Studies on the POU gene Oct-6 in ectoderm cell differentiation

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Studies aan het POU gen Oct-6 in ectodermale cell differentiatie

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Introduction

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1. General introduction

The development of multicellular organisms is a complex process of cell proliferation, cell differentiation and cell death. It results in a great diversity of different cell types, each of which fulfils a specific function in the adult organism. The developmental pathway of an individual cell is dependent on the combined temporal expression of a specific set of genes. Gene expression is primarily regulated by transcription factors, which bind in a sequence specific manner to distal enhancer elements, and interact with other proteins to modulate the activity of the basal transcription factors and the RNA polymerase which are assembled at the proximal gene promoter (109, 140).

Several sequence specific DNA binding motifs have been described that are conserved among the members of different transcription factor families. One of these contains with the homeobox DNA binding domain which was identified as a highly conserved 60 amino acid region in the homeotic gene products of the fruitfly *Drosophila melanogaster*. The homeodomain forms a helix-turn-helix DNA binding motif and is both structurally and functionally conserved in all animals (2, 113). Disruption of homeobox gene function leads to homeotic transformations of the body plan, presumably by impairing the transcriptional regulation of genes which further specify the regional identity of cells along the body axis (84).

Structural variants of the helix-turn-helix motif are present in e.g. the winged-helix and POU DNA binding domains (29, 86). The POU DNA binding domain was first described as a conserved region of 150 to 160 amino acids in the mammalian Pit-1, Oct-1 and Oct-2 proteins and the *Caenorhabditis elegans* protein Unc-86 (67). The Unc-86 protein is required for specification of the neuronal identity of several neuroblast cell lineages, whereas the Pit-1 gene product is essential for the development of the thyrotrope, somatotrope and lactotrope cell lineages of the anterior pituitary gland (45, 101). The ubiquitously expressed protein Oct-1 and the B-cell specific protein Oct-2 were identified on the basis of their ability to bind *in vitro* the critical *cis*-regulatory octamer element of the immunoglobulin gene promoters and enhancers (164). Despite this, they differ in their ability to interact with other cellular and viral proteins, providing Oct-1 and Oct-2 with distinct properties in transcriptional regulation (30, 54, 105, 165, 170).

The POU domain transcription factors thus appear to form an important family of developmental regulators. Based on the strong conservation of the sequences coding for the POU DNA binding domain additional POU genes have been isolated and characterized. This chapter reviews the POU family members and their role in neuronal cell differentiation and development.

2. POU transcription factors

2.1 The POU protein family

POU domain proteins have been identified in a variety of species ranging from insects and worms to mammals. Outside the POU domain no sequence similarities are apparent, except for a number of single amino acid stretches for which no function has been firmly assessed (147). Based on the conservation of primary amino acid sequences of the POU DNA binding domain, the family of POU transcription factors has been subdivided into seven classes (63, 185). A phylogenetic tree of the POU protein

family is presented in figure 1 and shows the relative homologies among the different class members and between the different classes of POU proteins. Classes II, III and IV contain both vertebrate and *Drosophila* POU proteins, while genes encoding POU proteins belonging to classes I, V, VI and VII are not present in the insect genome. This suggests that genes of the class II, III and IV POU proteins are the oldest in evolutionary terms and that the other POU genes have appeared later in evolution.

Alternative splicing has been shown to result in the synthesis of variant POU proteins with potentially very distinct regulatory properties, thus increasing the functional complexity of this family of transcriptional regulators. Proteins produced through alternative splicing of the pituitary- and epidermis-specific genes Pit-1 (GHF-1) and Skn-1a/i (Oct-11) have been identified to differentially regulate transcription of the prolactin and keratin 10 (K10) gene promoters, respectively (3, 173). In contrast, six alternatively spliced Oct-2 gene products have been identified in the developing B-cell lineage. They all retain an intact POU domain and are able to activate an octamer containing promoter in transient assays. Furthermore, they show overlapping expression patterns in the differentiating B-cell lineage, making it difficult to envisage functionally distinct roles for the Oct-2 splice variants in B-cell development (189).

In contrast to the POU genes named above, the mammalian class III proteins Brn-1, Brn-2, Brn-4 and Oct-6 are encoded by intronless genes. However, it has been suggested that the Brn-2 gene encodes three Brn-2 protein isoforms through the use of alternative in frame AUG start codons (160). Whether these proteins are present in Brn-2 gene expressing cell types *in vivo* remains to be substantiated. The notion that the class III genes are devoid of introns has led to the hypothesis that these genes are duplication products of a common ancestor gene which had integrated into the genome as a retrotranscription product (93, 55). Like the other POU genes, the class III POU genes are dispersed throughout the genome suggesting that they have acquired independent mechanisms of gene expression regulation (9, 190).

