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

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SUMMARY

The totipotential 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 germline. Embryos infected with recombinant retroviruses during preimplantation development were analyzed for viral integration at later stages. At least three cells form the germline 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 (TNAP) (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 with high frequency to the germline of chimeras (Evans and Kaufman, 1981; Martin, 1981). EC cells have been established following transplantation of early to mid-gastrulation embryos

into nude mice. P19 EC cells are derived from a teratocarcinoma formed after transplantation of a 7.5 d.p.c. embryo into testis (McBurney, 1982). P19 EC cells induced by retinoic acid (RA) or dimethyl sulfoxide differentiate into neuroectodermal, endodermal or mesodermal derivatives (reviewed by McBurney, 1993). P19 EC cells are also used to generate embryonic chimeras, but attempts to colonize the germline with P19 EC cells have failed. EG cell lines have been described more recently (Dolci et al., 1991; Godin et al., 1991; Matsui et al., 1991, 1992; Resnick et al., 1992). Derived from primordial germ cells, these stem cells can contribute to the germline of chimeras as do ES cells (Labosky et al., 1994b; Stewart et al., 1994). In addition to colonizing the germline, both ES and EG cells can be induced to differentiate extensively in culture, and also to form teratocarcinomas when injected into nude mice (reviewed by Hogan et al., 1994). EG cells serve here also as an example of how strikingly a cell line can differ from the embryonal counterpart. One contrasting feature is that EG cells have apparently lost their ability to migrate (reviewed by Wylie, 1993).

In order to address the question to what elements might be critical for gene expression in the germline, we have asked whether the *Oct-4* gene contains *cis*-regulatory elements that specifically and continuously drive its expression throughout the mouse totipotent cycle. Regulation of the murine *Oct-4* gene expression was studied both in developing embryos and in various embryonal cell lines expressing *Oct-4*. We identify two elements within the *Oct-4* gene which differentially control its expression during embryogenesis, one of which specifically drives *Oct-4* expression in the germline. This is the first report of a *cis*-regulatory element that is specifically active in totipotent mammalian cells during their cycle of differentiation and regeneration.

MATERIALS AND METHODS

DNA methods
GOF-32, GOF-18, GOF-12

Insertion of *lacZ* into Genomic *Oct-4* Fragments of 32, 18 or 12 kb in length. *Oct-4*/*lacZ* reporter constructs were prepared in two major steps. First, a 2.07 kb genomic *Hin*dIII fragment of the *Oct-4* gene was subcloned into pBluescript KS (Stratagene, USA). This fragment contains about 170 bp of the first exon of *Oct-4* and about 1.8 kb of upstream sequences (see Fig. 1B). By PCR-based mutagenesis a unique *Mlu*I site was introduced immediately adjacent to the initiator codon of *Oct-4*. This procedure inserts six additional nucleotides resulting in the sequence ATGacgcgtGCT. Then *lacZ* was inserted as a reporter gene into the modified *Hin*dIII fragment. The *lacZ* gene was derived as a 3.53 kb *Kpn*I/*Bam*HI fragment from pCH110 and carried the SV40 polyadenylation signal (Pharmacia, Sweden). The *lacZ*/SV40 fragment was linkered and inserted in frame into the *Mlu*I site. Linkers used for this purpose were: 5′-CGCGCGGAGACCTC-CTCGTAC-3′ and 5′-GAGGAGGTCTCCG-3′ for the N terminus of *lacZ*, and 5′-GATCCATGGATCCAAGCTTCC-3′ and 5′-CGCGGGAACCTTGGATCCATG-3′ for the C terminus. Second, the *Hin*dIII fragment containing the *lacZ* fusion was used to replace the original 2.07 kb genomic *Hin*dIII fragment in the different genomic clones of *Oct-4*. All three genomic clones were transcloned in pBluescript KS in which the *Hin*dIII site in the polylinker was deleted by *Hin*dIII digestion and filling-in with Klenow enzyme followed by religation. In the final clones the *Oct-4*/*lacZ* fusion genes run in the Bluescript vector: T7: 5′ to 3′: T3.

GOF-6 was prepared by cloning the *lacZ*-containing *Hin*dIII fragment of *Oct-4* (see above) into the *Hin*dIII site of the 5 kb *Bam*HI fragment of genomic *Oct-4* that has been subcloned in pBluescript KS (T7: 5′ to 3′: T3). GOF-5 was derived from GOF-6 by deleting the 1.08 kb upstream *Sal*I/*Aat*II fragment by partial digestion, followed by religation in the presence of a linker with *Sal*I/*Aat*II overhang. The linker used for this purpose consisted of: 5′-TCGACGGACACCT-GACGT-3′ and 5′-CAGGTGTCCG-3′. GOF-9 and GOF-13 were derived from GOF-12 and GOF-18, respectively, by taking the 5′-side *Sal*I/*Aat*II fragments of the latter constructs to replace the corresponding 5′-side *Sal*I/*Aat*II fragment in GOF-6. In this exchange cloning process, the DNAs had to be partially digested with *Aat*II after a complete digestion with *Sal*I.

