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Molecular Cloning, Genetic Mapping, and Developmental Expression of Bovine *POU5F1*[1]

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ABSTRACT

We describe isolation and characterization of the bovine ortholog of *POU5F1* (*bPOU5F1*) encoding octamer-binding transcription factor-4 (Oct-4). The organization of *bPOU5F1* is similar to its human and murine orthologs, and it shares 90.6% and 81.7% overall identity at the protein level, respectively. Transient transfection of luciferase reporter constructs in murine P19 embryonal carcinoma cells demonstrated that *bPOU5F1* has a functional promoter and contains two enhancer elements, of which one is repressed by retinoic acid. *bPOU5F1* was mapped to the major histocompatibility complex on chromosome 23. *bPOU5F1* mRNA was detected by nested reverse transcription-polymerase chain reaction in immature oocytes and in in vitro-produced preattachment-stage embryos. Oct-4 in oocytes and in vitro-produced preattachment-stage embryos was demonstrated by indirect immunofluorescence. Confocal laser scanning microscopy revealed Oct-4 in both the inner cell mass and trophoblast cells of the blastocyst until Day 10 of development. Immunofluorescence performed on the outgrowths formed at Day 13 postfertilization from in vitro-produced Day 8 blastocysts showed Oct-4 staining in all cells. This expression pattern suggests that *bPOU5F1* acts early in bovine embryonic development but that its expression is not restricted to pluripotent cells of the blastocyst.

INTRODUCTION

Octamer-binding transcription factor-4 (Oct-4) belongs to the POU family of transcription factors that was first defined on the basis of sequence similarities between the three mammalian transcription factors Pit-1/GHF, Oct-1, and Oct-2 and the nematode regulatory protein Unc-86 [1]. Oct-4 was first detected in mouse F9 embryonal carcinoma (EC) cells by gel-shift analysis and named NF-A3 [2]. The corresponding cDNA was cloned independently as Oct-3 [3, 4] and Oct-4 [5]. A differential translation product from the same gene was described as Oct-5 [5]. Nomenclature committees have assigned the names *POU5F1* and *Pou5f1* to the gene encoding Oct-4 in humans and mice, respectively.

In the mouse, *Pou5f1* is the earliest expressed gene known to encode a transcription factor, and in vivo its protein is present at low levels in unfertilized oocytes and is localized predominantly in the nuclei of preimplantation embryos at all cleavage stages. After differentiation of the trophectoderm lineage from the late blastocyst, Oct-4 is found only in inner cell mass (ICM) cells [6]. In direct contrast to earlier RNA localization studies [7, 8], Oct-4 was found at high levels in primitive endoderm cells that differentiate and migrate along the inner surface of the trophectoderm [6]. In the postimplantation mouse embryo, *Pou5f1* transcripts were detected in embryonic ectoderm at Day 7 but not in endoderm, allantois, and other extraembryonic tissues or in mesoderm forming at Day 7.5 [7]. *Pou5f1* mRNA in the mouse was further detected in male and female primordial germ cells after Day 8.5 of gestation but was absent in sperm and the testis, suggesting that its expression becomes restricted to the female germline at later stages of germ cell development [9].

Consistent with its embryonic expression pattern in vivo, Oct-4 is expressed in vitro in undifferentiated mouse embryonic stem (ES) cells and in mouse and human EC cells [3, 10], but it is strongly down-regulated during retinoic acid (RA)-induced differentiation in (murine) P19 and F9 EC cells [3, 11]. Analysis of the transcriptional control region by deletion mutagenesis revealed the presence of a *cis* RA-responsive element located between nucleotides −1132 and −889, which is composed of two domains (1A and 1B) and functions as an RA-repressible enhancer [12].

A second, more distantly located transcriptional control element was identified by transfection of a series of constructs composed of mouse *Pou5F1* upstream sequences coupled to a LacZ reporter gene in mouse ES, EC, and embryonic germ (EG) cells [13]. This distal enhancer (DE) was shown to be critical for expression in ES and EG cells, although the level of activation was lower in EG than in ES cells. Consistent with the results of Okazawa and coworkers [12], these studies showed that expression in EC cells was controlled by a proximal enhancer (PE), likely to

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be the RA-responsive element located between nucleotides −1132 and −889. The PE was dispensable for *Pou5f1* expression in ES and EG cells [13]. A third regulatory element of approximately 230 base pairs (bp) in the promoter region immediately upstream of the initiation codon was shown to be essential for expression in ES and EC cells (and presumably also EG cells), as deletion of this fragment abolished expression completely [13, 14]. Consistent with this regulation in vitro, *Pou5f1* expression in preimplantation mouse embryos was dependent on the DE or germline enhancer with no reduction in LacZ expression upon deletion of the PE, whereas postimplantation expression was driven by the PE or epiblast enhancer [13]. Footprint analysis in EC and ES cells provided refinements of the location of critical regulatory elements in the promoter region to the Sp1 site (between nucleotides −52 and −42), domain 1A of the PE (between −1120 and −1100), and a site named 2A in the DE (between −2027 and −2010) which is similar to 1A of the PE, although situated in opposite orientation [15].

Despite extensive knowledge of expression and regulation of *Pou5f1* in the mouse, to date only its human ortholog has been cloned and characterized [16]. No reports have been published describing cloning of *POU5F1* orthologs in farm animal species despite its obvious importance in early embryonic development, probably at the top of a cascade of regulatory genes that induce and regulate differentiation, and despite relatively intensive but as yet only partially successful attempts to isolate pluripotent stem cells [17] or ES cells (reviewed in [18]). Especially in the light of the long generation interval of farm animals in comparison with mice and the high costs associated with in vivo validation of putative ES cells for germline transmission in these species, there is a need for (molecular) markers for a first screen for the pluripotency of such cells in vitro. The objective of the present study was to isolate the bovine ortholog of *POU5F1* and evaluate its potential as a stem cell marker by following expression during early embryonic development.

MATERIALS AND METHODS

Isolation of a Bovine Genomic POU5F1 Clone

A bovine genomic DNA library constructed in the *Bam*HI sites of phage EMBL3 SP6/T7 (Clontech, Palo Alto, CA) was screened according to standard procedures [19] using a full-length mouse *Pou5f1* cDNA clone [4] as probe. After three rounds of screening at increasing stringency, a single phage clone (λbPOU5F1) with an approximately 11-kilobase (kb) insert was isolated, which contained the entire coding sequence and intervening introns of *bPOU5F1* as well as 2.9 kb of upstream sequence.

Restriction Mapping, Subcloning, and Sequencing of λbPOU5F1

A restriction map of λbPOU5F1 was produced by (double) digestion with restriction endonucleases *Bam*HI and *Xba*I (Gibco-BRL, Gaithersburg, MD). Restriction fragments harboring *bPOU5F1* coding sequences were identified after probing the Southern blot with the full-length mouse cDNA and cloned in appropriately digested and dephosphorylated pBluescript SK- (Stratagene Inc., La Jolla, CA) or pGEM-7Zf(-) (Promega, Madison, WI) vector. Plasmid DNA suitable for automated sequencing was purified using a commercial miniprep kit (Qiagen, Chatsworth, CA). Subclones containing coding regions and upstream regulatory sequences were completely sequenced in both directions by primer walking using an automated nucleotide sequencer (ABI Systems, Foster City, CA). The resulting sequences were aligned and analyzed using IGsuite software (Intelligenetics, Leek, Belgium), LASERGENE software (DNASTAR Inc., Madison, WI), and the online Basic Local Alignment Search Tool (BLAST; [20]).