2.2 The POU DNA binding domain

Sequence-specific DNA binding of the POU proteins is mediated by a 150-160 amino acid sequence called the POU domain. It consists of two highly conserved regions, a carboxy-terminal 60 amino acid POU homeodomain (POU_{HD}) and an amino-terminal 75-82 amino acid POU specific (POU_{SP}) domain. Both domains are connected by a linker sequence of variable length which is conserved only among the class II and III POU proteins (reviewed in references 69 and 185). The structure and amino acid sequence of the POU homeodomain is closely related to the homeobox DNA binding domain of the homeotic gene products. The POU homeodomain has a characteristic cystein residue at position 9 of the third, DNA recognition helix of the helix-turn-helix DNA binding motif, while other classes of homeobox containing proteins are characterised by a serine (Paired proteins) or glutamate (Hox proteins) residue at this position. The amino acid sequence conservation of the POU specific domain among members of one class of POU proteins is higher than the sequence conservation in the linked POU homeodomain. Elucidation of the solution structure of the POU specific domain has shown that it forms a tetra-α-helical structure which bears strong similarity to the DNA binding domains of the bacteriophage lambda and 434 repressors (7, 33).

The POU homeodomain, like the DNA binding domain in other homeobox genes, binds with high affinity to A/T rich sequences, whereas the entire POU domain

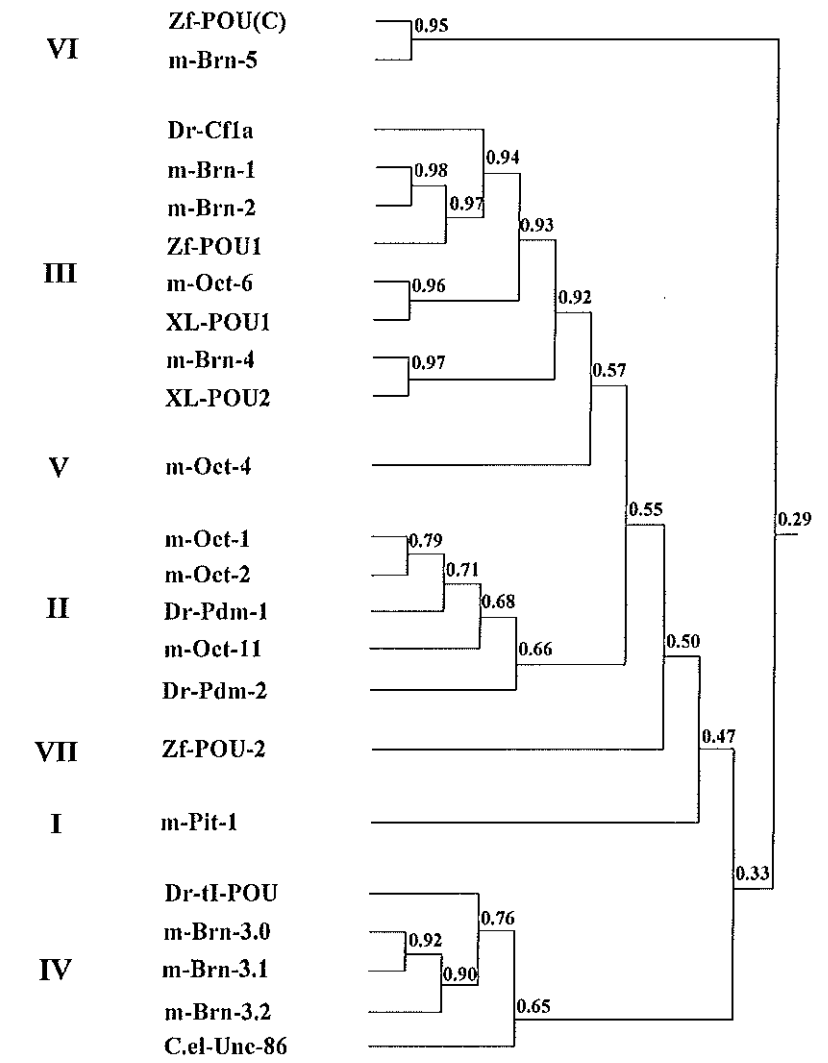

Figure 1. Phylogenetic tree of the POU protein family based on the homology in the amino acid sequence of the POU DNA binding domains. The prefixes indicate the species from which the protein was isolated: C.el, Caenorhabditis elegans; Dr, drosophila; m, mouse; XL, xenopus laevis; Zf, zebrafish. The sequences were aligned with the DNAMan Multiple sequence alignment program. The figures denote the relative sequence conservation.