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 *Hin*dIII fragment of *Oct-4* that had been cloned as above into pBluescript KS. For ΔPP the above plasmid, a 236 bp *Bst*EII/*Avr*II fragment was deleted, followed by filling-in with Klenow enzyme and religation. For ΔPE the plasmid was digested completely with *Bst*EII and partially with *Bam*HI to delete a 986 bp *Bam*HI/*Bst*EII 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 *Hin*dIII fragment in GOF-18 with the *lacZ*-containing *Hin*dIII fragment modified as above. GOF-18ΔDE was prepared from GOF-18 by partial digestion with *Bam*HI to delete the 3.29 kb fragment.

Cloning of *Bam*HI genomic *Oct-4* fragments in front of TK*lacZ*

Upstream and downstream *Bam*HI genomic *Oct-4* fragments were isolated and cloned individually in both orientations into a unique *Bam*HI site of TK*lacZ* (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-4*/*lacZ* constructs for generating transgenic animals were isolated as linear *Not*I/*Sal*I vector-free fragments by fractionating the restriction enzyme-digested plasmid DNA on an agarose gel. After electroelution, 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×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% CO$_2$ 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™; 10^3 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% gelatine 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 2×10^3 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).

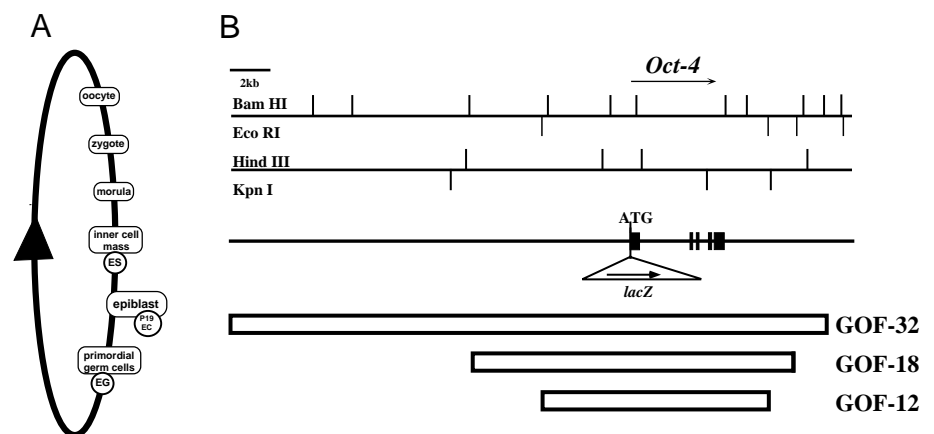For transfections 10 μg of doubly CsCl-purified DNA was used for

Fig. 1. *Oct-4* expression in the totipotent cycle. (A) *Oct-4* expression during mouse development. Cells and tissues expressing *Oct-4* are boxed. The totipotent cycle is indicated by the circular arrow. Embryonic stem (ES), P19 embryonal carcinoma (EC) and embryonic germ (EG) cell lines representing cells of different developmental stages as indicated. The epiblast is not centered on the cycle to indicate that only some epiblast cells are in the totipotent cycle, namely the precursors of the primordial germ cells (Lawson and Hage, 1994). Embryogenesis is divided here into pre- and postimplantation stages. Expression data were compiled from the in situ hybridization results reported previously (Rosner et al. 1990; Schöler et al., 1990a; Yeom et al., 1991). (B) Genomic organization and physical maps of the *Oct-4* gene in the 32 kb, 18 kb and 12 kb fragments (GOF-32, GOF-18 and GOF-12, respectively). Black boxes represent exons of *Oct-4*. The position of the *lacZ* insertion is indicated. Arrows show the direction of transcription.

