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Oct-6: a POU transcription factor expressed in embryonal stem cells and in the developing brain

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A family of octamer binding proteins is expressed during mouse development. Oct-4 and Oct-6 have been identified as two octamer binding proteins present in embryonal stem cells. Here we report the complementary DNA cloning and characterization of the mouse *Oct-6* gene. The protein of 448 amino acids contains a glycine/alanine-rich amino terminal region, a histidine-rich sequence with homology to a region of kininogen associated with clotting, a POU domain and a short proline/histidine-rich carboxy terminal region. Expression of Oct-6 in HeLa cells is sufficient for transcriptional activation from the octamer motif, identifying Oct-6 as a transcription factor. The *Oct-6* expression is downregulated upon embryonic stem cell differentiation increasing again during brain development. Expression in brain is present in certain areas of telecephalon, mesencephalon and brain stem with abundant expression in the cortex anlagen and in the developing colliculi. Thus Oct-6 is a new octamer binding transcription factor specifically regulated during mouse development.

Key words: brain development/embryogenesis/octamer/POU gene/transcription factor

Introduction

Development begins with a fertilized oocyte that gives rise to diverse cells with differing fates. Current models of development are based primarily on the molecular embryology of *Drosophila* and postulate a hierarchy of regulatory genes guiding the transfer of genetic information into embryonic structures (reviewed in Akam, 1987; Ingham, 1988). Establishment of diverse cell fates during embryogenesis requires differential gene expression that is modulated by specific regulatory proteins (reviewed in Davidson, 1990). The identification of murine regulatory proteins specific for early stages of embryogenesis might unravel important members of a regulatory hierarchy in the mouse.

Members of a family of Oct-proteins bind to the octamer motif (Schöler *et al.*, 1989a), a *cis*-acting regulatory element found in many promoters and enhancers and stimulate transcription via the octamer motif (reviewed in Falkner *et al.*, 1986; Hatzopoulos *et al.*, 1988; Schaffner, 1989; Schreiber *et al.*, 1989). In undifferentiated embryonal carcinoma (EC) cells, the octamer motif mediates both transcriptional activation (Schöler *et al.*, 1989b) and repression (Lenardo *et al.*, 1989). Three octamer binding proteins might account for these opposite activities, namely Oct-4, Oct-5 and Oct-6 (Schöler *et al.*, 1989a). Oct-4 and Oct-5 are encoded by the *Oct*-4 gene (Schöler *et al.*, 1990a; 1990b).

Here we report the cDNA sequence of the third EC octamer binding protein Oct-6 as well as its expression during mouse development. The cDNA encodes a protein of 448 amino acids containing a POU domain (Herr *et al.*, 1988) identical to Tst-1 and SCIP (He *et al.*, 1989; Monuki *et al.*, 1989). A stretch of 8 amino acids between the potential helices 1 and 2 of the POU homeodomain has been conserved between both EC proteins Oct-6 and Oct-4, although both proteins otherwise diverge. Ectopic expression of *Oct*-6 cDNA was sufficient to activate transcription from the octamer motif, indicating that Oct-6 is a transcription factor. Expression of the *Oct*-6 gene during embryogenesis was analyzed by *in situ* hybridization, Northern blot analysis, RNase protection and DNA binding studies. We demonstrate *Oct*-6 expression in embryonal stem cells and in developing brain and adult testis. With the proteolytic clipping assay the presence and identity of Oct-6 in brain cortex is shown. These studies suggest that the transcription factor Oct-6 might control events at very early and late stages of murine development.

Results

Identification of cDNA clones encoding Oct-6

Four DNA-protein complexes are detectable with the octamer motif in undifferentiated F9 EC cells in the electrophoretic mobility shift assay (EMSA) (named Oct-1, Oct-4, Oct-5 and Oct-6 according to Schöler *et al.*, 1989a). A cDNA library prepared from F9 cells was screened with a probe spanning the mouse *Oct*-2 POU domain (Hatzopoulos *et al.*, 1990). Three groups of clones encoding octamer binding proteins were isolated. Clones of the first group encoded Oct-1 and those of the second Oct-4 and Oct-5 (Schöler *et al.*, 1990a). About one-third of the recombinant clones, comprised a third group and were tentatively assigned as incomplete *Oct*-6 clones.

These cDNAs encode a protein with a POU domain identical to rat Tst-1 and SCIP (He *et al.*, 1989; Monuki *et al.*, 1989). Probes from both, *Tst*-1 and *SCIP*, detect brain mRNA (He *et al.*, 1989; Monuki *et al.*, 1989). The POU domain of Tst-1/SCIP is almost identical to Brn-1 and Brn-2 (He *et al.*, 1989). Thus, although the POU domains for Tst-1/SCIP and our F9 clones are identical, they may be derived from different genes. To test this and in order to obtain longer cDNA clones, we screened a mouse brain cDNA library with a probe spanning the 5′ region of our