Transient Transfection of bPOU5F1 Reporter Constructs

For construction of *bPOU5F1* luciferase (Luc) reporter constructs, first a blunt-end *Ava*I fragment (−1710/+48, Fig. 1a) was cloned in pBluescript SK-. A *Bam*HI-*Xba*I (−1021/+ 48) fragment from this clone and a *Bam*HI-*Xba*I fragment (−2892/-1021, Fig. 1a) were subsequently cloned in both orientations in the *Bam*HI site of a promoterless reporter pLuc. Digestion of the sense *bPOU5F1*-Luc construct with *Stu*I followed by ligation resulted in the construct ΔPE (−1467/−889, Fig. 1a). ΔDE,PE was made by ligating the *Bam*HI-*Xba*I-digested *bPOU5F1*-Luc reporter. The *bPOU5F1* proximal promoter construct was made by cloning the *Sau*IIIA (−156/+20) fragment in the *Bam*HI site of pLuc. Cell culture, transient transfections, and β-galactosidase assays were performed as described before [21].

Genetic Mapping of bPOU5F1

Nine bulls that sire paternal half-sib families collectively known as the Illinois Reference and Resource Families (IRRF; [22]) were screened for a polymorphism in *bPOU5F1* by single-strand conformational polymorphism (SSCP) analysis as described [23]. Polymerase chain reaction (PCR) products for SSCP analysis were generated using oligonucleotide primers OCT27 (5′-GGAAATTGGGAACACAATGGG-3′, coding strand) and OCT28 (5′-AACAGCAACCTTCGTTTCGG-3′, noncoding strand), which are both located downstream of the predicted stop codon of *bPOU5F1* (see Fig. 1b). Offspring of heterozygous sires were subjected to SSCP analysis, and dams were included if available. Pairwise and multipoint linkage analysis of *bPOU5F1* genotypes with IRRF markers across the genome [24] were performed as described [25].

RNA Isolation and Northern Blot Analysis of bPOU5F1

Steady-state RNA levels of *bPOU5F1* in somatic tissues were determined on a Northern blot containing 10 μg of mRNA isolated from tissues collected from a still-born calf (brain, colon, heart, kidney, lung, and small intestine) or from adult animals (liver, ovary, placenta, testis) as described before [25]. The blot was hybridized essentially as described [26] with a 2127-bp *Bam*HI-*Xba*I insert from a λbPOU5F1 plasmid subclone containing exons 2, 3, 4, and 5 of the coding region (Fig. 1b). A separate Northern blot containing 10 μg of mRNA from undifferentiated and 10^{-6} M retinoic acid (RA)-treated Tera2cl13 human EC cells [27] was included as a control for hybridization. Blots were subsequently hybridized with a human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA probe as a control for RNA loading.

Reverse Transcription (RT)-PCR of bPOU5F1 in Oocytes and Preattachment-stage Bovine Embryos

Bovine ovaries were collected at a local abattoir in a thermoflask and transported to the laboratory. Cumulus oo-

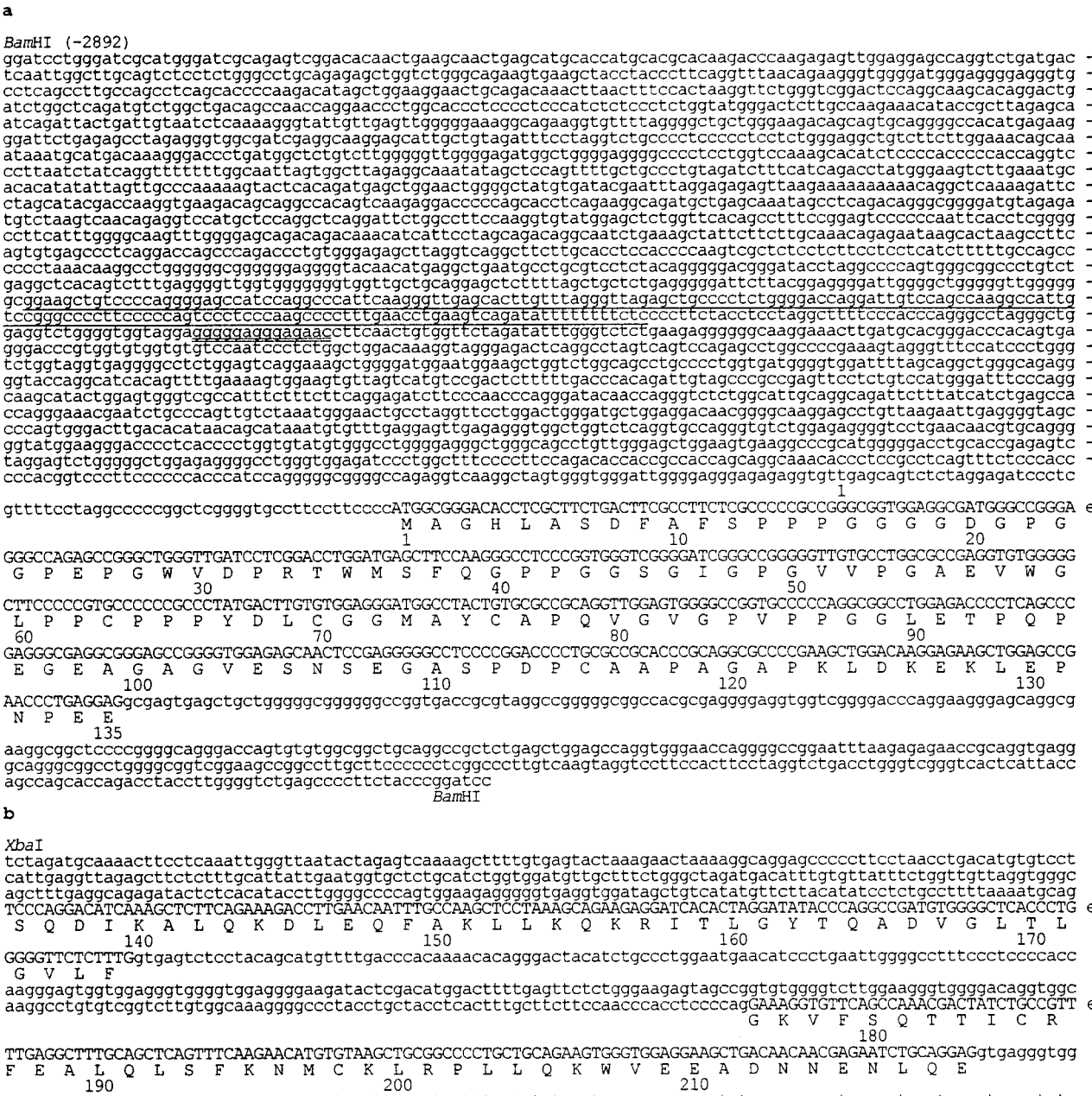

FIG. 1. Nucleotide sequence of *bPOU5F1*. Shown are **a**) 3721-bp *Bam*HI fragment (GenBank accession no. AF022986) and **b**) a 2127-bp *Xba*I-*Bam*HI fragment (GenBank accession no. AF022987). Upstream nucleotide sequences are indicated on the right and numbered relative to the transcriptional start site of mouse *Pou5f1* [12]. Exons are indicated on the right, and coding sequences are printed in uppercase. Amino acids are indicated in one-letter code and numbered below the nucleotide sequence. The underlined region in **a** is 96% identical to part 1B of the mouse PE. The double-underlined 13-bp region in **a** is 100% conserved in the mouse. Underlined regions in **b** indicate the position of primers OCT27 and OCT28 used for mapping of *bPOU5F1* by SSCP. Double-underlined in **b** is putative polyadenylation signal.