$1.5\text{-}1.8\times10^5$ 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 embryogenesis 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-galactosidase 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-
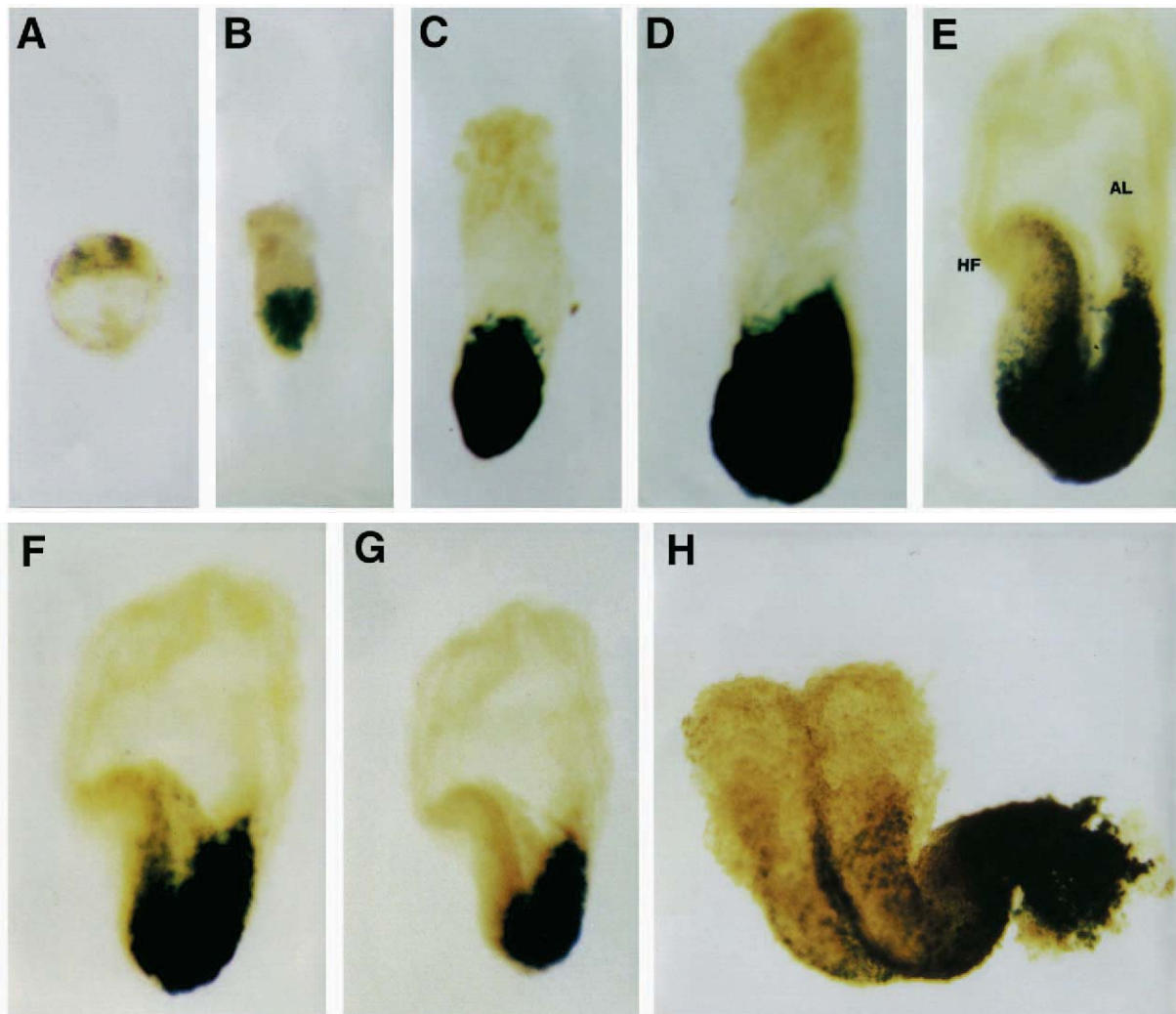

Fig. 2. Oct-4/*lacZ* transgene expression during early mouse development. Expression pattern of GOF-18 during pre- and early postimplantation development in stable transgenic animals. Mouse lines were generated which contained the GOF-18 construct. Embryos at 3.5 days through 7.5 days post-coitum (d.p.c.), 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, headfold; AL, allantois. The embryos shown in C-G were cleared after staining for β-galactosidase.