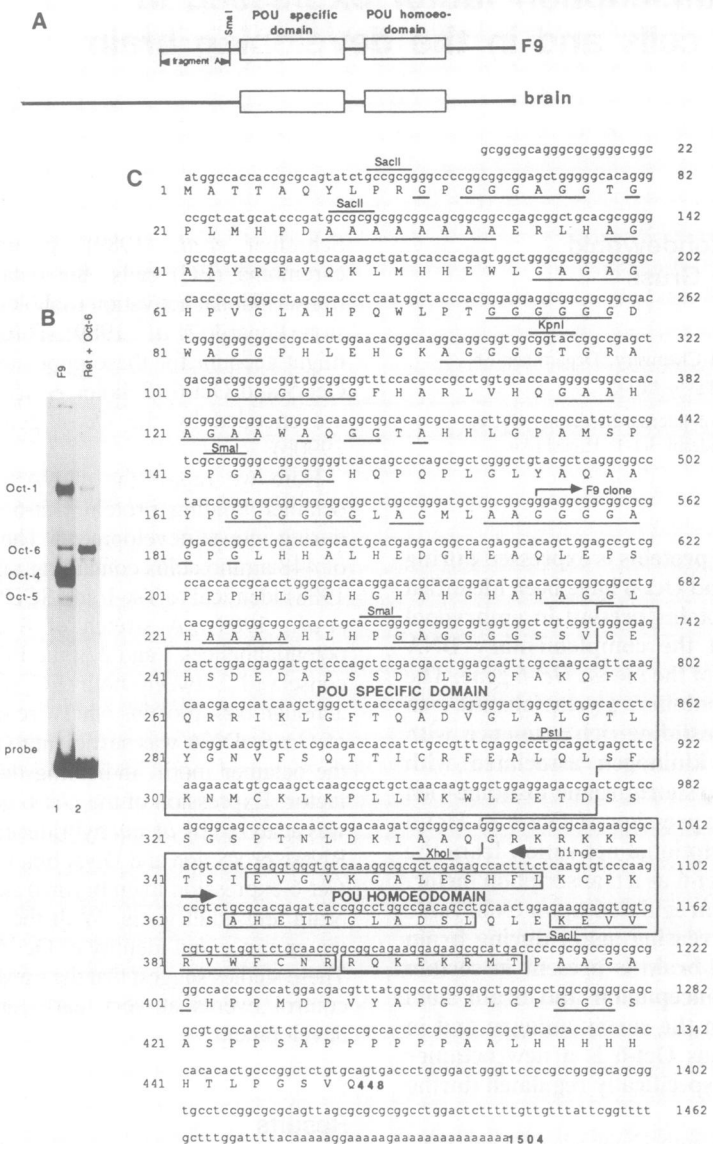

Fig. 1. Structure and sequence of mouse *Oct*-6. (**A**) Structure and length of the longest *Oct*-6 cDNA clones derived from F9 (960 bp) and brain (1504 bp) libraries are shown. The POU-specific domain, the POU-homoeodomain and the fragments used for the analysis of the *Oct*-6 gene are indicated. Fragment A: nt 549−nt 708 (160 bp). (**B**) Comparison of the product of the brain cDNA cone with F9 octamer binding proteins. Radiolabeled octamer probe was incubated before electrophoresis with a F9 whole cell extract (lane 1) or with *in vitro* translated product of the brain cDNA clone (lane 2). Before transcription the clone was restricted at the unique *Bam*HI site in the linker 3′ of the coding sequence. For generation of anti-sense RNA the clone was cut at the unique *Hin*dIII site in the linker 5′ of the coding sequence. The sense and anti-sense RNA was translated with a reticulocyte lysate and analyzed in the EMSA as described in Materials and methods (anti-sense RNA translation not shown). The nomenclature of the octamer binding proteins is according to Schöler *et al.* (1989a). (**C**) Nucleotide sequence of the entire cDNA sequence derived from the longest brain cDNA clone. Numbering (right, nucleotide sequence; left, amino acid sequence in single-letter code) begins at the putative initiation codon. The POU specific domain, the POU homoeodomain and the four possible helices of the POU homoeodomain are boxed. The 5′ end of the longest F9 clone, the possible hinge region between helices 1 and 2 of the POU homoeodomain and certain restriction sites are indicated. Alanine/glycine-rich sequences are underlined.

clones and lacking any POU sequences (probe A; Figure 1A).

The inserts of the clones isolated by this procedure were analyzed by sequential *in vitro* transcription, translation and finally by DNA binding in the EMSA. The product of the longest clone (brain, Figure 1A) had the same mobility as Oct-6 (Figure 1B). Nucleotide sequence comparison (Figure 1C) and Southern blot analysis of mouse genomic DNA (not shown) demonstrated that the F9 and brain clones are derived from the same gene. Therefore the brain *Oct*-6 cDNA clone was used in subsequent experiments.

Expression pattern of the Oct-6 gene

The expression pattern of the *Oct*-6 gene was examined first by Northern blot analysis. Given the strong sequence similarity between the POU domains of the *Oct*-genes, this domain was excluded from hybridization probes. Fragment A (Figure 1A), hybridized to transcripts of 3.6 and 2.8 kb length in RNA from F9 stem cells, testis and brain (Figure 2A). These transcripts were not detected in any other tissue. In F9 cells the signal obtained for *Oct*-6 RNA is weaker than that of *Oct*-4 RNA and thus correlates with Oct-6 and Oct-4 binding activity found in EMSAs (not shown). Additionally,

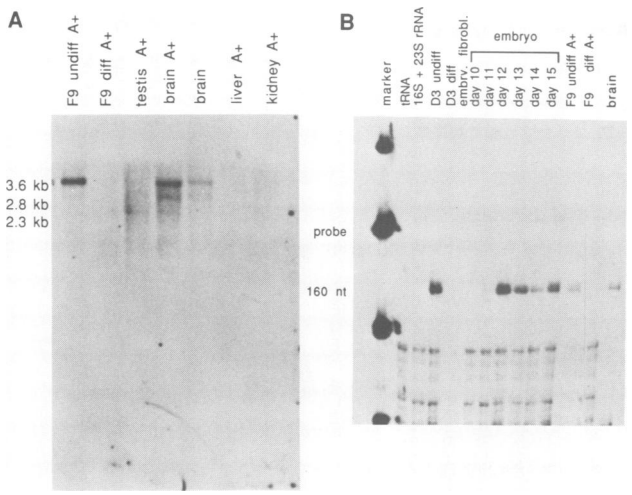

Fig. 2. Expression pattern of the Oct-6 gene. (**A**) Northern blot analysis with F9 cell RNA and RNAs of a limited number of adult tissues. Poly $(A)^+$ RNAs (20 μg) prepared from the indicated samples were run on a formaldehyde–agarose gel and were analyzed by Northern blot hybridization. In case of brain RNA both, poly $(A)^+$ RNA and whole cell RNA were analyzed. RNAs in each lane were intact based on ethidium bromide staining and hybridization with a *ras* DNA probe. (**B**) Autoradiograph of RNase protection assays with RNAs representing different embryonic stages and from adult brain as indicated at the top of the figure. The position of the full probe and the protected probe (152 nucleotides) are indicated. 30 μg total RNA was used in the experiments, except for 5 μg F9 RNA.

the probe hybridized to a 2.3 kb mRNA, in brain and testis RNA but not in F9 RNA. The 3.6 kb RNA represents the major *Oct*-6 mRNA in F9 stem cells and brain. In testis each RNA species is found less abundant and in approximately equimolar amounts. The lengths of these mRNAs are distinct from those found in testis and brain of adult rat where they are 4.4 and 7.6 kb, respectively (He *et al.*, 1989). The predominant 3.6 kb *Oct*-6 mRNA might correspond to the predominant Tst-1/SCIP species (3 kb) in postnatal and young adult rats (Monuki *et al.*, 1989).

RNase protection assays confirmed the identity of the reactive RNAs as authentic transcripts of the *Oct*-6 gene (Figure 2B). Anti-sense RNA was prepared from the same fragment as used for the Northern blot analysis. Again, *Oct*-6 transcripts were detected in F9 cells, in brain and in testis (Figure 2B; testis RNA not shown), again no signal was obtained with RNA from differentiated F9 cells (Figure 2A and B). Thus the decrease of *Oct*-6 RNA correlates with the decrease of Oct-6 binding activity during F9 stem cell differentiation (Schöler *et al.*, 1989a). Using the RNase protection assay the analysis was extended to the embryonal stem (ES) cell line D3 (Doetschman *et al.*, 1985) (Figure 2B). Because ES cells are totipotent stem cells derived from the inner cell mass of murine blastocysts (Gardner and Rossant, 1979; Evans and Kaufman, 1981; Martin, 1981), these cells represent a very early embryonic stage. As in F9 cells, a protected band was obtained with D3 stem cell but not with D3 cells differentiated *in vitro* (Figure 2B). Finally, expression was examined in mouse embryos from day 10 to day 15. Weak expression was detected at embryonic day 10 and day 11, respectively. By day 12 a strong signal was obtained, indicating that *Oct*-6 expression increases significantly at about embryonic day 11 (Figure 2B).