binds a longer core motif, resembling the consensus octamer sequence ATTTGCAT. Footprinting analysis with purified POU and POU_{HD} peptides of the Oct-1 and Pit-1 proteins on the octamer sequence of the Adenovirus serotype 2 (Ad2) origin and the Pit-1 response element in the rat prolactin gene promoter revealed that the POU_{HD} contacts the A/T rich left half of the octamer sequence whereas the POU_{SP} is required for base contacts with the right half of the octamer motif (73, 183). Although the POU_{SP} domain, like the lambda repressor DNA binding domain, has only a weak DNA binding affinity, it enhances the binding affinity and selectivity of the entire POU domain in comparison with POU_{HD} alone (184). A crystal structure of the Oct-1 POU domain bound to the H2B octamer sequence confirmed the positions of the Oct-1 POU subdomains on the DNA (86). Furthermore, it showed that no protein-protein contacts are made between the two POU subdomains indicating that the POU DNA binding domain consists of two separate DNA binding structures which form a single functional unit. This is further strengthened by the recent observation that two unlinked POU_{HD} and POU_{SP} subdomains are able to bind cooperatively to the octamer consensus sequence (87). DNA binding of a POU domain thus consists of three independent structural units and it has been proposed that the linker sequence tethers the two POU subdomains in high local concentrations near the DNA.

Studies on the mechanism of DNA binding revealed strong differences between members of different classes of the POU protein family (fig.2). The class III Brn-2 protein permits a spacing of 0, 2 or 3 nucleotides within the bipartite DNA sequence motif, whereas DNA binding of the class IV Brn-3 protein depends on a 3 nucleotide spacing between the two halfs of the octamer sequence (102). Furthermore, the recognition sequence of the Brn-2 and Brn-3 POU_{SP} domains is in an inverted position when compared to the orientation of the POU_{SP} binding sequence in the Pit-1, Oct-1 and Oct-2 DNA binding motifs. This results in an altered position of the Brn-2 and Brn-3 POU specific domains on the DNA and changes the relative orientation of the Brn-2 and Brn-3 POU specific and homeodomains (figs.2a and 2b). The ability of POU domain proteins to bind DNA sequence motifs with different spacing and orientation of the half sites thus results in clear differences to bind enhancer elements of a variety of different genes. In addition, protein modification has been shown to strongly affect DNA binding characteristics. Oct-1 becomes hyperphosphorylated on a serine residue at position 7 of the POU_{HD} when a cell enters mitosis, reducing the Oct-1 DNA binding affinity (161). Phosphorylation of Pit-1 on a threonin at the same position leads to a conformational change of Pit-1 on the DNA decreasing its DNA binding affinity for one binding site (growth hormone promoter), but increasing it for the other (prolactin promoter) (82). The modifiable serine/threonin is located in the N-terminal part of the POU_{HD} which makes critical minor groove contacts (86). This and the strong conservation of the serine residue among the POU family members indicate that phosphorylation might be a more general mechanism to regulate DNA binding.

2.3 POU-protein interactions

Despite the high degree of sequence homology the POU domains contribute to the specificity in DNA binding site selection and binding affinity of the individual POU proteins to regulate expression of a specific set of affector genes. Apart from the above mentioned characteristics the POU domain provides an important interface for protein-protein interactions which increases the cell-specific activity of POU proteins. Both POU-POU homodimerization as well as interactions with heterologous proteins have been shown to have profound effects on the