18 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 extra-embryonic 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-
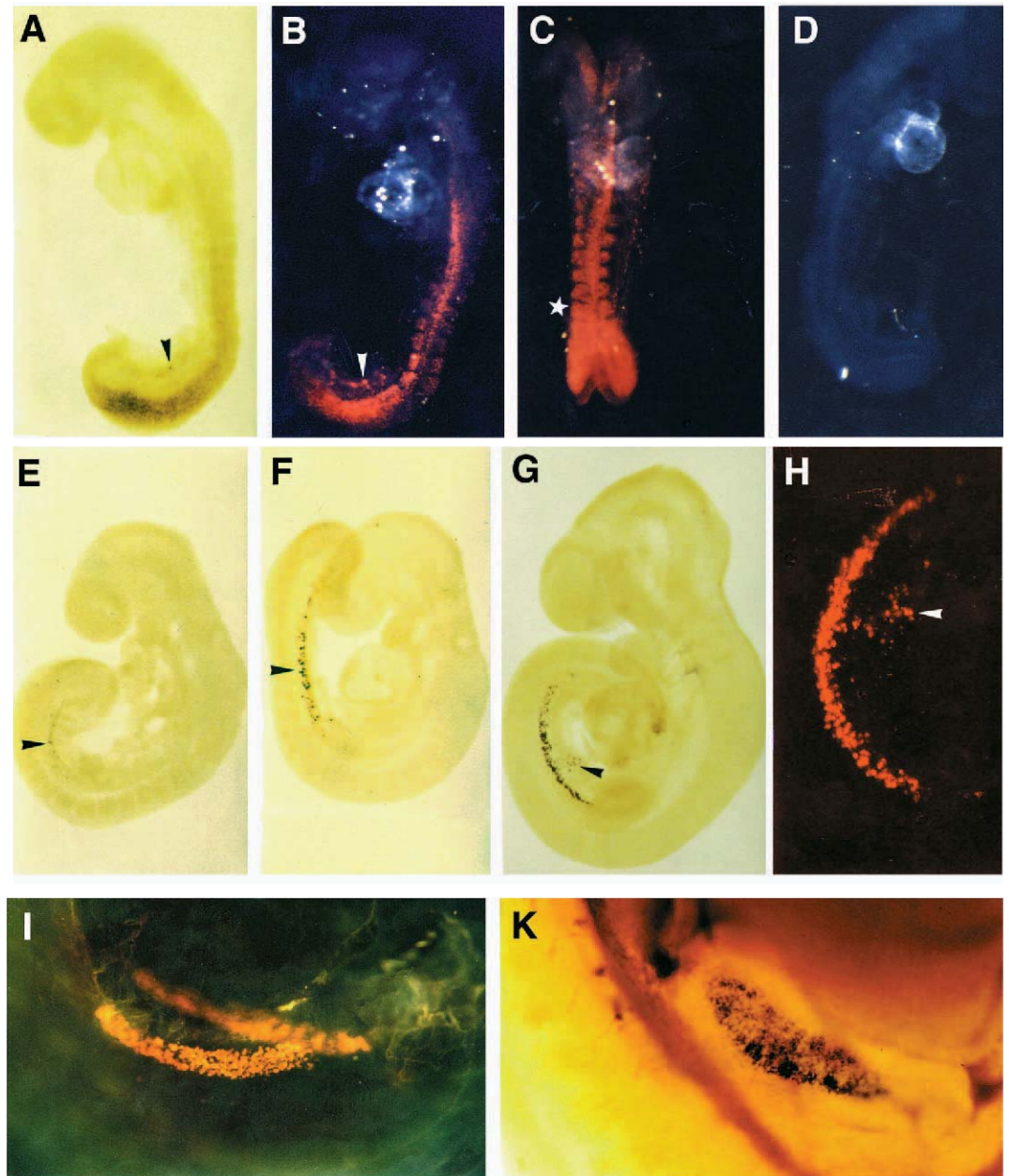