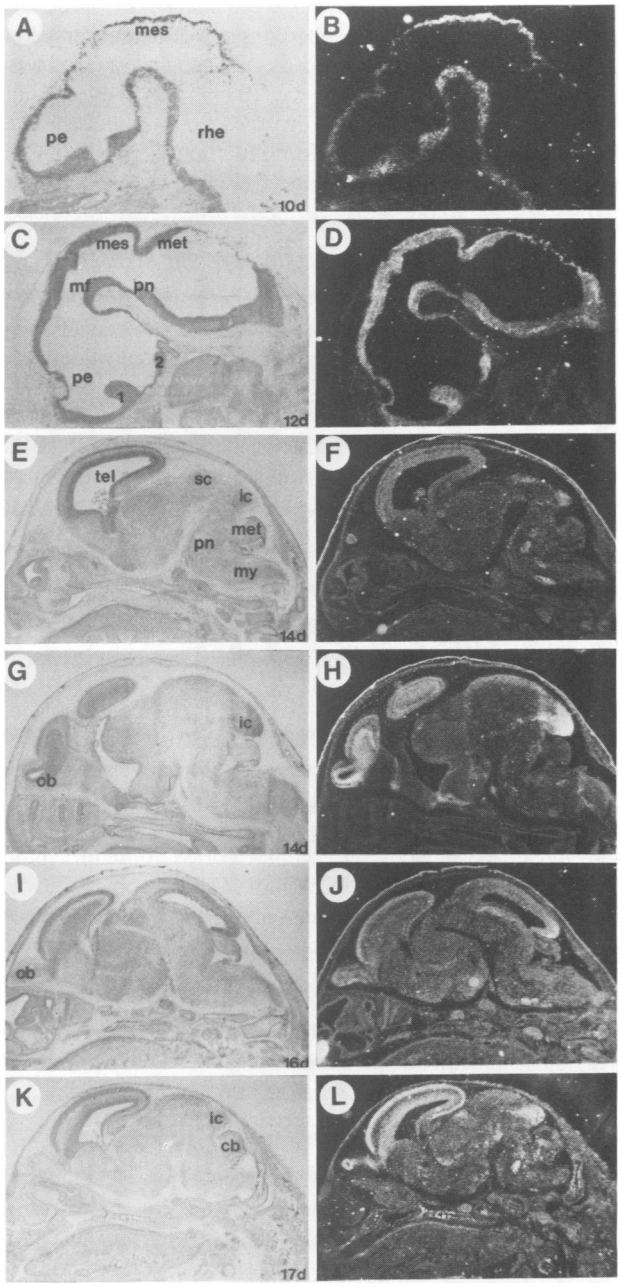

Fig. 3. Localization of *Oct*-6 transcripts within the developing brain. Mid-sagittal and para-sagittal sections through brains of different embryonic days were hybridized with an Oct-6 specific anti-sense RNA probe. Bright-field (left) and dark-field images (right) show brain sections of day 10 (A, B), 12(C, D), 14 (E, F, G, H), 16 (I, J) and 17 (K, L) embryos. (cb) indicates cerebellum, (ic) inferior colliculus, (mes) mesencephalon, (met) metencephalon, (mf) mesencephalic flexure, (my) myelencephalon/medulla, (ob) olfactory bulb, (pe) prosencephalon (pe 1) prospective striatum, (pe 2) prospective thalamus, (rhe) rhombencephalon and (sc) superior colliculus.

Spatial expression of the Oct-6 gene during mouse development

The distribution of *Oct*-6 transcripts during mouse embryogenesis was determined by *in situ* hybridization. Sense and anti-sense RNA probes were transcribed from linearized plasmids containing a 160 bp *Oct*-6 cDNA insert (Figure 1A, Fragment A).

To determine the precise expression pattern in the developing central nervous system, sagittal sections of

embryos from day 10 to day 17 were analyzed (Figure 3). Continuous expression in defined regions of prosencephalon (pe), mesencephalon (mes) and rhombencephalon (rhe) was observed (Figure 3). Brains of day 10 embryos showed faint expression in cell clusters in the floor plate of the prosencephalon, mesencephalon and rhombencephalon as well as in the mesencephalic roof (Figure 3A and B).

By day 12 expression in these areas had increased significantly (Figure 3C and D). Two distinct cell groups were visible in the prosencephalic floor, one in the ventral half of the prospective striatum (pe 1), the other in a region that gives rise to the thalamus (pe 2). The prosencephalic roof, which develops into the diencephalic roof plate, was also positive for *Oct*-6 expression. In the mesencephalon, expression included the ventral mesencephalic flexure. A signal was detectable in the mesencephalic roof, which gives rise to superior (sc) and inferior (ic) colliculi and also in the metencephalon which gives rise to the cerebellum (cb). *Oct*-6 expression was found also in the developing pons (pn) and myelencephalon/medulla (my). All zones of expression are within the intermediate layer of the developing central nervous system, where neurons and glioblasts start to differentiate (Rickman and Wolff, 1985).

At day 14 the number of *Oct*-6 positive cells in the intermediate layer had increased and formed aggregates. Expression was found within the ventral pons and the ventral medulla. Positive cell aggregates were found ventral of the prospective striatum, along the mesencephalic flexure, and within the thalamus (not shown). In addition, expression was detected in the developing cerebral cortex, olfactory bulb (ob), but not within the cerebellum. *Oct*-6 expression was also found in the superior colliculi and strongly in the inferior colliculi of the mesencephalic roof. Both colliculi serve as centers for visual and auditorial correlation. The superior colliculi are primary receptive centers for the optic tracts and are linked to the inferior colliculi which, in turn, connect with the cochlear nuclei of the pons.

A similar pattern was observed for day 16 and day 17, with increased expression in the cerebral cortex (Figure 3 I to L). Expression continued in the cerebral cortex and the olfactory bulb of adult mice, whereas superior colliculus and the thalamus were negative for *Oct*-6 expression (not shown).

In situ hybridization was also used to investigate *Oct*-6 transcription at the blastocyst stage. However, in contrast to results obtained with the *Oct*-4 gene (Schöler *et al.*, 1990b), *Oct*-6 transcripts could not be detected (data not shown).

Expression of Oct-6 protein in embryonal stem cells and in the developing brain

In a previous report we described a DNA protein complex specfic for the frontal cortex and subcortical regions of rat brain (Schöler *et al.*, 1989a). Prolonged electrophoresis showed that the mobility of this complex was identical to that of F9 Oct-6 and slightly higher than that of brain Oct-3. The brain-specific protein had been originally named Oct-3 by us (Schöler *et al.*, 1989a) but unfortunately the same term is now used by others (Okamoto *et al.*, 1990; Rosner *et al.*, 1990) for the Oct-4 protein (Schöler *et al.*, 1989a, b; 1990a, b). To distinguish F9 Oct-3 from brain Oct-3 and to avoid further ambiguities, those octamer-binding proteins that are expressed in the nervous system will be named NOct proteins.