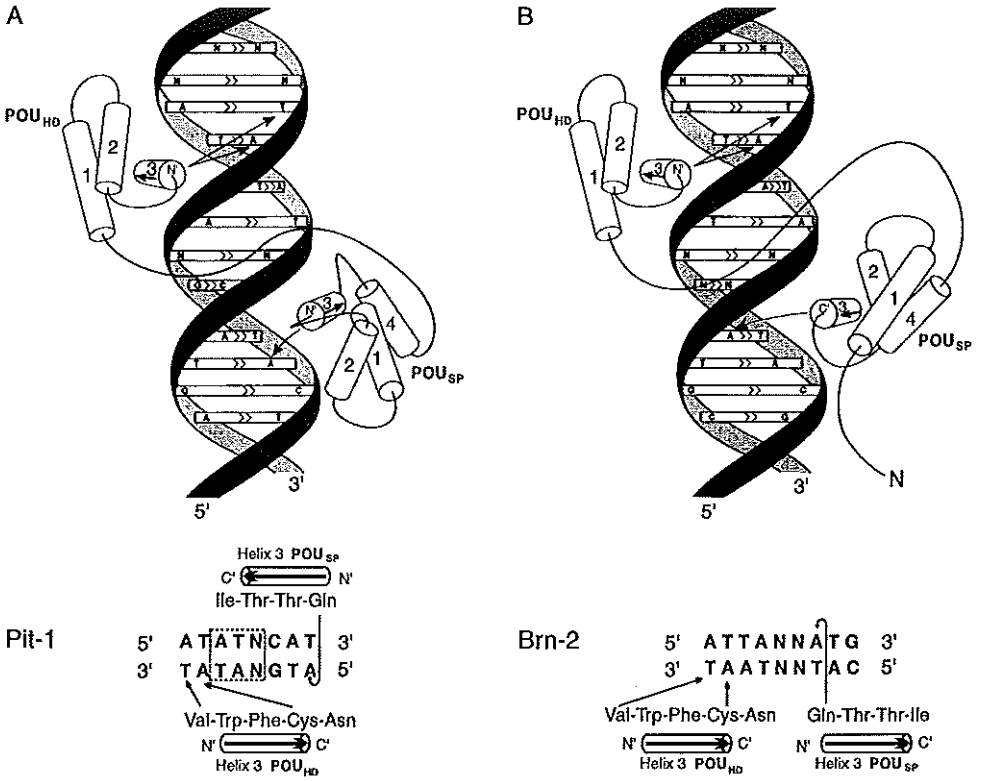

Figure 2. Diagrams showing the position of the Pit-1 (A) and Brn-2 (B) POU domains on their respective DNA recognition motifs. The α-helices present in the POU specific domain (POU_{SP}) and POU homeodomains (POU_{HD}) are depicted as cylinders. DNA-contacts made by POU_{SP} and POU_{HD} in the major grooves are indicated with arrows, as is the orientation of the DNA recognition helices. Below, a more schematic presentation is drawn. Notice that unlike the POU_{SP} domain in Pit-1, the Brn-2 POU_{SP} domain binds DNA on the opposite strand, but is oriented in the same direction as the POU homeodomain (This figure is taken from reference 102).

regulation of gene expression (see Table 1).

Functional POU-POU homodimer formation has been described for the pituitary-specific protein Pit-1. Pit-1 is monomeric in solution, but binds DNA as a dimer. Mutational analysis showed that the Pit-1 POU_{SP} domain is necessary for cooperative binding of the two monomers (73). Functional analysis of the thyrotrope specific splice variant Pit-1T showed that interaction with Pit-1 is required for activation of the thyrotrope stimulating hormone ß (TSHß) gene promoter in thyrotrope cells, providing evidence that

POU-POU interactions can alter the potential of POU proteins to activate transcription (61).

Interaction of Pit-1 and a LIM protein that is specifically expressed in the pituitary (P-LIM/Lim-3), also results in a synergistic activation of the prolactin and TSHß gene promoters in transient assays (10). This interaction requires both an intact Pit-1 POU domain and LIM domain of P-LIM. Functional interaction between two other members of these protein families was described previously. In the worm *Caenorhabditis elegans* the POU protein Unc-86 is required for differentiation of touch cell receptor precursors and is involved in activation of the *mec-3* gene (45, 192). The LIM domain protein MEC-3 is essential for specification of the touch cell receptor developmental fate in neuroblast precursors. MEC-3 heterodimerizes with Unc-86 to increase the Unc-86 DNA binding affinity which results in a maintenance of *mec-3* gene expression (193). These observations show that POU/LIM complexes form functional transcriptional regulators and could represent a prelude to the identification of additional POU-LIM protein interactions involved in tissue-specific gene expression.

B-cell specific expression of the Oct-2 protein and the importance of the octamer sequence for B-cell specific activition of the immunoglobulin promoters strongly suggested that Oct-2 is responsible for transactivation of these genes in B-cells (151). However, this model was challenged by the observations that the immunoglobulin genes are transcribed normally in B-cell lines containing no or very low Oct-2 protein levels, as in Oct-2 deficient mice which have been generated by homologous recombination (31, 42). Recently, a protein was isolated from a B-cell fraction which could transactivate both Oct-1 and Oct-2 mediated transcription in an *in vitro* assay (104). This protein called OCA-B/OBF-1/Bob1 is expressed only in B-cells and associates equally well with the POU domains of the ubiquitously expressed Oct-1 and B-cell specific Oct-2 proteins, but not with the Brn-2, Oct-4 or Oct-6 proteins (54, 105, 170). It does not increase the DNA binding affinity of the Oct-1 and Oct-2 proteins, arguing that it functions as a B-cell specific co-activator of octamer bound proteins, possibly by interaction with components of the basic transcription machinery. This would implicate the existence of homologs of the OCA-B/OBF-1/Bob1 protein which could act in concert with other POU proteins in a cell specific manner.

Besides interaction with cellular proteins, POU proteins are involved in complex formation with viral proteins to mediate viral gene regulation. After infection of a cell with the herpes simplex virus (HSV) the viral transactivator protein VP16 recruits Oct-1 and a second host cell factor (HCF) into a multiprotein complex onto the DNA of the regulatory elements of the HSV immediate early (IE) genes (reviewed in 69). Association with VP16 alters the binding affinity of Oct-1 for a TAATGAGAT recognition sequence present in the promoters of the viral IE genes and provides Oct-1 with a potent transactivation domain to regulate expression. The ability of Oct-1 to bind an altered DNA sequence motif is accomplished by the flexibility of the Oct-1 POU_{SP} to adopt different positions on the DNA (30). Helix 2 of the Oct-1 POU_{HD} is essential for complex formation with VP16 (165). VP16 does not interact with Oct-2 unless an alanine amino acid at position 22 in helix 2 of the Oct-2 POU_{HD} is substituted for a glutamate, which is present at that position in the Oct-1 POU_{HD} (95).