Fig. 3. Oct-4/*lacZ* transgene expression in germ cell lineage. Expression pattern of GOF-18 in 9-12.5 d.p.c. mouse embryos of stable transgenic lines. (A) Bright field side-view of an 9 d.p.c. somite-stage embryo. (B,C) Dark-field image of the embryo in A is shown from the side and from ventral views, respectively. (D) Dark-field image of a non-transgenic 9 d.p.c. embryo. Arrowheads in A and B, presumptive primordial germ cells in the hind gut area of the embryo, and star in C indicates the most posterior pair of somites. E-G show 9.25 d.p.c., 9.5 d.p.c. and 10.5 d.p.c. embryos, respectively. (H) Dark-field image of a second 10.5 d.p.c. embryo. The arrowheads in E-F point to primordial germ cells moving towards the position where the genital ridges are forming. I and K show dark-field image of female, and bright-field image of male genital ridges, respectively. All embryos were cleared after staining for β-galactosidase activity. All stages shown in this figure were derived from one transgenic line. The pattern of staining is identical to GOF-32 and to three different GOF-18 lines at the four stages tested.
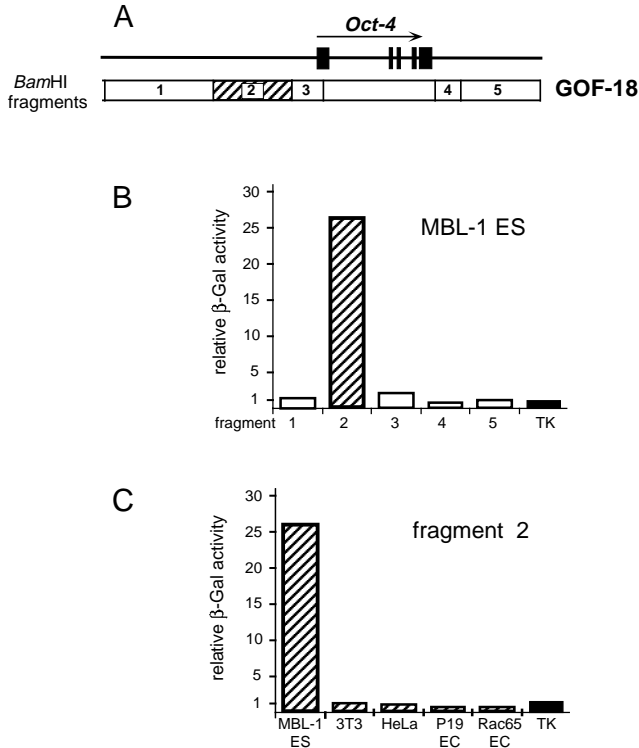
Fig. 4. The *Oct-4* gene contains a transcriptional enhancer specifically active in MBL-1 ES cells. (A) Physical map of *Oct-4* showing *Bam*HI restriction fragments in the 18 kb (GOF-18) clone. Numbers in the boxes indicate the respective *Bam*HI fragments cloned upstream of the TK minimal promoter. The size of the *Bam*HI 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 *Bam*HI 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 β-galactosidase activities. Relative β-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.

Fig. 5. Enhancers specific to ES and EC cells. (A) Location of the *Bam*HI restriction fragments 2 (hatched) and 3 (white) in the *Oct-4* gene. 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 β-galactosidase activity was calculated by dividing by the activity of TK-*lacZ* which was set at 1.

field photography also showed that signals in the neural groove and paraxial mesoderm were not yet completely extinguished (Fig. 3C, ventral view of the same embryo as in A and B). Expression was strongest in the unsegmented presomitic mesoderm and decreased anteriorly as the somites age. The transgene is expressed in a tail-fin configuration because the presomitic mesoderm does not meet along the midline and therefore is seen as two stripes of diverging tissue (Fig. 3C). Weak staining was also detected below the presomitic mesoderm in the neuroepithelium of the neuropore resulting in a similar tail-fin configuration but with a less pronounced notch. The heart, easily detected by its white color, and the most posterior somite (Fig. 3C, star) are embryonic landmarks for the expression in the neural groove. Weak staining is also
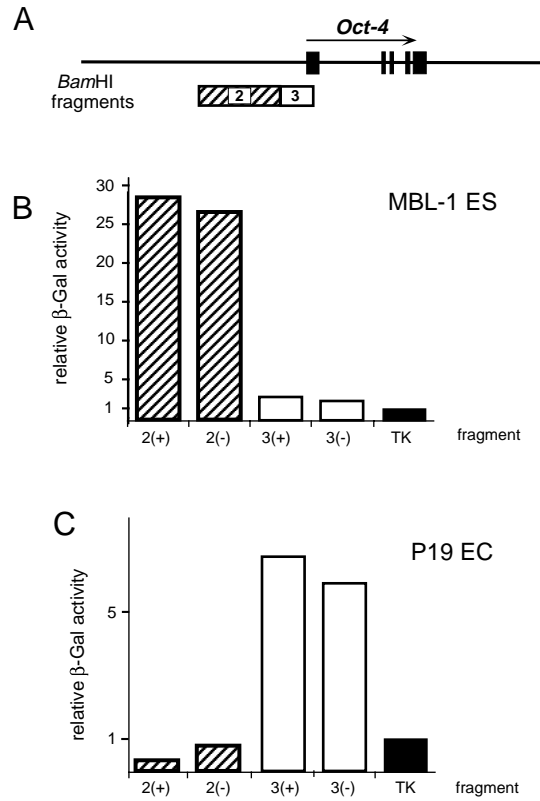
detected in the head folds. In addition, more positive spots in the hindgut area were visible (Fig. 3B, white arrowhead). The dark-field image of a stage-matched, non-transgenic embryo was an opaque white shadow (Fig. 3D), demonstrating that the red color in the transgene analysis resulted from β-galactosidase.

β-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 β-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 β-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

pattern (Fig. 3I) and the wavy pattern (Fig. 3K) indicate that these are female and male genital ridges, respectively. The relative position of the positive cells in the embryo during development, their increasing number, the reproduction of the same pattern by several independent lines, and the fact that PGCs express *Oct-4*, strongly suggest that the *Oct-4* transgene is expressed from approximately day 9 onward in the germ cell lineage. In conclusion, the detailed analysis of the GOF-18 transgenic strains revealed that the 18 kb fragment follows the endogenous *Oct-4* pattern described previously (Schöler et al., 1990a).