TABLE 1. Oligonucleotide primers used for analysis of bPOU5F1.

Primer	Sequence (5′–3′)	Strand	Aminoacid position
OCT12	TGGCGCCGGTTACAGAACCA	Noncoding	276–283
OCT15	CGTTCTCTTTGGAAAGGTGTTC	Coding	173–179
OCT26	ACACTCGGACCACGTCTTTC	Noncoding	270–277
OCT29	GTTCAGCCAAACGACTATCG	Coding	178–185

cyte complexes were obtained by aspiration of 2- to 8-mm follicles and selected on the basis of the presence of a multilayered cumulus investment. In vitro maturation and in vitro fertilization (IVF) of the cumulus oocyte complexes as well as in vitro embryo culture of the fertilized oocytes was performed as previously described [28]. Under the culture conditions employed, approximately 25% of fertilized oocytes are at the blastocyst stage at Day 9 postfertilization. Pools of 20 immature denuded oocytes (germinal vesicle [GV] stage) or embryos at the 2-, 4-, or 8-cell, morula, or Day 9 blastocyst stages of development, and one Day 14 and one Day 16 blastocyst were collected in 200 μl Ultraspec solution (Biotecx, Veenendaal, The Netherlands). ICM were obtained from Day 8 blastocysts produced in vitro as described [29], prepared by immunosurgery as described previously for mice [30], and collected individually in 200 μl Ultraspec solution. Total RNA was isolated as described previously in detail for human oocytes and individual preimplantation embryos [31]. RNA was treated with ribonuclease (RNase)-free deoxyribonuclease (DNase), dissolved in 10 μl RNase-free water, and reverse-transcribed in a 20-μl volume as described [31], with the exception that 1 μl of 0.5 mg/ml oligo dT12–18 (Pharmacia, San Francisco, CA) was used as primer. Two microliters of cDNA (4 μl in case of the Day 14 blastocyst) was used as template for amplification of *bPOU5F1* in two rounds of PCR with hemi-nested primers.Primers for the first round were OCT15 and OCT26 (Table 1), which were both designed across splice junctions to favor amplification of cDNA over genomic DNA. OCT15 was designed from the murine nucleotide sequence [32] and contains a primer:template C:G mismatch at its most 5′ base. Second-round PCR was performed using primers OCT26 as above and OCT29 (Table 1), which was modified at the 3′ base to introduce an *Eag*I restriction site into PCR products derived from *bPOU5F1* but not into products derived from a *bPOU5F1*-related sequence (see *Results*). Reactions were carried out as described [31] with the exception that 0.6 units of Goldstar polymerase (Eurogentec, Seraing, Belgium) was used. This polymerase lacks 3′–5′ exonuclease activity. The thermal cycling profile for the first round was initial denaturation for 5 min at 94°C, followed by 40 cycles of 15 sec at 94°C, 30 sec at 55°C, and 45 sec at 72°C. Final extension was for 7 min at 72°C. Subsequently, 2 μl of first-round product was transferred and amplified for 30 cycles according to the same profile, except that annealing was at 60°C. Water controls and RNA samples that had not been reverse-transcribed were included and transferred as described to monitor contamination and amplification from genomic DNA. Five microliters of second-round product was resolved and visualized as described [31], and another 5-μl aliquot of second-round PCR product was digested with 5 U *Eag*I (New England Biolabs, Beverly, MA) for 1 or 16 h at 37°C in a 15-μl volume before gel electrophoresis to confirm the genuine origin from *bPOU5F1*. RNA isolations and RT-PCR were performed in duplicate for all stages examined. Genomic DNA was amplified in a single round of PCR as described using primers OCT15 and OCT12 (Table 1) to confirm the existence of *bPOU5F1*-related sequences.

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         1
Bovine   MAGHLASDFAFSPPPGGGGDGPGGPEPGWVDPRTWMSFQGPPG 43
Human    ------------------------------------L------- 43
Murine   ------------------:--SA-L----------L------- 42

GSGIGPGVVPGAEVWGLPPCPPPYDLCGGMAYCAPQVGVGPVPPGGLETPQ 94
-P------G--S----I--------EF-------G------L--Q-----S- 94
-P-----::::S--L-IS----A-EF-------G----L-L--QV-V--L- 89

PEGEAGAGVESNSEGASPDPCAAPAGAPKLDKEKLEPNPEESQDIKALQKD 145
------V------D----E--TVTP--V--E-----Q-------------E 145
---Q---R-------T-SE---DRPN-V--E-::V--T------M-----E 138

                    POU-specific domain
LEQFAKLLKQKRITLGYTQADVGLTLGVLFGKVFSQTTICRFEALQLSFKN 196
---------------------------------------------------- 196
-----------------------------------------------L-- 189

MCKLRPLLQKWVEEADNNENLQEICKAETLVQARKRKRTSIENRVRGNLES 247
-----------------------------------------------N 247
--------E----------------S-------------------WS--T 240

                    POU homeo domain
MFLQCPKPTLQQISHIAQQLGLEKDVVRVWFCNRRQKGKRSSSDYSQREDF 298
L------------T-------------------------------A----- 298
---K----S----T---N-----------------------IE-----EY 291

EAAGSPFTGGPVSSPLAPGPHFGTPGYGGPHFTTLYSSVPFPEGEVFPSVS 349
-------S-----F--------------S----A-----------A--P-- 349
--T-T--P--A--F--P-----------S--------:-------A----P 341