The papovavirus JC specifically infects glia cells in humans, causing progressive multifocal leukoencephalopathy (PML), a demyelinating disease in the nervous system. The POU protein Oct-6 is expressed in Schwann cells and stimulates glia specific transactivation of the early and late genes. This indicates that Oct-6 may be involved in progression of JC virus infection

(186). Oct-6 transcriptional activity is synergistically increased upon interaction with the viral large T antigen, which associates with the Oct-6 POU domain (142). A similar increase in transactivation is observed when Oct-6 and the high mobility group protein HMG-I/Y are co-expressed (97). HMG-I/Y binds with low affinity to the viral regulatory element, but strongly enhances the DNA binding affinity of the POU protein Oct-6 for the viral *cis*-acting sequences. Thus a number of different proteins seem to be required to establish a functional complex involved in viral gene regulation.

Apart from Oct-6 and HMG-I/Y, other protein-protein interactions have been described for POU proteins with members of the high-mobility-group (HMG) family of DNA binding proteins. Members of this family share a conserved HMG domain which mediates low-affinity DNA binding. In a search for proteins that physically interact with Oct-2 the gene encoding the high mobility group 2 (HMG2) protein was isolated (200). HMG2 binds the Oct-2 POU_{HD}, as well as the POU domains of Oct-1 and Oct-6, and increases Oct-2 DNA binding affinity. Expression of a reporter gene under the control of four wild type octamer motifs is repressed two-fold after transient co-transfection with an anti-sense HMG2 expression vector in S194 plasmacytoma cells. The reporter gene is activated 4 to 5 fold upon co-transfection with a HMG domain-VP16 fusion protein. These data have been interpreted to be indicative for a direct interaction between HMG2 and the endogenous Oct-2 protein present in the S194 cell line, but this has not been directly tested, e.g. by examination of a synergistic activation of the reporter gene upon HMG2 and Oct-2 overexpression (200). More solid evidence exists for a functional interaction between Oct-4 and Sox2 in regulation of fibroblast growth factor 4 (FGF4) gene expression. FGF4 is an essential growth factor for the developing embryo and shows overlapping patterns of expression with the POU factor Oct-4 in the inner cell mass (ICM) of a blastocyst embryo and in the primitive ectoderm and in the primordial germ cells (43, 134, 148). Both proteins are also highly expressed in undifferentiated embryonal carcinoma (EC) and stem cells (ES). Expression of fibroblast growth factor 4 (FGF4, kFGF) in F9 EC cells is regulated by an enhancer element present in the 3' untranslated region of the FGF4 gene (159). In band shift assays Oct-4, and Oct-1, bind the octamer DNA sequence motifs present in the critical *cis*-acting element. However, in the absence of dIdC protein complexes with altered mobility for both Oct-1 and Oct-4 were detected with a cell-specific factor, which was identified as the HMG protein Sox2 (32, 197). In transient assays expression of a reporter gene under control of the FGF4 enhancer element is synergistically activated upon co-transfection with Sox2 and Oct-4 expression vectors. This activation is abolished when the C-terminal part of Sox2 is deleted, although this deletion does not result in a loss of DNA binding. Furthermore, no activation of the reporter gene is observed when Sox2 and Oct-1 or Sox5, a close homolog of Sox2, and Oct-4 are overexpressed (197). These data argue that a cell-specific combination of transcription factors of different protein families may form a transcriptional complex with unique regulatory properties.

POU protein	dimerization partner	cooperative DNA binding	increase in transcription activation
Pit-1	P-LIM	0	+
	Pit-1T	n.d.	+
	Pit-1	n.d.	n.d.
Oct-1	VP16	+	+
	OCA-B/OBF-1/Bob1	+	+
	HMG2	+	?[b]
	Sox2	+	0
Oct-2	OCA-B/OBF-1/Bob1	0/+[a]	+
	HMG2	+	?[b]
Oct-6	JC T-antigen	n.d.	+
	HMG-I/Y	+	+
	HMG2	n.d.	n.d.
Cf1a	I-POU	-	-
Unc-86	Mec3	+	+
Oct-4	Ad5 E1A	+	+
	Sox2	+	+

Table 1. Overview of POU-protein interactions and its effects on DNA binding and transcriptional activation. +, -, 0: positive, negative or no effect of protein interactions on DNA binding or transcriptional activation; n.d.: not determined. [a]): contradictory results have been published on the absence (104) or the presence (170) of an OCA-B/OBF-1/Bob1 induced novel Oct-2 complex in a band shift assay, [b]): only indirect evidence has been reported for a synergistic interaction between HMG2 and Oct-2 (200). For other references: see text.