As a first step to determine the relationship of F9 Oct-6,

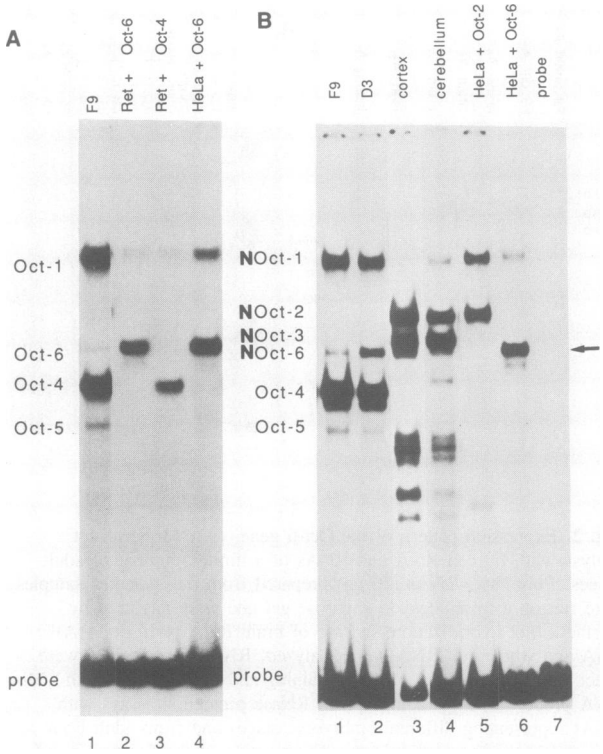

Fig. 4. Oct-6 is present in ES cells and brain. (**A**) Comparison of Oct-6 translated *in vitro* with Oct-6 ectopically expressed in HeLa cells. Before application to the EMSA, Oct-6 was either expressed by *in vitro* transcription and subsequent translation in a rabbit reticulocyte extract (lane 2) or by a SV40 promoter-driven expression vector in HeLa cells (lane 4). As controls, Oct-4 translated *in vitro* (lane 3), and F9 octamer binding proteins are shown (lane 1). For details of expression see legend of Figure 7. (**B**) Comparison of embryonal stem cell Oct-6 and NOct-6. Radiolabeled octamer probe was incubated before electrophoresis with whole cell extracts of either undifferentiated F9 cells (lane 1), D3 stem cells (lane 2), cortex (lane 3) or cerebellum (lane 4) of day-3 newborn mice, and with Oct-6 (lane 6) or Oct-2A (lane 7) expressed in HeLa cells. The octamer binding proteins are named according to Schöler *et al.* (1989a).

NOct-6 and NOct-3 we examined if Oct-6 is modified *in vivo*. For this purpose, Oct-6 prepared by *in vitro* translation of the *Oct*-6 cDNA was compared in the EMSA with Oct-6 produced ectopically in HeLa cells (for expression details see below). In both cases the protein gave a band at the same position, indicating that Oct-6 is either not modified in HeLa cells or that the modification is not detectable in the EMSA (Figure 4A).

Next we examined if cells and tissues containing *Oct*-6 RNA give a complex with identical mobility in the EMSA. Therefore, the Oct-6 protein was expressed in HeLa cells and compared with F9, D3 and cortex extracts (Figure 4B). Cerebellum of newborn mice was used as a negative control, as *Oct*-6 RNA had not been found by *in situ* analysis. Moreover, cerebellum extract gives most of the Oct complexes in the EMSA; the complexes not found in cerebellum, are in extracts of F9, D3 or cortex. Each source that had been shown to be positive for *Oct*-6 RNA gave a complex at the expected position (compare lane 6 with lanes 1 to 3), whereas the cerebellum is negative for both *Oct*-6 RNA and NOct-6 protein (lane 4). In this experiment the presence of *Oct*-6 RNA correlates with that of the NOct-6 complex.

The analysis of the NOct-6 complex was initially hindered

because in addition to NOct-3 another complex was comigrating with NOct-6 in the EMSA. The protein is found in virtually every cellular extract with a slightly higher mobility than NOct-6 (for example Figure 8A, where this complex is indicated with an arrow). Competition experiments and binding to fragments with the mutated octamer motif defines it as an unspecific binding protein (not shown). The unspecific protein is either found after long exposure times or when low amounts of competitor are used (Figure 8A).

Identification of brain Oct-6 by the proteolytic clipping assay

The presence and identity of Oct-6 in brain was confirmed by the proteolytic clipping assay (Schreiber *et al.*, 1988; Schöler *et al.*, 1989a). The octamer binding proteins of cortex and cerebellum from newborn mice were compared with Oct-1, Oct-2A and Oct-6 expressed in HeLa cells (for expression see below). Whole cell extracts were first incubated with the octamer probe and subsequently digested with different amounts of the nonspecific endoprotease dispase (Figure 5A to C; Oct-1 and Oct-2 not shown). A comparison of the proteolytic clipping intermediates showed that the pattern of cloned Oct-6 (A) is missing in the experiment with cerebellum extract (C), but could be identified in the experiment with cortex extract (B). Actually, the cortex pattern appears to be a combination of that of both other extracts as schematically presented in Figure 5 D to F.

Although extracts of different brain regions contain quite a complex set of proteins, the proteolytic clipping assay demonstrates that NOct-6 is encoded by the *Oct*-6 gene and therefore the term Oct-6 will be subsequently used for brain and F9 Oct-6. This analysis had been simplified by almost missing NOct-1 and NOct-2 degradation intermediates. When cloned Oct-1 and Oct-2 expressed in HeLa cells are proteolytically degraded, definite bands are only seen for the final DNA binding domains (not shown). Fortunately, these have a higher mobility than that of NOct-3 and Oct-6 as such do not obscure the pattern of NOct-3 and Oct-6 clipping (not shown).

The Oct-6 protein is found in the developing cortex but not in the cerebellum

The expression pattern of Oct-6 during brain development was further analyzed by EMSA using extracts of brain regions at different developmental stages of the mouse. Cortex, metencephalon (at later stages cerebellum) and brainstem of embryos, newborn and adult mice were analyzed (Figure 6). Only a weak Oct-6 signal is detectable at embryonic day 12 or 15, much less prominent than NOct-3 (lanes 1 to 6). Subsequently, a complex is found both in cortex and brain extracts, whereas Oct-6 remained absent in every extract derived from cerebellum (lanes 7 to 19). In summary, the Oct-6 complex is faintly detectable in brain of day 12 embryos, increasing significantly thereafter in cortex and brainstem. Thus the appearance of the Oct-6 complex follows that of *Oct*-6 RNA. In addition to the Oct-complexes described previously (Schöler *et al.*, 1989a) a complex with the same mobility as Oct-2B could be identified in brainstem and metencephalon (Figure 6).