VTALGSPMHAN 360
--T------S- 360
---------S- 352
```

FIG. 2. Comparison of the amino acid sequences of bovine, human, and murine Oct-4. Human and murine sequences are from [16] and [12], respectively. Only deviations from the bovine sequence are indicated. Dashes (-) indicate amino acid identity to bovine Oct-4. Gaps introduced to maintain alignment are indicated by a colon (:). The POU-specific and POU homeo domains are printed in bold. The linker region connecting these domains is underlined.

Polyclonal Oct-4 Antiserum

Polyclonal antisera were raised against peptide NH_2-NNENLQEICKSETLVQ-COOH corresponding to the linker region connecting the POU-specific and POU-homeodomains of *Pou5f1* [3–5, 32]. The murine and bovine protein sequences in the region of the peptide are identical with the exception of a substitution of serine at position 216 in the mouse for alanine at the corresponding position 223 in cattle (Figs. 1b and 2). Peptide synthesis and (repeated) immunization of New Zealand White rabbits were performed as described [33]. Affinity-purified antibody was prepared by coupling the peptide to CnBr-activated Sepharose 4B by standard procedures (Pharmacia Biotech, St. Quentin-Yvelines, France), equilibrating the gel with PBS, loading the antiserum, and eluting peptide binding antibodies at acidic pH in 100 mM acetic acid, 300 mM NaCl, pH 3.0. The specificity of the affinity-purified fraction was determined by indirect immunofluorescence on P19 EC cells that were either undifferentiated or treated with 10^{-6} M RA

for 5 days and on STO murine fibroblast cells (American Type Culture Collection).

Immunocytochemical Localization of Oct-4 in Bovine Oocytes, Preattachment-stage Embryos, and Blastocyst Outgrowths

Whole-mount immunocytochemistry was performed on bovine oocytes, embryos, and blastocyst outgrowths. The following developmental stages were examined: GV-stage oocytes; metaphase-II (M-II)-stage oocytes; pronuclear-stage zygotes; 2-cell, 4-cell, 8-cell, and 16-cell embryos; morulae; and early, expanded, hatched, and elongated blastocysts at Days 10, 14, and 16 postfertilization. Elongated blastocysts were obtained from in vitro-produced 2- to 4-cell-stage embryos [34]. For the generation of Day 10 blastocysts, embryos at the 2- to 4-cell stage were transferred in sheep oviducts and recovered 8 days later. To obtain Day 14 and Day 16 blastocysts, 2- to 4-cell-stage embryos were transferred in ligated sheep oviducts and recovered on Day 7 post-IVF. Embryos developed to the blastocyst stage were then transferred into the uterus of synchronized cows and recovered on Day 14 and Day 16 postestrus [29]. In order to determine Oct-4 expression in blastocyst outgrowths, in vitro-fertilized oocytes were cultured until the blastocyst stage at Day 8 on oviduct epithelial cells, transferred to buffalo rat liver-conditioned medium [35], and allowed to attach on gelatin-coated coverslips (Sarstedt, Numbrecht, Germany). At Day 13 postfertilization, four outgrowths were fixed and processed as for the other specimens.

A minimum of 10 specimens for each developmental stage were fixed at room temperature for 20 min in 2.5% (w:v) paraformaldehyde in PBS (pH 7.4), and for an additional 10 min in the same fixative with the addition of 0.3% (v:v) Triton X-100. Fixation was followed by an incubation in 50 mM ammonium chloride for 60 min to remove remaining free aldehyde groups. Samples were incubated with the primary antibody diluted 1:10 overnight at 4°C, followed by fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Labs., West Grove, PA) secondary antibody. After several washes, samples were mounted on slides in Vectashield Mounting Medium (Vector Labs., Burlingame, CA). Controls were performed using the primary antibody neutralized with a 10-fold excess of the peptide or by omission of the primary antibody. Oocytes, embryos, and outgrowths were examined and photographed with a Nikon Diaphot TMD fluorescence microscope (Nikon Instruments, Garden City, NY).

Confocal Laser Scanning Microscopy

Immunofluorescence localization of Oct-4 in expanded and hatched blastocysts was observed using a Meridian InSight Point Confocal Laser Scanning Microscope (CLSM) assembled on a Zeiss (Oberkochen, Germany) Axioscope microscope equipped with Zeiss ICS oil immersion lenses. FITC samples were observed using an argon laser with an excitation filter at 488–10 nm, a dichroic mirror at 505 nm, and a barrier filter LP 515 nm. Images of serial optical sections were recorded every 1 μm along the z-axis of each embryo. Three-dimensional reconstructions were made from each series of optical sections using the computer work station and the software provided by the manufacturer.

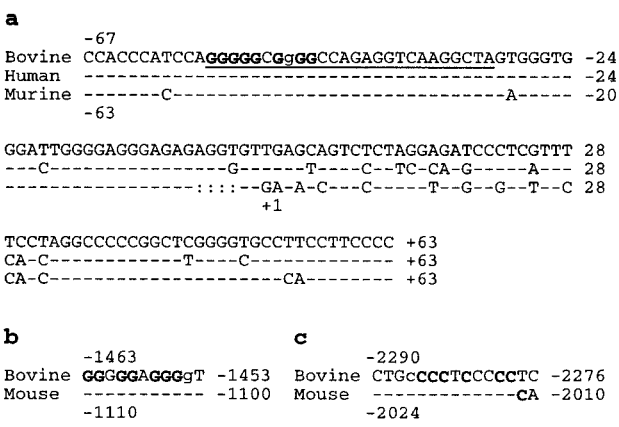

FIG. 3. Alignment of the *bPOU5F1* promoter sequence with its mouse and human orthologs (**a**) and alignment of putative *bPOU5F1* proximal (**b**) and distal (**c**) enhancer sequences with the corresponding mouse sequence. The human sequence is from [16] (GenBank accession no. Z11900), the mouse promoter sequence is from [12] (GenBank accession no. S58422), and the mouse enhancer sequences are from [15]. The putative Sp1 binding site (bovine sequence −56/−46) and overlapping hormone responsive element (bovine sequence −49/−31) in **a** are underlined. Residues that were protected by footprint analysis in the mouse are printed in bold; those that were hypermethylated are printed in lowercase and are from [15]. Nucleotides have been numbered relative to the transcriptional start site (+1) of the mouse [12] and are indicated at the beginning of the alignment at the top left for the bovine sequence, at the bottom left for the murine sequence, and at the right hand side for the bovine and mouse (**a–c**) and human (**a**) sequence.

RESULTS

