cell marker (see above). To test if the gene itself can serve as a marker, mice carrying a lacZ-disrupted TNAP allele were examined for embryonic TNAP expression from the blastocyst through embryonal day 14 (MacGregor et al., 1995). β-Galactosidase activity was detected in the germ cells after 7 d.p.c. However, β-galactosidase activity was not found before gastrulation in any cells that would give rise to the embryo proper. Therefore, TNAP is not expressed in the germline before gastrulation and thus cannot serve as a germline marker before gastrulation. GOF-18ΔPE lines show an expression pattern in which the germline is specifically stained in the mouse life cycle. Thus, the germline enhancer (DE) of Oct-4 is the first described element which appears to be specific for the totipotent cycle.

Primordial germ cells differentiate along male and female pathways into the highly specialized sperm and oocyte, respectively, and eventually regenerate new germ cells. The germline enhancer is active in preimplantation embryos and the germ cell lineage, and is inactive in non-totipotent cells, strongly suggesting the presence of a totipotent-specific regulatory condition. We speculate that the germline enhancer is activated by transcription factors that are specific for the totipotent cycle and thus should help to identify germline-specific factor(s). The DE is active in ES and EG, the PE in EC cell lines and in the particular cell types of the embryo that these cell lines resemble. The correlation between the in vitro and in vivo activities of both enhancers provides strong evidence that ES, EC and EG cell lines are suitable model systems for further study of the germline and epiblast enhancer functions at the molecular level. Hopefully, such an analysis will also help to unravel the ‘determinants’ of the mammalian germline alluded to by August Weismann 110 years ago.
We are most grateful to Patricia Laboski and Brigid Hogan for providing six different embryonic germ cell lines. We thank Heike Hess for her input on the embryo clearing; Dirk Bohmann, Iain Mattaj, Henk Stunnenberg and Karin Sturm for comments on the manuscript, Francis Stewart for discussions on the totipotent cycle and Petra Riedinger for artwork. G. F. was supported by the CNRS, K. O. and M. G. were supported by an EC biotechnology grant. Y. I. Y., A. B., C. E. O. and the project were supported in part by a grant from the Deutsche Forschungsgemeinschaft (DFG Grant Scho 340/2-1).

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(Y. I. Yeom and others (Accepted 27 November 1995)