Structure of the Oct-6 protein

The sequence of the Oct-6 protein was derived from the longest open reading frame of the brain *Oct*-6 cDNA clone

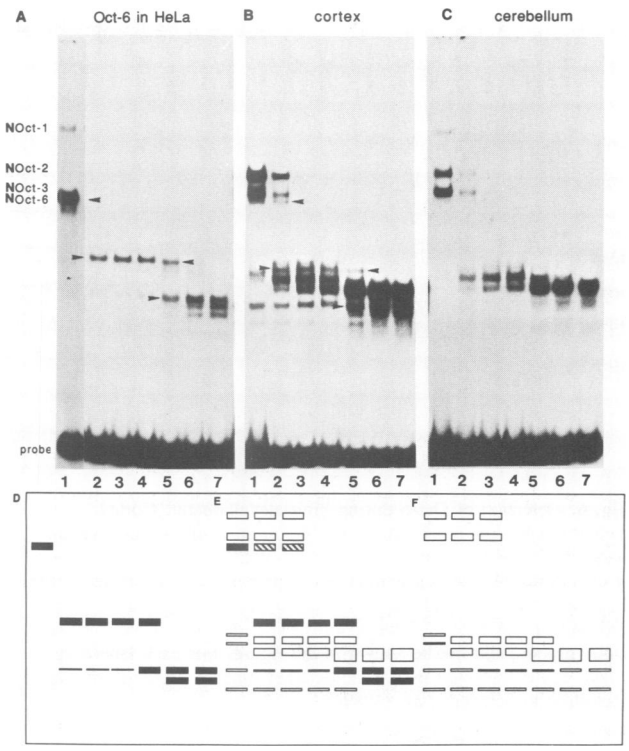

Fig. 5. The degradation pattern of cloned Oct-6 is found with cortex but not with cerebellum extracts. Whole cell extracts of HeLa cells containing ectopically expressed Oct-6 (**A**), extracts of cortex (**B**) and of cerebellum (**C**) from day-3 newborn mice were incubated in parallel with radiolabelled octamer probe. The extracts are identical to those used for lanes 9 and 10 of Figure 6. Prior to electrophoresis increasing amounts of dispase were used. The first lane of each set is without dispase, the following lanes are with 0.5 (lanes 2), 1 (lanes 3), 2 (lanes 4), 4 (lanes 5), 6 (lanes 6) and 8 (lanes 7) μg dispase. The positions of cloned Oct-6, NOct-6 and their respective degradation intermediates are indicated with arrows. Below each analysis the clipping patterns of cloned Oct-6 (**D**) and the octamer binding proteins found in cortex (**E**) and cerebellum (**F**) are shown schematically. Black or cross-striped boxes represent Oct-6 and respective degradation intermediates, white boxes representing complexes and degradation intermediates that are found in cerebellum. For each lane 2 μg protein were used, except for the very first lane (Oct-6 in HeLa, without dispase), where 8 μg was used.

(Figures 1C and 7). *Oct*-6 cDNA encodes a protein of 448 amino acids containing a POU domain that is identical to Tst/SCIP and thus belongs to POU class III (He *et al.*, 1989). The POU domain is located in the carboxy-terminal half of the protein with 239 and 53 amino acids at the amino- and carboxy-termini, respectively. As with other POU homeodomains, the POU homeodomain of Oct-6 is predicted to contain four helices with clusters of basic amino acids preceding the first helix, and overlapping the end of the third and most of the fourth helix (Otting *et al.*, 1988; Garcia-Blanco *et al.*, 1989; Qian *et al.*, 1989). Another region with basic amino acids is located in the center of the POU-specific domain. Sequence comparison shows that both ES proteins, Oct-6 and Oct-4, contain an identical stretch of eight amino acids. This stretch is located between helix one and two of the POU homeodomain and thus might form a hinge between both helices (Figure 1C; Schöler *et al.*, 1990a).

The Oct-6 sequence has four cysteine residues all located in the POU domain, two in the POU-specific domain and two in the POU homeodomain. Unexpectedly, binding of Oct-6 was lost when stored for several weeks at −70°C,

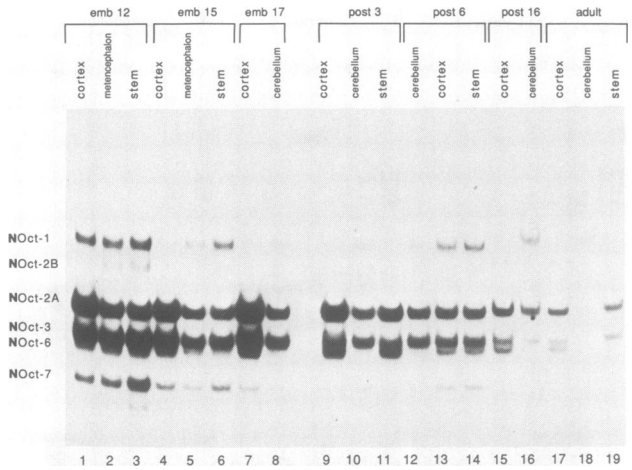

Fig. 6. Detection of Oct-6 during brain development. Cortex, metencephalon (or cerebellum) and brainstem of the developmental stages indicated above the figure were isolated according to Hogan *et al.* (1986). Whole cell extracts were prepared and incubated before electrophoresis with radiolabeled octamer probe. To separate NOct-3 and NOct-6 the time of electrophoresis was doubled. Therefore the DNA probe could not be shown in the figure. For each lane 6 μg whole cell extract was used. The octamer binding proteins are named according to Schöler *et al.* (1989a).

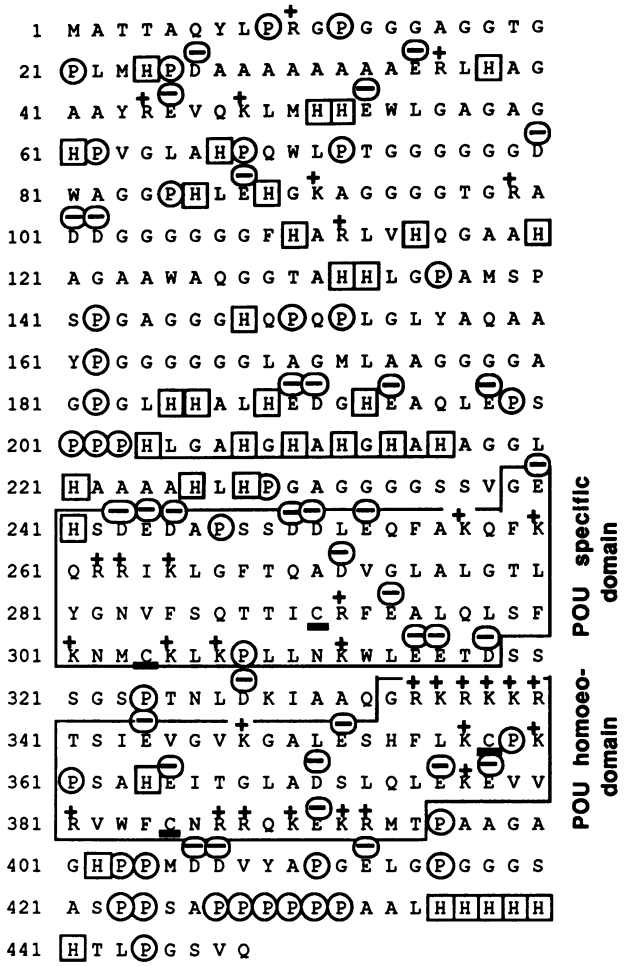

Fig. 7. Predicted amino-acid sequence (single-letter code) of Oct-6. The POU specific domain and the POU homoeodomain − and + indicate acidic and basic amino acids, respectively. Histidine residues are boxed and proline residues are circled. The cysteine residues are underlined with thick bars.

whereas binding of Oct-1 and Oct-2 was not affected. However, Oct-6 binding was restored completely when preincubated with DTT, suggesting that the disulfide bridges need to be disrupted before binding (not shown).