A single phage clone λbPOU5F1 with an insert of approximately 11 kb harboring the entire bovine *POU5F1* gene was isolated by screening of a genomic DNA library with a full-length murine *Pou5f1* cDNA clone as a probe. Two fragments were subcloned and sequenced in both directions: a 3721-bp *Bam*HI fragment containing exon 1 (405-bp coding sequence), 2955 bp of promoter and upstream regulatory sequence, and 361 bp of the intron between exons 1 and 2 (Fig. 1a); and a 2127-bp *Bam*HI-*Xba*I fragment containing exons 2 (121 bp), 3 (131 bp), 4 (159 bp), and 5 (267-bp coding sequence) and flanking sequences (Fig. 1b). The numbering of bovine exons is according to that of *Pou5f1* [12], and the boundaries of exons 2–5 conform with the GT/AG consensus sequence of splice donor/acceptor sites [36]. The splice donor site following exon 1 is GC instead of GT (Fig. 1a), which is the most commonly found alternative splice donor sequence [37] and is also present at the corresponding position in the human *POU5F1* ortholog [16]. The predicted Oct-4 protein encoded by these five exons consists of 360 amino acids (Fig. 2) and has 90.6% and 81.7% overall identity with its human [16] and murine [3–5, 32] counterparts, respectively. Broken down by exon, the percentages of amino acid identity between bovine and human Oct-4 are 84.4, 97.5, 100, 94.3, and 89.7 for exons 1 through 5, respectively. For the comparison between cattle and mice, these percentages are 71.0, 95.1, 95.4, 84.9, and 82.8, respectively.

Alignment of the promoter sequence with the orthologous human [16] and mouse [12] sequences revealed extensive conservation in the region upstream of the transcription initiation site: the putative Sp1 binding site and overlapping hormone responsive element at nucleotides −56/−31 in the bovine sequence are identical in all three species (Fig. 3a). In addition, there is complete sequence

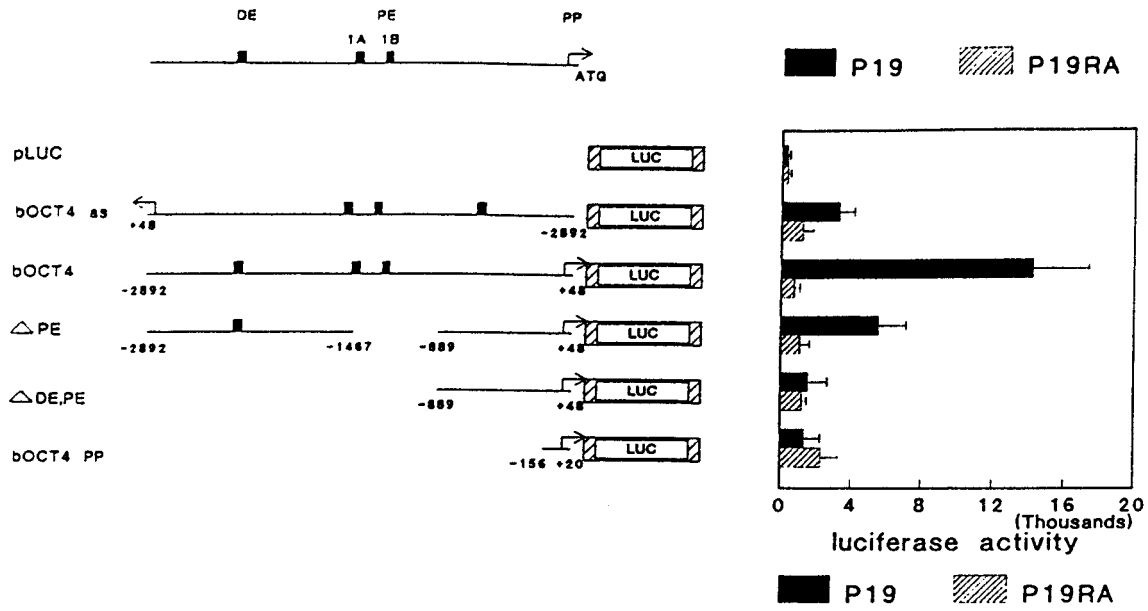

FIG. 4. Transient transfection of *bPOU5F1* luciferase (Luc) reporter constructs in P19 EC cells. PP indicates the proximal *bPOU5F1* promoter (−156/+20). The putative DE and parts 1A and 1B of the putative PE are indicated by black boxes, and the transcription start site by an arrow. ΔPE and ΔDE,PE represent constructs with deletions of the putative PE or PE and DE, respectively. Luc activities and standard deviations were the result of four experiments.

conservation between the bovine and mouse sequence at sites that showed protection or hypermethylation in part 1A of the PE (Fig. 3b) and strong conservation at those we believe are the bovine equivalent of the DE (Fig. 3c). The corresponding sequence in humans at these positions is not known. Alignments of the bovine and murine upstream sequences further indicated several other regions of strong homology, of which a 171-bp region defined by the bovine sequence at nucleotides −1270/−1100 (Fig. 1a) with 96% identity is most significant. This region contains part 1B of the PE as defined by Okazawa and coworkers [12]. Another 13-bp region, 5′-GGGGGAGGGAGAA-3′, at −1037/−1024 is 100% conserved with a mouse sequence downstream of the PE (Fig. 1a) and closely resembles the critical region of part 1A of the PE (Fig. 3b). BLAST analysis (data not shown) revealed a 183-bp region with 86% identity to the bovine Short Interspersed Nuclear Element (SINE) Bov-tA consensus sequence [38] at positions −697/−517 (Fig. 1a), which is absent in the mouse.

TABLE 2. Pairwise recombination frequencies (θ) and LOD scores (*z*) between *bPOU5F1* and markers on bovine chromosome 23.

Locus pair	n[a]	θ at z_{max}	SD[b]	z[c]
bPOU5F1-BM1818	56	0.07	0.034	10.00
bPOU5F1-RM185	54	0.04	0.020	11.94
bPOU5F1-PRL	30	0.07	0.047	5.54
bPOU5F1-CYP21	43	0.00	0.034	12.64
bPOU5F1-TAMULS113	35	0.00	0.042	10.24
bPOU5F1-BoLA-A	34	0.00	0.043	9.63
bPOU5F1–DRB3	79	0.01	0.011	20.55
bPOU5F1-BM1258	60	0.10	0.036	8.69
bPOU5F1-UWCA1	23	0.09	0.052	3.37
bPOU5F1-MGTG7	35	0.03	0.026	7.96
bPOU5F1-BM47	67	0.21	0.051	4.35

[a] Number of coinformative meioses for the pair of loci.
[b] Standard deviation of θ.
[c] LOD, Log_{10} of the likelihood ratio.

Transient transfection experiments in P19 EC cells using luciferase (Luc) reporter constructs were performed to determine whether the *bPOU5F1* promoter is functional and harbors regulatory elements mediating down-regulation in response to RA. Highest Luc activity (average 14 500 arbitrary units) was observed when the entire promoter and upstream sequences (−2892/+48) were cloned in correct orientation upstream of the Luc gene, which was down-regulated to basal level upon treatment with RA. Control transfections of constructs with *bPOU5F1* promoter and regulatory elements in opposite orientation showed much less Luc activity (average 3800 units). Deletion of the putative PE resulted in reduced Luc expression in comparison with the full-length construct (average 5500 units), but this activity was still repressible by RA (average 1088 units), demonstrating that the PE functions as an enhancer but that down-regulation in response to RA also occurs without the PE. Deletion of the PE and DE together showed low levels of Luc activity (average 1500 units) comparable to that of a construct with only the proximal (−150/+48) *bPOU5F1* promoter, and was independent of treatment with RA (Fig. 4).

The genetic localization of *bPOU5F1* was determined by segregation analysis of an SSCP in a 257-bp PCR product amplified from the region downstream of the predicted stop codon of the gene. Linkage analysis performed on the genotypes of offspring from three heterozygous IRRF sires placed *bPOU5F1* on bovine chromosome 23 in the region of the major histocompatibility complex (MHC), also known as the bovine leukocyte antigen (BoLA) complex. No recombinants were observed between *bPOU5F1* and the gene for 21-steroid hydroxylase (*CYP21*), the MHC class I locus *BoLA-A*, and microsatellite marker TAMLS113.3, with more than 30 informative meioses for each pair of loci (Table 2; [24]). As a result, the order of *bPOU5F1* with respect to these three closely linked loci could not be resolved. However, multipoint analysis (data not shown) resulted in the order (centromere) − *BoLA-*

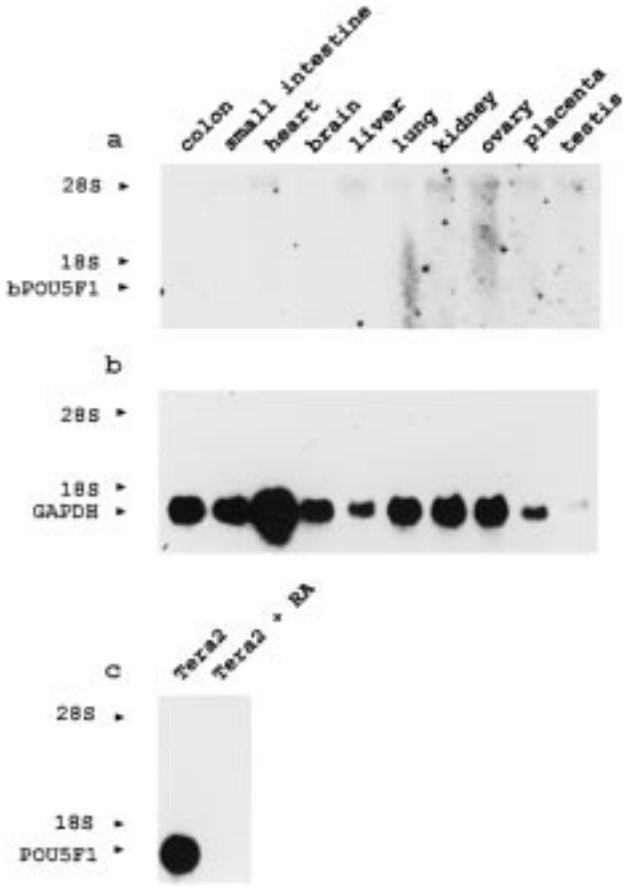

FIG. 5. Northern blot analysis of *POU5F1* in (**a**) bovine somatic tissues and (**c**) undifferentiated and RA-treated Tera2 EC cells. (**b**) GAPDH hybridization as control for RNA loading.