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 *Bam*HI fragments from either up- 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 *Bam*HI 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-
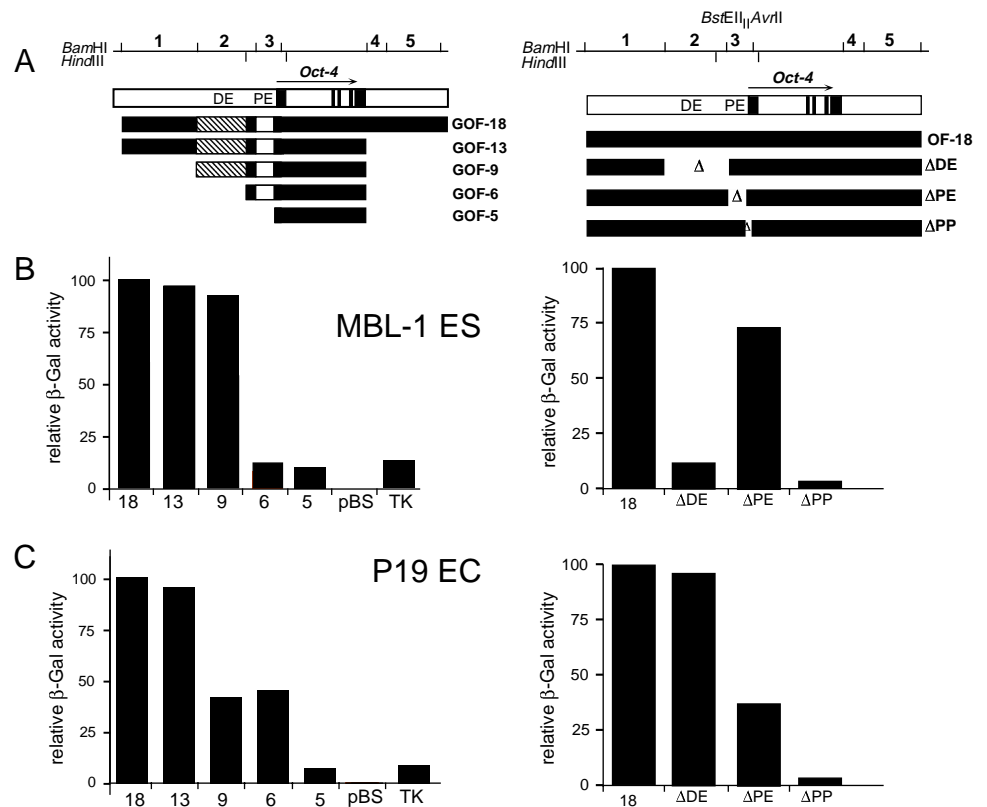

Fig. 6. Contribution of the distal and the proximal enhancer to *Oct-4* gene activity. (A) Physical map of *Oct-4* indicating *Bam*HI and *Hin*dIII restriction sites within the 18 kb (GOF-18) clone. Reporter constructs containing decreasing length of flanking sequence of the *Oct-4* gene (left) or three internal deletions (right) are shown. The positions of the distal enhancer (DE) and the proximal enhancer (PE) are indicated in the genomic map. The *lacZ* insertion is not shown but is identical to that in Fig. 1B. The black bars below the genomic map represent fragments of *Oct-4*, non-boxed areas represent deletions (Δ). The hatched area (DE) lies within *Bam*HI fragment 2; the white boxed area (PE) within *Bam*HI fragment 3. GOF-18ΔDE, GOF-18ΔPE and GOF-18ΔPP harbor deletions of the DE-, PE- or the promoter (PP). *Bst*EII and *Avr*II sites are given to indicate deletion boundaries in GOF-18ΔPE and GOF-18ΔPP (see Materials and Methods). The 5′ boundary of GOF-5 is located at an *Aat*II site between the *Bst*EII and CAP sites. Thus all upstream sequences except for PP are deleted. (B) Activity of the different *Oct-4/lacZ* constructs in MBL-1 ES cells and (C) in P19 EC cells. The prefix GOF- is omitted below the bars; pBS, pBluescript KS; TK, TK-*lacZ*, the parent construct. β-Galactosidase activities were normalized to the activity of an internal standard, RSV-luciferase. Relative β-galactosidase activity was calculated based on a GOF-18 activity of 100.