Several sequence features outside the POU domain are noteworthy, but of unknown significance. First several contiguous glycine-alanine stretches of up to eight residues are present. Polyalanine stretches of various lengths are in *Drosophila* developmental control genes such as engrailed, even-skipped and caudal (Poole *et al.*, 1985; McDonald *et al.*, 1986; Frasch *et al.*, 1987).

Second, a histidine rich region (30%) is found from position 185 to 228 amino-terminal to the POU domain. This includes alternating histidine and glycine (or alanine) residues (HLGAHGHAHGHAHA) and a cluster of four acidic amino acids. A similar alternating sequence motif (HGLGHGHKHGHGHG) is found also in the histidine-rich region of high molecular weight (HMW) kininogen (Kato *et al.*, 1983; Lottspeich *et al.*, 1985). Interestingly, this region is associated with the clotting activity of HMW kininogen. In bovine HMW kininogen a 41 amino acid fragment (fragment 2; 27% histidine) with this motif is released by the action of plasma kallikrein, and hereby the clotting activity is lost (Iwanaga *et al.*, 1979).

Third, proline residues distributed throughout the amino-terminal half of Oct-6 are interspersed with twelve acidic and seven basic amino acids. Another region harboring both proline and acidic amino acid residues follows the POU domain. Whether any of these acidic regions participate in transcriptional activation, as has been described for other acidic domains, remains to be tested (reviewed in Mitchell and Tjian, 1989).

Finally, contiguous stretches of six proline and of six histidine residues each are found at the carboxy-terminus of Oct-6. Even longer rows of proline and histidine residues are found in other proteins, although, again their significance is unknown. For example, two stretches of ten and eight histidine residues are found in *Drosophila* polycomb (R.Paro, personal communication). Polyproline stretches are found in several viral proteins. For example, the activity of the HSV transactivator VP16 is increased by another viral protein with a polyproline at the carboxy-terminus (McKnight *et al.*, 1987).

Transactivation by cloned Oct-6

Depending on the context of other transcription elements, the octamer motif confers transcriptional activation (Schöler *et al.*, 1989b) or repression (Lenardo *et al.*, 1989) in F9 cells.

The transactivation activity of Oct-6 was compared with that of Oct-2A (Müller-Immerglück *et al.*, 1988) and of Oct-4 (Schöler *et al.*, 1990b). For this purpose, the *Oct-6* coding sequence was cloned in both orientations into an eukaryotic SV40 expression vector (Figure 8C). The expression plasmids were transfected into HeLa cells together with β-globin reporter plasmids containing either a normal or a mutated octamer site in front of the β-globin TATA box (Figure 8D). Both target vectors carry a SV40 enhancer downstream of the β-globin gene. This particular arrangement of TATA box and octamer motif is transactivated by Oct-2A (Müller-Immerflück *et al.*, 1988; 1990).

Expression of Oct-6, Oct-4 and Oct-2A were confirmed by the EMSA (Figure 8A). In the absence of a functional

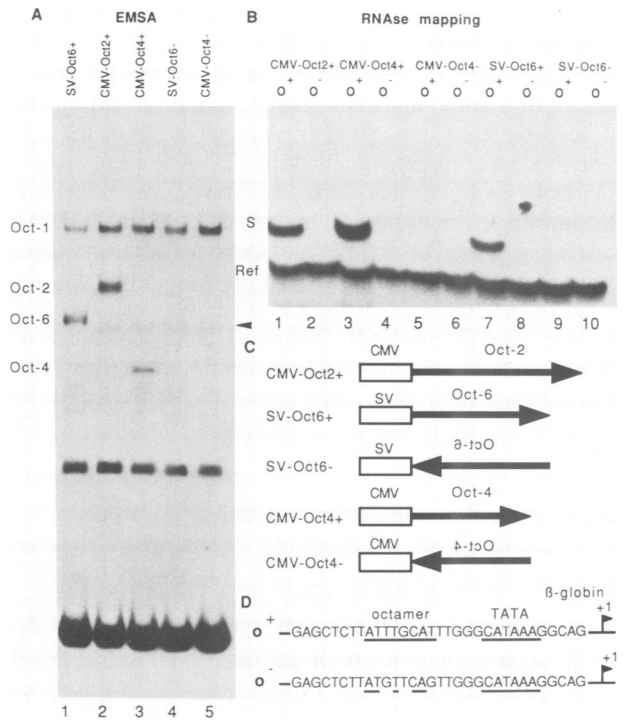

Fig. 8. Transcriptional activation via the octamer motif by cloned Oct-6. (**A**) EMSA of ectopically expressed octamer binding proteins. Whole cell extracts of HeLa cells with cotransfected Oct-2A, Oct-4 or Oct-6 expression vectors were prepared and incubated before electrophoresis with radiolabeled octamer probe. The vectors are indicated above the figure and are outlined in (C). Lanes 1 to 3 are from experiments with expression vectors with sense-orientated DNA, lanes 4 and 5 with DNA orientated in anti-sense. In contrast to the standard EMSA procedure half the amount of unspecific competitor was used. A unspecific complex with a slightly higher mobility than Oct-6 is marked with an arrow at the right side of the figure (for discussion see text). (**B**) Transactivation via the octamer site as determined by RNase protection. Lanes with uneven numbers: cotransfection with an unmutated octamer motif (o^+). Lanes with even numbers: cotransfection with a mutated octamer motif (o^-). The position of the testgene (S) and of the reference gene (Ref) are indicated at the left side of the figure. The expression vectors and reporter vectors are indicated above the figure and outlined in (C) and (D), respectively. (**C**) Expression vectors used for transactivation: CMV-Oct2+, SV-Oct6+, SV-Oct−, CMV-Oct4+, CMV-Oct4−. + represents sense-orientation, − represents antisense-orientation of the respective gene. Oct-2 and Oct-4 are driven by the CMV enhancer/promoter, Oct-6 by the SV40 enhancer/promoter. (**D**) The reporter plasmids contain either octamer motif sequences (o^+) or a mutated octamer motif (o^-) in front of the β-globin gene. A SV40 enhancer is located in both vectors downstream of the β-globin gene. The reporter plasmids and their analysis are described by Müller-Immerglück *et al.* (1988; 1990).

transactivator (Figure 8A, lanes 4 and 5), β-globin expression was not detectable (Figure 8B, lanes 5, 6, 9 and 10). However, cotransfection of functional Oct-6 induced gene expression significantly via the octamer motif (lanes 7 and 8). This stimulation is comparable to that of Oct-2A and Oct-4 (lanes 1 and 3), clearly demonstrating that Oct-6 functions as a transactivator.