```
OCT29  5'-GTTCAGCCAAACGACTATCG-3'
                 EagI  5'-CGGCCG-3'
            180
bPOU5F1     AGCCAAACGACTATCTGCCGTTTTGAGGCTTTGCAGC 192
bPOU5F1rs1  -------------------A-----------------
bPOU5F1rs2  ---T---TT--A------T---------T----AG--

TCAGTTTCAAGAACATGTGTAAGCTGCGGCCCCTGCTGCAGAAGTGGGTGG 208
----------------------------------------------------
----------G------A-------T-------------G------A-
                 **
AGGAAGCTGACAACAACGAGAATCTGCAGGAGATATGCAAGGCAGAGACCC 226
----------------------------------------------------
-----A-------G--T-----G------------C--------A-AC---

TTGTGCAGGCCCGAAAGAGAAAGCGGACGAGTATCGAGAACCGAGTGAGAG 243
-------------------------------------------A--------
CCA--------T-G----------AAG-------G----G-T---------

GCAACCTGGAGAGCA::::::::::::::::TGTTCCTGCAGTGCCCGAAG 254
---------------:::::::::::::::::--------------------
----------C----ACAGGCAGAACAGAGGCC--------C-----A---

CCCACCCTGCAGCAAATTAGCCACATCGCCCAGCAGCTCGGGCTGGAGAAA 271
----------------------------------------------------
--------------:::::---TAG-G--------TT-G-----T-----G
  **
GACGTGGTCCGAGTG 276                          3'-CTTT
---------------
--T-::::::::::::