ing several deletions into the GOF-18 construct (Fig. 6). In transient transfection experiments, deletion of the downstream *Bam*HI 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 *Bam*HI fragment 1 (Fig. 6B; compare GOF-13 and GOF-9). However, further deletion of the 2.7 kb BamHI-*Hind*III 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, ΔPE 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 those 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, demonstrating 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
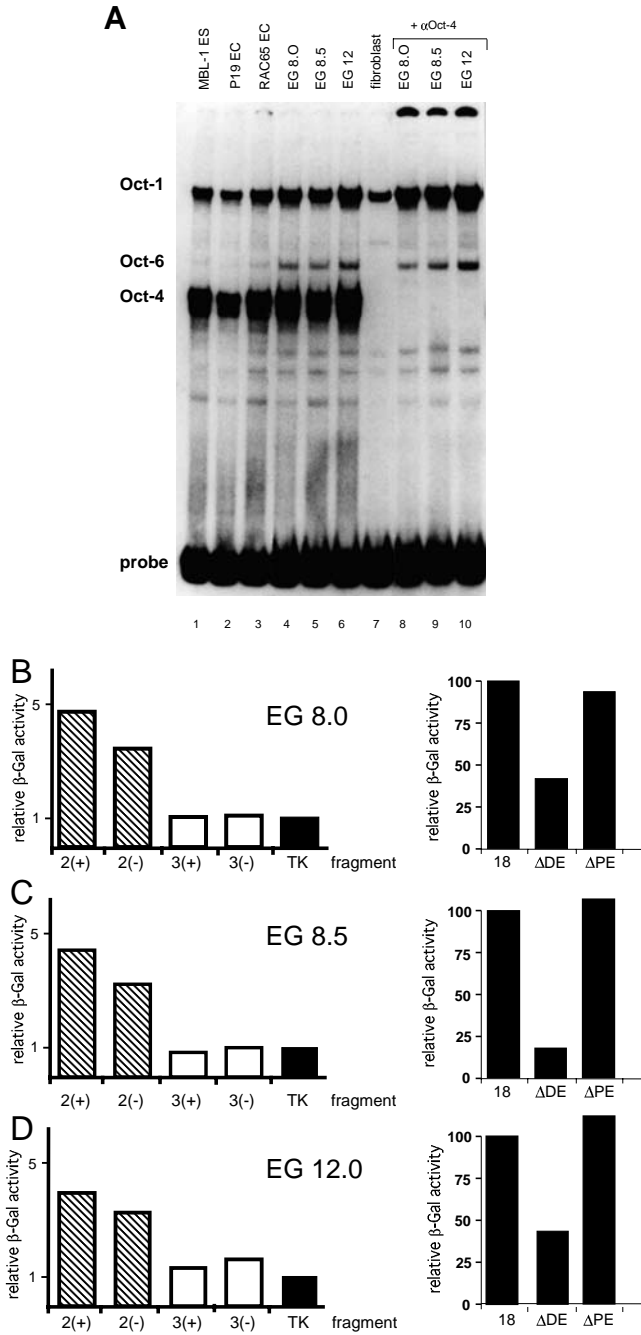
Fig. 7. Enhancer function in embryonic germ cell lines. (A) Presence of Oct-1, Oct-4 and Oct-6 proteins in various embryonal cell extracts and fibroblasts (lane 7). Band-shift analysis of ES (lane 1), EC (lanes 2 and 3) and EG (lanes 4-6 and 8-10) extracts. Whole cell extracts (10 μg) were analyzed with 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 *Bam*HI 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).

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
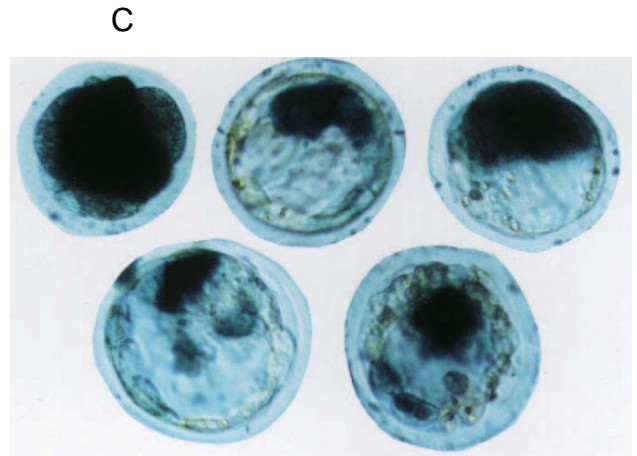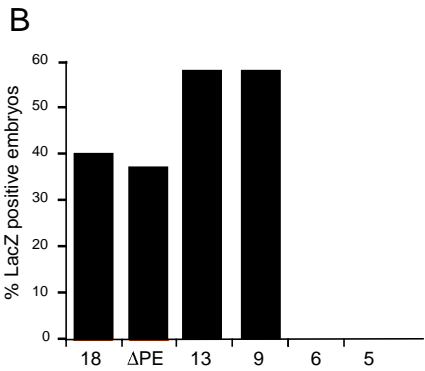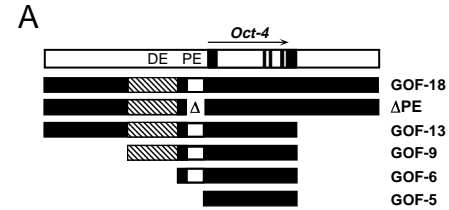