The 6W enhancer, containing six tandem repeats of a mouse immunoglobulin heavy chain gene enhancer fragment, is highly active in F9 stem cells where Oct-4 and Oct-6 are present (Schöler *et al.*, 1989b). Because transactivation experiments in HeLa cells with cloned *Oct*-4 were not successful (Schöler *et al.*, 1990b), we wanted to determine if Oct-6 is responsible for enhancer activation. For this purpose, we used the same reporter vectors as for the Oct-4 analysis (Schöler *et al.*, 1990b), but again transactivation of the 6W enhancer could not be achieved (not shown). Therefore, none of the cloned octamer binding porteins show enhancer activation but can only activate via an octamer motif near the TATA box (Müller-Immerglück *et al.*, 1988; 1990; Schöler *et al.*, 1990b; Tanaka and Herr, 1990; Okamoto *et al.*, 1990).

Discussion

We have reported cloning of a cDNA encoding the new transcription factor Oct-6 activating transcription via the octamer motif. As with the other embryonal stem cell protein, *Oct*-4, *Oct*-6 is downregulated upon differentiation, but is later expressed again in cells of the developing brain and in testis. The temporal and spatial expression of *Oct*-6 was analyzed by Northern blot analysis, RNase protection, *in situ* hybridization and DNA binding studies. A clear correlation of Oct-6 RNA and Oct-6 protein is shown. The proteolytic clipping assay confirmed the presence and identity of Oct-6 as one of several octamer binding proteins in brain.

Oct-6 is encoded by the isolated cDNA

Several lines of evidence indicate that the cDNA described in this study encodes Oct-6. First, four octamer binding proteins (Oct-1, Oct-4, Oct-5 and Oct-6) were detected in F9 stem cells. 18 clones from the F9 cDNA library fall into three different groups, two encoding Oct-1, and Oct-4/Oct-5 (Schöler *et al.*, 1990a). The third group comprises five cDNA clones of a new gene coding for an octamer binding POU protein. Second, the RNA detected with an *Oct*-6 specific probe is in agreement with the presence of DNA binding activity. Third, brain Oct-6 is identified due to its clipping pattern as the protein encoded by the cloned *Oct*-6. Finally, the protein encoded by the cDNA has the same mobility in the EMSA as F9 Oct-6 and brain Oct-6. The brain and F9 *Oct*-6 cDNA clones are identical, except for 500 bp missing at the 5′end of the F9 cDNA. Thus we cannot exclude the different exons are used for generating the amino-terminus, although F9 and brain Oct-6 have the same size in the EMSA, and the 3.6 and 2.8 kb *Oct*-6 mRNAS are found both in brain and in F9 cells.

Relationship of mouse Oct-6 and rat Tst-1/SCIP

The identity of the POU domain sequence indicate that *Oct*-6 and *Tst*-1 (He *et al.*, 1989) and *SCIP* cDNAs (Monuki *et al.*, 1989) are derived from homologous genes in mouse and rat. A comparison of the restriction maps of Oct-6 and SCIP supports this notion. The POU domain of both proteins is located close to the carboxy terminus. Furthermore, each restriction site in the SCIP coding region (*ApaI, PstI, SacII*) is found at a similar position in that of Oct-6, although Oct-6 has additional sites not found in SCIP (*SacII, KpnI*). The different mRNA sizes and expression patterns might be due to species specific differences, but might also indicate expression from a different gene. This is not unlikely, because several members of POU class III are almost identical (He *et al.*, 1989).

In the EMSA brain Oct-6 and NOct-3 almost have the same mobility. However, the proteolytic clipping pattern of both clearly demonstrate that both proteins are different (Figure 5). Moreover, NOct-3 is present where *Oct*-6 RNA

is not found (Figures 3 and 6). Therefore both proteins are most likely derived from different genes and not due to different modifications of the same protein. Candidates for genes encoding NOct-3 are *Brn*-1 and *Brn*-2 which are both expressed in most regions of the rat brain (He *et al.*, 1989). NOct-3 is probably not encoded by *Brn*-3, because *Brn*-3 RNA is not found in rat cortex, whereas NOct-3 protein is.

Oct-6 expressed in embryonal stem cells, germ cells and in brain

The embryonal stem cell line D3 and several other ES cell lines (not shown) derived from the inner cell mass (ICM) of blastocysts, contains Oct-6 protein, as does the embryonal carcinoma cell line F9. Although Oct-6 RNA and protein is definitely present in embryonal stem cells we could not demonstrate *Oct*-6 expression in the blastocyst by *in situ* hybridization. In contrast, *Oct*-4 expression was readily detectable (Schöler *et al.*, 1990b). This might be due to a low level of *Oct*-6 expression as suggested by comparing Oct-6 and Oct-4 expression in ES and EC cells, or to limited transcription in only a few cells of the ICM. Moreover, although we have detected Oct-6 in all of six different ES and EC cell lines tested our experiments cannot rule out that Oct-6 expression is induced by the very act of culturing EC and ES cells. However, to our knowledge this has not been shown for any gene expressed in ES cells. Oct-6 is also expressed in primordial germ cells as determined by the EMSA (not shown), furthermore in testis as shown by Northern blot analysis (Figure 2).

Oct-6 expression during the migratory phase of cortical neurogenesis is readily detectable in the cortical plate, subependymal zone and in the olfactory bulb. The widespread distribution of expression at embryonic day 12 and 14 and its expression in the cortical plate at day 17 indicate that *Oct*-6 is expressed in differentiating neurons. This is similar to *Tst*-1/*SCIP* expression in rat brain (He *et al.*, 1989). However, at least two differences are found between rat *Tst*-1/*SCIP* and mouse *Oct*-6 expression. In contrast to decreasing *Tst*-1/*SCIP* expression during brain development, *Oct*-6 expression increases during the same period. Furthermore, in contrast to *Tst*-1/*SCIP* expression in rat cerebellum, no *Oct*-6 expression was detectable in this brain region. *Tst*-1/*SCIP* is expressed by myelinating glial cells (Schwann cells and oligodendrocytes), but not by nonmyelinating glia (astrocytes). Because expression of *Tst*-1/*SCIP* precedes induction of the major myelin genes, it might play a role in cell-type specific expression of these genes (Monuki *et al.*, 1989).

Oct-6 protein is present in embryonal stem cells and in the developing brain. Therefore this transcription factor could be regulating different aspects of development. Since *Oct*-6 expression is turned down upon differentiation of ES cells and is strongly increased at about day 11, the *Oct*-6 gene might be expressed in a biphasic manner. In the first phase it might act prior to other putative control genes, such as Hox or Pax genes (reviewed in Dressler and Gruss, 1988; Holland and Hogan, 1988; Kessel and Gruss, 1990). In the latter case it might take part in setting up the temporal and spatial patterns in mammalian brain development. Moreover, because Oct-6 is also found in cells of the adult brain, it might also be required for the maintenance of certain cellular functions and thus act, similar to Oct-2, as a cell-specific transcription factor.