CTGCACCAGGCTCACA-5' OCT26
```

FIG. 6. Partial nucleotide sequences of *bPOU5F1*-related sequences *bPOU5F1rs1* (GenBank accession no. AF022988) and *bPOU5F1rs2* (GenBank accession no. AF022989) aligned with the predicted cDNA sequence of *bPOU5F1*. Only deviations from *bPOU5F1* are shown. Dashes (-) indicate identity with *bPOU5F1*. Double stars (**) indicate nucleotides that are interrupted by introns in *bPOU5F1*. Gaps introduced to maintain alignment are indicated by a colon (:). Corresponding amino acid positions of bovine Oct-4 are indicated on the right. The sequence of RT-PCR primer OCT29 and the resulting *Eag*I site in *bPOU5FI* are aligned at the top. The position of primer OCT26 is aligned at the bottom.

DRB3 (recombination fraction (θ) = 0.01; (log likelihood ratio (Llhr) = 6.62) − [*bPOU5F1, BoLA-A, CYP21*, TAMLS113.3] (θ = 0.01; Llhr = 0.39) − MGTG7 (θ = 0.05; Llhr = 5.10) − [RM185, prolactin (*PRL*)] − (tel) as most likely, which places *bPOU5F1* with strong statistical confidence between *BoLA-DRB3* and *PRL*.

Steady-state RNA levels of *bPOU5F1* in tissues from a newborn calf and adult animals was determined by Northern blot analysis using an homologous probe. No specific signal was detected in colon, small intestine, heart, brain, liver, lung, kidney, ovary, placenta, and testis tissues after 5 days of exposure (Fig. 5a) or after 12 days of exposure (data not shown). By contrast, *POU5F1* mRNA could be readily detected in undifferentiated Tera2 cells on a separate blot that was hybridized simultaneously and exposed for 2 days (Fig. 5c). GAPDH mRNA was detected in all bovine samples (Fig. 5b). These results suggest that there are very low levels of *bPOU5F1* mRNA present in these tissues or none at all.

The presence of *bPOU5F1* transcripts in oocytes and during preattachment development was determined by RT-PCR. Control PCR experiments using primers OCT12 and OCT15 and bovine genomic DNA as a template resulted in PCR products in the approximate size range of the 333 bp expected from *bPOU5F1*-derived cDNA, indicative of the existence of related (pseudo)genes (data not shown). Cloning of these products and sequencing of twelve randomly chosen clones revealed two distinct sequences, *bPOU5F1rs1* and *bPOU5F1rs2*, with 99.3% and 72.9% nucleotide identity, respectively, to the corresponding coding regions of *bPOU5F1* (Fig. 6). On the basis of this observation and the assumption that these *bPOU5F1*-related sequences could be transcribed, an RT-PCR assay specific for *bPOU5F1* was developed; primer OCT12 was omitted to eliminate amplification of *bPOU5F1rs2*, and primer OCT29 was designed to introduce an *Eag*I restriction site in PCR products derived from *bPOU5F1* but not *bPOU5F1rs1*, because of a nucleotide difference between these sequences at position 186 of the alignment depicted in Figure 6. Using this RT-PCR assay, specific amplification products were obtained from cDNA but not from control RNA of GV-stage oocytes and in vitro-fertilized and cultured 2-, 4-, and 8-cell-, morula-, and Day 9 blastocyst-stage embryos, ICM cells isolated from a Day 8 blastocyst, and Tera2cl13 cells as control (Fig. 7A). Digestion of these PCR products with *Eag*I resulted in partial cleavage in all samples (Fig. 7B), indicative of the presence of *bPOU5F1* transcripts. The origin of undigested PCR products that were also observed in all cDNA samples but not in RNA controls is not clear. The results of duplicate experiments were consistent for all stages examined. No amplification products were obtained from Day 14 and Day 16 blastocysts or from water controls (data not shown).

Immunocytochemical evaluation of Oct-4 expression in oocytes and embryos revealed expression in the cytoplasm and the nucleus of all stages examined until Day 10 of development (Figs. 8 and 9). Evaluation of stained specimens showed a marked nuclear localization of Oct-4 during segmentation but a more diffuse distribution upon formation of the blastocoel (Fig. 8). Oct-4 was detected with CLSM in ICM and trophectoderm cells of expanded and hatched blastocysts at Days 8, 9, and 10 after fertilization (Figs. 9 and 10). Oct-4 protein could not be detected in

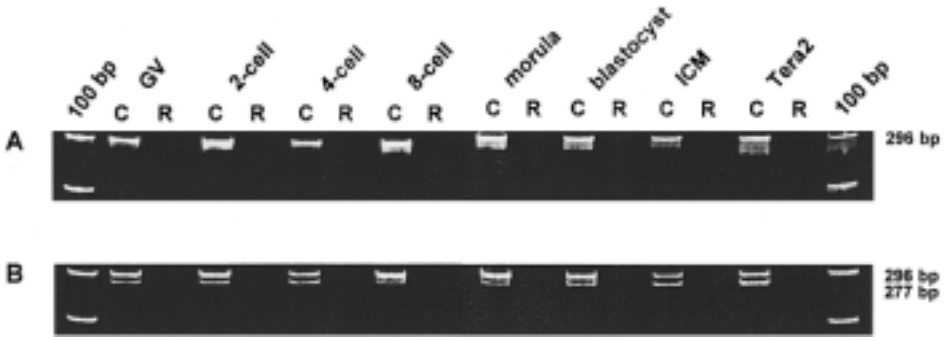

FIG. 7. RT-PCR analysis of *bPOU5F1* transcription in bovine GV-stage oocytes, in preattachment embryos at the 2-, 4-, 8-cell, morula, and blastocyst stages, and in inner cell mass (ICM) cells. Tera2 cells are included as positive control. Lanes containing cDNA (C) and equivalent amounts of RNA (R) are shown. Lanes labeled with 100 bp were loaded with a 100-bp ladder (Gibco-BRL) as reference for fragment size. **A**) 296-bp second-round PCR products; **B**) the same products after digestion with *Eag*I, which is expected to cleave off 19 bp from PCR products derived from bovine and human *POU5F1*. In all samples, a 277-bp product is generated, indicating that the *bPOU5F1* transcript is expressed.

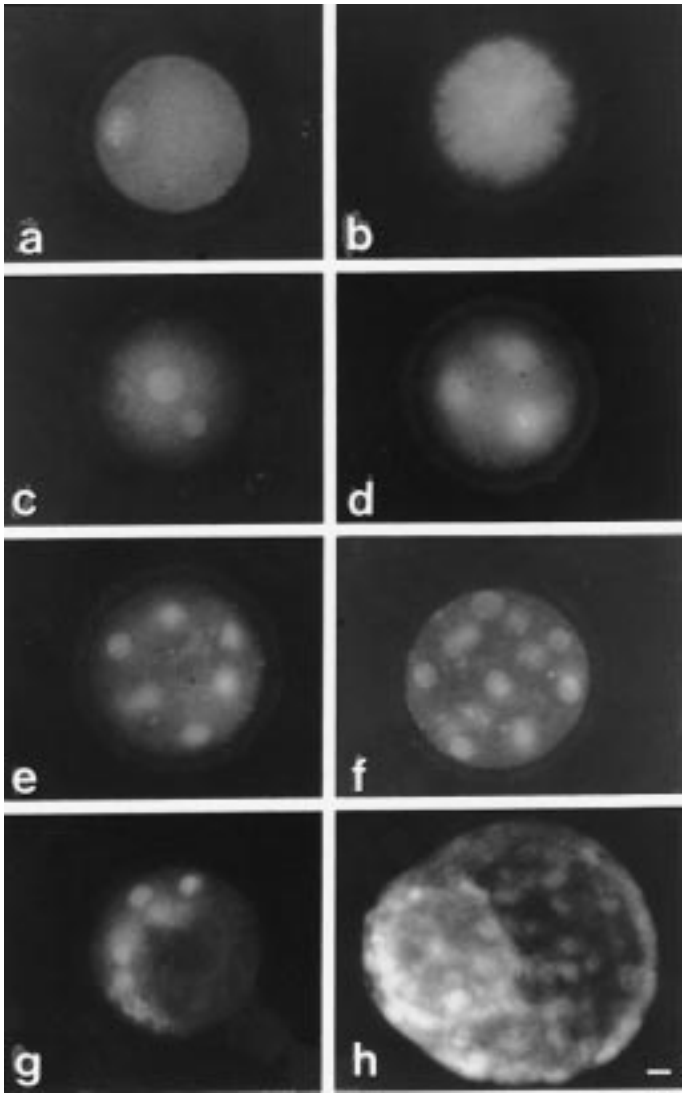

FIG. 8. Immunofluorescence analysis of Oct-4 in preattachment-stage bovine embryos. **a**) Primary oocyte (GV stage); **b**) secondary oocyte (metaphase-II stage); **c**) zygote; **d**) 4-cell embryo; **e**) 8-cell embryo; **f**) morula; **g**) early blastocyst (Day 6 post-IVF); **h**) hatched blastocyst (Day 8 post-IVF). Bar = 22 μm.

blastocysts at Days 14 (Fig. 10) and 16 (data not shown) of development. Oct-4 staining was visible in putative ICM cells and cells attached to the culture surface when blastocyst outgrowths were analyzed (Fig. 11). Control experiments resulted in nuclear staining of almost all undifferentiated P19 EC cells but not of somatic cells (data not shown). No staining was observed in control experiments using Oct-4 antibody preincubated with excess peptide or omitting the primary antibody (data not shown).

DISCUSSION

In this study, we describe the cloning and characterization of the bovine ortholog of *POU5F1*, with the aim of establishing its value as a marker for pluripotency of early bovine embryonic cells. Our results demonstrate that strong conservation of gene structure and sequence exists between *bPOU5F1* and its murine and human counterparts and that *bPOU5F1* is expressed in the early embryo. Further support for *bPOU5F1* as the true ortholog of the previously described mouse and human genes derives from the fact that *bPOU5F1* maps to the MHC in a region of conserved synteny between cow, human, and mouse [39], to which human *POU5F1* [40] and mouse *Pou5f1* [7, 16, 32] have been mapped before.