Fig. 8. *Oct-4* expression in preimplantation embryos requires the distal enhancer. (A) Deletion constructs used in the transient transgenic analyses to define the contribution of the distal and proximal enhancer for *Oct-4* gene activity in preimplantation embryos. Constructs are the same as in Fig. 3. (B) Quantitative analysis of the transcriptional enhancer activity of *Oct-4* flanking sequences in transient transgenic preimplantation embryos. Oocytes 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 transgenics 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 DE-driven *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 after 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* (Sl), 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 interactions 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.
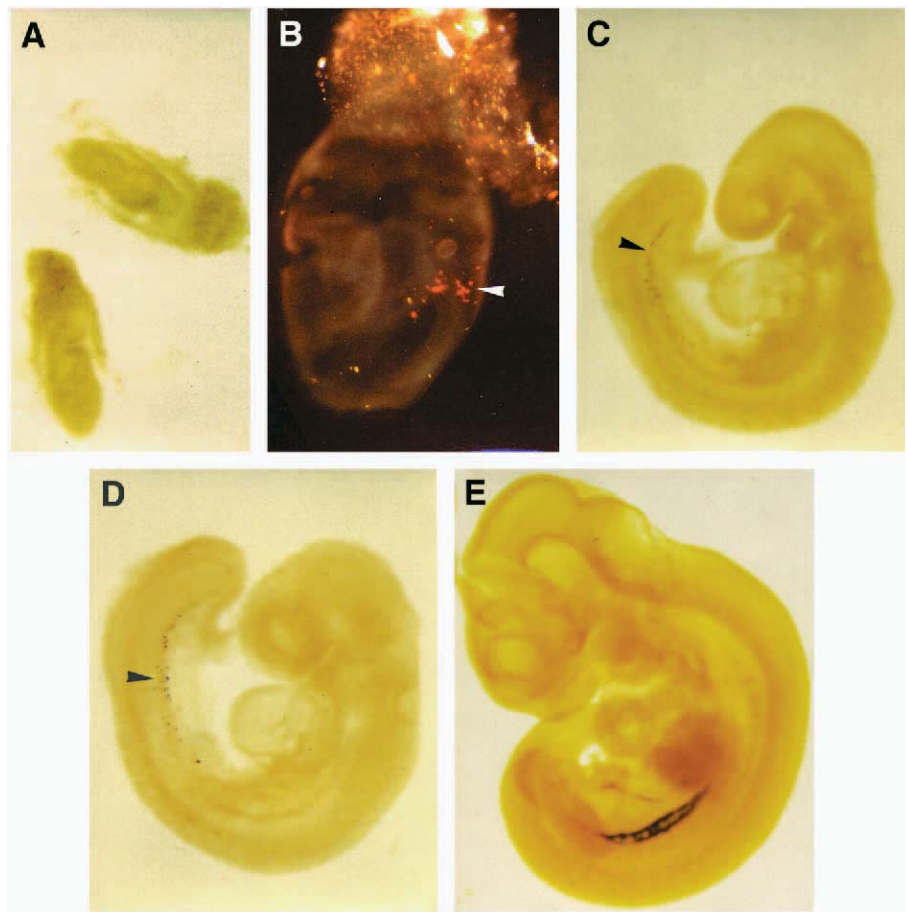

Fig. 9. Specific expression of *Oct-4/lacZ* transgenes in the germ cell lineage. Several stable transgenic lines were established with the GOF-18ΔPE construct. Embryos at 6.5-11.5 d.p.c. were stained for β-galactosidase activity and cleared. (A) 6.5 d.p.c., early streak; (B) 8 to 8.25 d.p.c., head-fold; (C) 9.25 d.p.c., somite-stage; (D) 9.5 d.p.c.. (E) 11.5 d.p.c. primordial germ cells located in the genital ridges. Arrows in B-D mark position of presumptive primordial germ cells. All panels show bright-field images except B in which illumination was required to detect germ cell staining.

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