3. POU proteins as developmental regulators

3.1 Drosophila POU genes

Four different POU genes have been isolated from the Drosophila genome and extensively analysed with respect to their function in cell differentiation. Considering the functional conservation between the mammalian and Drosophila homeobox genes, the *Drosophila* POU genes provide an important paradigm concerning the functions of the mammalian counterparts.

The Cf1a POU protein was isolated as a regulator of dopadecarboxylase (Ddc) gene expression (81). The Ddc gene product catalyses the synthesis of the dopamin and serotonin neurotransmitters. Although Ddc is expressed in a multiplicity of cell types, mutation of the Cf1a binding site in the distal enhancer element of the Ddc gene eliminated expression in only a selected set of dopaminergic neurons, arguing that Cf1a regulates Ddc expression in a distinct cell population. Its function as a transcriptional regulator is further complicated by the finding that Cf1a forms a stable heterodimeric complex with another POU protein called I-POU. I-POU lacks two basic amino acids in the N-terminal arm of the POU_{HD}, preventing the I-POU/Cf1a heterodimer from binding DNA (177). The dimerization interface of Cf1a/I-POU encompasses the basic N-terminal region and helices 1 and 2 of the POU homeodomains. This contrasts with the requirement of POU_{SP} domain for Pit-1 homodimerization (see section 2.3). Dimerization of I-POU and Cf1a is highly specific as I-POU does not interact with POU proteins that are closely related to Cf1a, such as Brn-1, Brn-2 and Oct-6 (178). It had been hypothesized that the requirement of the POU_{HD} in dimerization may reflect the existence of additional inhibitory proteins in the homeobox gene family of transcription factors (68). The action of these proteins could be analogous to the function of I-POU. However, the hypothesis has not yet been confirmed by the identification of such proteins. The function of Cf1a was assessed by isolating mutations in the Cf1a locus (5). Deletion of the Cf1a locus leads to alterations in both the CNS and in the tracheal system. In the latter, migration of cells of the tracheal placode is disturbed resulting in the absence of the dorsal branches of the tracheal tree and dorsal trunk formation. These phenotypical features caused the authors to rename Cf1a *drifter*. The tracheal defect could be rescued by the tissue specific expression of a wild-type Cf1a transgene, showing that it does not result from additional gene disruptions at the Cf1a locus. In the ventral nerve cord of the CNS of Cf1a mutant flies two commisures within a single neuromere are fused and abnormalities are present in the longitudinal connectives. Furthermore, the midline glia fail to migrate to their position at the ventral midline of the neuromere. Occasionally, the axon paths in the ventral nerves are disturbed. Whether this results from a cell autonomous defect or is an indirect result from the dispositioning of cells in the tracheal system, is not clear yet. The tracheal system is thought to play a role in the guidance of outgrowing axons. Cf1a could thus be involved in the regulation of genes encoding cell surface proteins which are involved in axon guidance and cell migration.

Two other *Drosophila* POU genes were identified by several groups which resulted in a confusing nomenclature. Both *pdm-1*/dPOU19/dOct1/*nubbin* and *pdm-2*/dPOU28/dOct2/*miti-mere* (further referred to as *pdm-1* and *pdm-2*) show an extensive and partially overlapping expression pattern during development. At early stages of development the *pdm-2* gene is expressed in a pattern resembling that of different classes of *drosophila* segmentation genes (15, 139). Transgenic overexpression of a truncated *pdm-2* protein induces abdominal malformations. The viability of transgenic embryos is reduced when the transgene copy