Expression of *bPOU5F1* at the RNA and protein levels was observed in bovine oocytes and embryos until Day 10 of development, consistent with a role in early embryo development, as has been postulated in the mouse [4, 5, 7, 8]. The most notable difference with expression of Oct-4 dur-

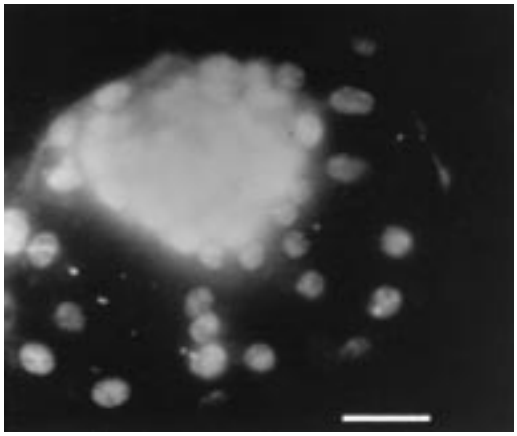

FIG. 9. Expression of Oct-4 in hatched bovine blastocyst (Day 9 post-IVF). Confocal image of single optical section in Z-axis. Bar = 23 μm.

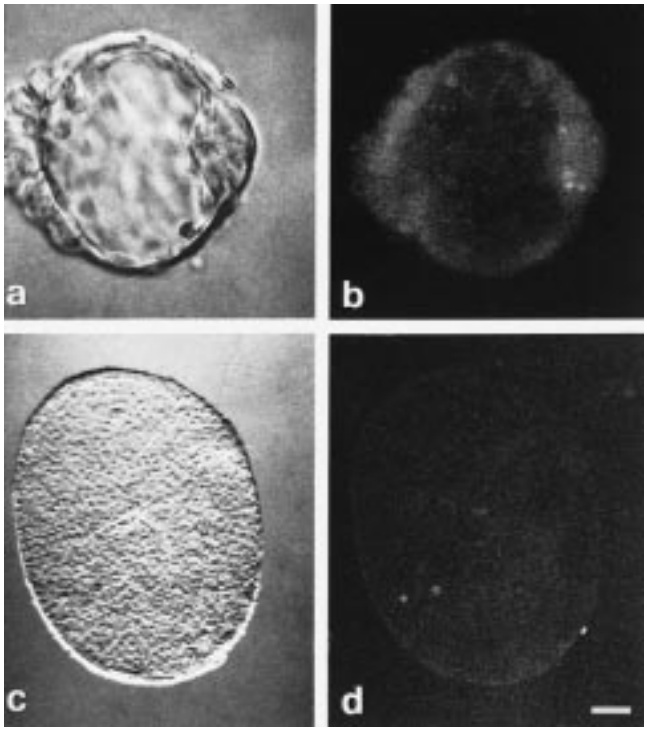

FIG. 10. Immunofluorescence detection of Oct-4 in Day 10 (**a, b**; bar = 29 μm) and Day 14 (**c, d**; bar = 120 μm) bovine blastocysts.

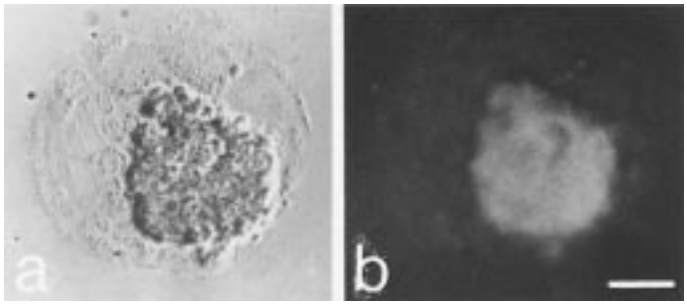

FIG. 11. Immunofluorescence detection of Oct-4 in bovine blastocyst outgrowth 13 days after fertilization. Bar = 85 μm.

ing murine preimplantation development [6] is that in cattle Oct-4 is detected at the (late) blastocyst stage in ICM and trophoblast cells; this is followed by coordinate down-regulation in these cell types around Day 10 postfertilization with no detectable expression after Day 12. Expression of Oct-4 in the bovine is thus not limited to pluripotent cells of the early embryo, which warrants careful interpretation when it is used as a marker for pluripotency of cultured (embryonic) cells.

Analysis of promoter and transcriptional control regions of *bPOU5F1* and transient transfections of Luc reporter constructs in P19 EC cells showed strong conservation of sequence and function with the orthologous regions in the mouse, especially at the proximal promoter and PE. The only exception is a bovine SINE element in *bPOU5F1*. It will be interesting to determine whether the different expression patterns of Oct-4 in trophoblast cells of cattle and mice are mediated by transcriptional regulatory elements or rather reflect other species-specific factors influencing *bPOU5F1* transcription or protein clearance. This hypothesis can be tested by microinjection of bovine reporter constructs in mouse embryos as described [13] and determining whether the expression follows that of the mouse or the cow.

McWhir and coworkers [41] described a method for derivation of ES cell lines from murine embryos with a nonpermissive genetic background using selective ablation of differentiating cells. In order to determine whether a similar approach might be feasible in cattle, the outgrowths formed at Day 13 from in vitro-produced Day 9 blastocysts were examined for expression of Oct-4 by immunofluorescence. Our observation of Oct-4 expression in all cells, including two morphologically distinct cell types that may account for the ICM with its immediate differentiated derivatives [42] and trophoblast cells, suggests that apart from the difficulty of producing transgenic animals, this approach might be more complicated in cattle than in mice. However, additional experiments with prolonged culture of blastocyst outgrowths or isolated ICMs, or the identification of independent specific markers for undifferentiated bovine embryonic cells, are needed to investigate this in more detail. Interesting in this respect is that alkaline phosphatase (AP) activity, which in the mouse is a marker for undifferentiated cells in vivo and in vitro, is also expressed in ICM and trophoblast cells of the bovine blastocyst but showed a heterogeneous staining pattern in plated ICM cells cultured for 2–4 days [42], suggesting that either bovine ICM cells rapidly differentiate in culture or that AP is not restricted to pluripotent cells in bovine embryos. Oct-4 thus seems to behave rather as AP in the early bovine embryo.

Brook and Gardner [43] presented an alternative strategy for the derivation of mouse ES cell lines by explanting blastocyst tissues that had been microsurgically isolated from the permissive 129 strain and several hitherto nonpermissive strains of mice. This study showed that ES lines originate from the epiblast and could be established with high efficiency when using epiblasts isolated from Day 5 blastocysts, either intact or as a single cell suspension. Efficiencies of ES line derivation even reached 100% when epiblasts from Day 9 delayed-implantation 129 strain blastocysts were used. The authors claimed that their method is superior to that described by McWhir and coworkers [41] on the basis of the high success rate, the fact that there was no question about the genetic background of the mouse strains involved, and the fact that the *neo* gene could still be used as selection marker of transfected cells once lines had been derived; they suggested that their approach may be applicable to other species. Our observation of a broader expression pattern of Oct-4 during bovine early embryonic development than in the mouse, together with the compelling results achieved by Brook and Gardner [43] argue that further attempts to derive pluripotent cell lines or ES lines in cattle should be directed at mimicking the latter approach using (isolated) ICM cells of preattachment embryos at different stages of development. A similar approach has earlier led to the birth of identical offspring following nuclear transfer from cultured bovine ICM cells but not to proven ES lines [17].

Cibelli and coworkers [44] recently reported the production of transgenic chimeric offspring from bovine ES-like cells derived from blastocysts resulting from nuclear transfer with transgenic fetal fibroblast cells. These ES-like cells, which could not be clonally propagated, lacked the differentiation markers vimentin and cytokeratin and were AP-negative. They were not investigated for Oct-4 expression. Although transgenic chimerism was demonstrated, includ-

ing in gonadal tissues, the authors refer to these cells as ES-like cells because germline transmission was not demonstrated. It therefore still remains to be seen to what extent differences in early embryological development between mice and cattle, which do not have a discrete epiblast, as well as culture conditions employed, will prove a surpassable barrier in establishing ES lines from early bovine embryos.

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