The expression of *Oct*-6 is reminiscent of Drosophila homeodomain genes such as *fushi tarazu* (*ftz*), which is expressed early in development to play a critical role in pattern formation, and later reappears in a limited number of neurons (Doe *et al.*, 1988). Another protein, the *Cf1a* gene product of *Drosophila* binds to a DNA element required for expression of the dopa decarboxylase in selected dopaminergic neurons (Johnson and Hirsch, 1990). Interestingly, the POU domains of Oct-6 and Cf1a are highly conserved differing only in 12 of 140 amino acids. This includes the linker of 17 amino acids between the POU specific domain and POU homoeodomain which usually differs greatly amoungst different POU domain proteins. Besides these similarities, their amino acid sequences differ, except for having histidine rich clusters surrounding the POU domain.

Oct-6: activator, repressor or both?

Oct-6 and Oct-4 are two positively regulating transcription factors present in ES and EC cells (Lenardo *et al.*, 1989; Schöler *et al.*, 1989a,b; Schöler *et al.*, 1990a,b; Okamoto *et al.*, 1990; Rosner *et al.*, 1990). Repression and activation via the octamer motif has been described in F9 cells (Lenardo *et al.*, 1989; Schöler *et al.*, 1989b). Repression was shown with an immunoglobulin heavy chain gene enhancer fragment harboring the μE3, μE4 and octamer sites placed in front of a truncated promoter of the *fos* gene (Lenardo *et al.*, 1989). The positive effect of μE3 on the promoter was blocked by binding of proteins to the octamer motif. It remains to be tested, which octamer binding protein is responsible for repression. However, it would not be surprising if one or both of the cloned Oct-genes is responsible for both activities, because activation and repression have been described for several other eukaryotic transcription factors (reviewed in Levine and Manley, 1989).

Oligomerization of the octamer motif creates a potent enhancer in ES and EC cells (Schöler, 1989b). However, transactivation of this enhancer in HeLa cells by cloned *Oct*-4 and *Oct*-6 could not be achieved. Therefore either an inhibitory factor present in HeLa cells or a stimulatory factor present in the ES and EC cells but missing in HeLa, might account for this effect. Interestingly, both ES proteins contain the same stretch of eight amino acids located between helix one and two of the POU homoeodomain and thus might form an identical hinge. Both helices surrounding this sequence show about 40% homology to Oct-4, the highest homology of the Oct-4/Oct-6 hinge to members of the other POU classes (according to He *et al.*, 1989) is 50%. We speculate that this conserved amino acid reflects an interaction with another protein. This would be similar to the interaction between the HSV protein VP16 with Oct-1 (Gerster *et al.*, 1988; Triezenberg *et al.*, 1988; Kristie *et al.*, 1989) at the next hinge between helix two and three (Stern *et al.*, 1989). Expression of VP16 in cells merely containing Oct-1 activates the octamer motif in otherwise inactive promoters (O'Hare and Goding, 1988; O'Hare *et al.*, 1988; Tanaka *et al.*, 1988). Whether a similar interaction between the conserved Oct-4/Oct-6 hinge with a (stem cell specific) factor exists, and particularly, if enhancer activation in stem cell is due to such an interaction, will be tested.

Oct-6 is present in ES cells, in the developing and in the adult brain. Therefore this transcription factor could be regulating very early developmental proceses, acting prior

to other putative control genes, such as *Pax* or *Hox* genes. It will be interesting to determine if Oct-6 regulates members of these families (Kessel and Gruss, 1990). Furthermore, Oct-6 may regulate late processes, such as brain development. Taken together, we think that *Oct*-6 is a good candidate for a gene involved in control events of murine development.

Materials and methods

Standard techniques of molecular biology were performed as in Sambrook *et al.*, (1989), standard techniques for mouse embryology were performed as in Hogan *et al.*, (1986).

Cell lines and transfections for transactivation

HeLa, F9 and D3 cells were grown and transfected as described elsewhere (Schöler and Gruss, 1984; Schöler *et al.*, 1989a, b). HeLa cells were transfected by 8 μg reporter plasmid, 4 μg expression vectors and 1 μg reference plasmid and 7 μg Bluescript as carrier DNA. After harvesting of the cells two days later, 1/10 of the cells were used for the microextraction procedure. The RNA of the rest was extracted and analyzed by RNase protection. The transactivation analysis was done twice.

Preparation of the F9 and brain cDNA library

A directional cloned F9 cDNA library in the λ expression vector λZAPII (Stratagene) was constructed as described (Schöler *et al.*, 1990a), a mouse brain cDNA library in the λ expression vector λZAPII was constructed with the cDNA synthesis kit (Pharmacia) in accordance with the manufacturer's specification. Sequencing was performed as described previously (Schöler *et al.*, 1990a).

Recombinant plasmids and oligonucleotides

SV-Oct6+ and SV-Oct6− were obtained by cloning the complete Oct-6 coding sequence in a SV40 expression vector based on pBR322. Expression is driven by the SV40 enhancer/promoter region (*Pvu*II to *Hin*dIII), one 72 bp deleted in the SV40 enhancer (Tooze, 1981). A 610 bp *Sau*3A fragment of SV40 with the small t-intron and a 257 bp SV40 fragment (*Bcl*I to *Bam*HI) with transcriptional termination sequences follow the promoter fragment. The *Hin*dIII between promoter and t-intron was converted to *Bgl*II. For cloning of *Oct*-6 the plasmid containing the *Oct*-6 cDNA was cut with *Hin*dIII and *Bam*HI in the polylinker upstream and downstream of the cDNA, respectively. The ends were blunt-ended and cloned in both orientations into the blunt-ended *Bgl*II site of the expression vector. The orientation of the insert was determined by restriction with *Xho*I and *Bam*HI. CMV-Oct4+ and CMV-Oct4− clone were described by Schöler *et al.* (1990b), CMV-Oct2A(+) by Müller-Immerglück *et al.* (1988). The octamer containing probe was also described previously (1W in Schöler *et al.*, 1989a).

In vitro translation in reticulocyte lysates

In vitro transcription and translation of *Oct*-4 and *Oct*-6 was performed as described previously (Schöler *et al.*, 1990a). Before *in vitro* transcription the *Oct*-6 cDNA clone was restricted with *Bam*HI to obtain sense RNA with T7 RNA polymerase or with *Hin*dIII to obtain anti-sense RNA with T3 RNA polymerase.

Microextraction procedure for the preparation of whole extracts, electrophoretic mobility shift assay (EMSA) proteolytic clipping

These methods were performed as described previously (Schöler *et al.*, 1989a; 1990b).

Northern blot analysis and RNase protection assay

Northern blot analysis was performed as described previously (Schöler *et al.*, 1990a). RNase protection assays were performed essentially as described by Balling *et al.*, (1989).

In situ hybridization

The techniques employed for *in situ* hybridization are in Schöler *et al.*, (1990b). A 160 bp *Oct*-6 cDNA fragment (fragment A in Figure 1A) was cloned into Bluescript (Stratagene) and linearized with an appropriate restriction endonuclease. RNA probes were transcribed using T3 and T7 polymerases according to the manufacturer's instructions (Promega).

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