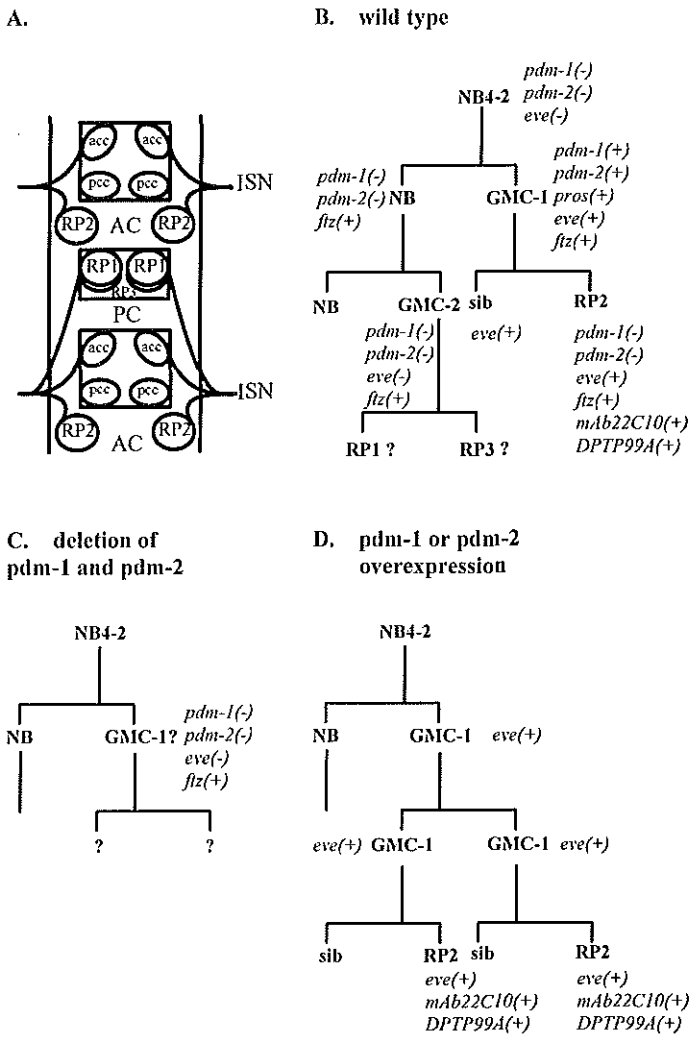

Figure 3. RP2 neurons develop from the NB4-2 lineage. (A) Schematic representation of the neuronal organization in the CNS. RP2 neurons are located in the anterior commisure (AC) of one neuromere. They project their axons ipsilaterally to the more anterior neuromere, forming the intersegmental nerve (ISN) with the axons of the acc and RP1 neurons. (B-D) Diagrams of the *Drosophila* neuroblast NB4-2 cell lineage in wild type (B) and mutant embryos (C-D). In the wild type situation NB4-2 divides to generate GMC-1 and a second NB. GMC-1 divides once more to give rise to the RP2 motoneuron and a sibling cell. The molecular markers that are expressed (+) or not expressed (-) in each cell type are indicated in italics. When both the *pdm-1* and *pdm-2* genes are deleted (C), GMC-1 does form, but does not express *eve*, and the RP2 motor neuron never develops. Overexpression of *pdm-1* or *pdm-2* in GMC-1 (D) leads to a duplication of GMC-1, resulting in the formation of two RP2 neurons.

number is increased or when the *pdm-1* and *pdm-2* gene doses are reduced (12), indicating that overexpression of a truncated *pdm-2* protein affects the function of *pdm-1* and *pdm-2*, or *pdm-2* alone, in segmentation. *Pdm-1* is expressed at lower levels and in a more ubiquitous pattern in the first 24 hours of development (15). Mutation of *pdm-1* results in a specific phenotype, which consists of almost a complete loss of wing structures, suggesting that *pdm-1* activity is required at the hinge region of the wing disc (131).

At later stages of development both genes are involved in cell fate specification of neuronal precursors, as has been studied in the neuroblast 4-2 (NB4-2) > GMC-1 > RP2/sib neural lineage (figure 3). The NB4-2 neuroblast can be identified on the basis of its invariant position in the neurogenic region. This identification has been further facilitated by the 5953 enhancer trap line, which expresses the ß galactosidase reporter gene in the NB4-2 neuroblast (35). NB4-2 divides asymmetrically into another NB and a ganglion mother cell (GMC); GMC-1. GMC-1 divides once more to generate a sibling cell and motor neuron RP2 (fig.3b). RP2 is located in the anterior commisure of one neuromere and projects its axons ipsilaterally to the intersegmental nerve of the more anterior neuromere (fig.3a). *pdm-1* and *pdm-2* are expressed at high levels in GMC-1, but not in its progeny (13, 194, 195). Previously however, assymetric distribution of decreasing *pdm-2* protein levels in the RP2 and sibling cells has been reported (12). Heat shock induced overexpression of transgenic wild-type *pdm-2* or *pdm-1* before GMC-1 cell division results in duplication of the RP2 motor neuron (12, 13, 194). The transiently elevated protein levels most likely forces GMC-1 in a self-renewing cell division yielding two identical GMC-1 cells which subsequently undergo an identical differentiation pathway as in the wild type situation (fig.3d). The pair-rule gene *even-skipped* is expressed in the GMC-1, RP2 and sibling cells, and was used in these experiments to identify the NB4-2 derivatives. The number of *eve* positive cells as well as the spatial position exclude the possibilities that either RP2 itself underwent an extra cell division or that overexpression led to a neomorphic mutation of a different GMC. Recently, genetic mutations that affect either the *pdm-1* or *pdm-2* gene, or both, have been reported, which made it possible to study the individual gene functions (195). Loss of either *pdm-1* or *pdm-2* results in a partial loss of the RP2 neuron, whereas deletion of both genes results in a complete block of NB4-2 differentiation at the GMC-1 stage (13, 195). The identity of the GMC-1 cells appears changed as *eve* expression is no longer observed in the double mutant (fig.3c). These data imply that the POU genes *pdm-1* and *pdm-2* function to specify the identity and developmental fate of the GMC-1 neuronal precursor cell.

3.2 POU genes in the mammalian nervous system

The mammalian nervous system is probably the most complex organ in its development, structure and function. Most POU genes that have been identified in mammals are expressed in the developing and adult nervous system, but their role in neuronal cell differentiation is largely unknown. However, the function of the pituitary specific protein Pit-1 in anterior pituitary gland development is firmly established (reviewed in 144). Pit-1 is expressed in the thyrotrope, somatotrope and lactotrope cell types and directly regulates the expression of the growth hormone (GH) and prolactin (PRL) genes. Mutations in the Pit-1 gene were identified in two strains of dwarf mice: the Snell and Jackson dwarf mice. In the Jackson mouse gross alterations in the Pit-1 locus eliminates transcription of the Pit-1 gene. In the Snell mouse a pointmutation results in a change of a tryptophan for a cystein amino acid residue in the DNA

recognition helix. This renders a protein that binds DNA with low affinity (101). In both mouse strains hypoplasia of the somatotrope, thyrotrope and lactotrope cells in the pituitary is apparent, resulting in a loss of GH, thyroid stimulating hormone ß (TSHß) and PRL gene expression (101). Recently, it was shown that also heriditary forms of dwarfism in humans are linked to mutations in the human Pit-1 gene. In one case a pointmutation in POU_{SP} disrupts Pit-1 dimerization and severely affects the expression of genes regulated by Pit-1 (138).

Recently published genetic analyses provide further evidence for the importance of POU genes as developmental regulators. Mutations in the human homolog of the Brn-4 gene have been linked to a heriditary case of deafness (34). The patients suffer from malformations of the internal acoustic canal and fixed stapes, suggesting that Brn-4 is necessary for the proper development of this structure. Patients were carrying small deletions in the POU_{HD} or POU_{SP} domains resulting in frame shifts and premature translation stops or were carrying non-conservative amino acid substitutions in POU_{HD}, probably interfering with DNA binding. The observed phenotype correlates with the high expression of Brn-4 mRNA in the otic vesicle throughout fetal development of the rat (100). In the mouse, disruption of the Brn-2 gene by homologous recombination results in a loss of neuronal cells in the paraventricular (PVN) and supraoptic (SO) nuclei of the endocrine hypothalamus (130, 158). The PVN and SON neurons project their axons to the posterior pituitary and secrete the neuropeptide hormones oxytocin (OT) and vasopressin (VP). In a Brn-2 homozygous mutant the PVN and SON neurons are severely reduced in number and appear immature. It is therefore possible that Brn-2 functions in the proliferation or survival of these neurons. Furthermore, the levels of VP and OT expression have dropped to 50% in morphologically normal Brn-2 heterozygous animals, implying a role in the cell-specific regulation of these neuropeptides (130).

Expression studies on the Brn-3 and Brn-5 POU genes have provided evidence for a role of these genes in neuronal differentiation. The class VI POU protein Brn-5/Cns-1 is expressed in the developing and adult brain, and in several other tissues like kidney, lung and testis (4). In cultures of postnatal cerebellar tissue Brn-5/Cns-1 expression is induced upon activation of the N-methyl-D-aspartate (NMDA) receptors, which is thought to play a role in the development and differentiation of cerebellar granule neurons (20). Brn-5/Cns-1 may thus represent one of the molecules which mediate the NMDA receptor signal that results in neuronal differentiation. The Brn-3.0 (Brn-3/Brn-3a) and Brn-3.1 (Brn-3c) genes share similar intron/exon structures, whereas Brn-3.2 (Brn-3b) is encoded by an intronless gene (175). This supports the hypothesis that the class IV Brn-3 genes, like the class III Brn-1, Brn-2, Brn-4 and Oct-6 genes, might be derived from a common ancestor gene, but have duplicated and diverged as a consequence of the increasing complexity of the mammalian nervous system (181). The class IV Brn-3 genes show limited overlap in their expression patterns, which is mostly confined to postmitotic neurons in the central nervous system. This suggests that the Brn-3 genes have a role in the later stages of neuronal cell differentiation (41, 132, 181, 191). Indeed, overexpression of Brn-3.0 in the neuronal cell line ND7 leads to neurite outgrowth and induction of SNAP25 gene expression, a presynaptic terminal gene (96).

Expression studies in cell cultures have shown that differential regulation of Brn-3 gene expression by different signaling pathways may be involved in establishment of the Brn-3 gene expression patterns. Brn-3.0 expression is induced and Brn-3.2 repressed in the neuronal cell line ND7 upon treatment with cAMP or removal of serum (19). cAMP together with retinoid acid (RA) represses Brn-3.2 expression in F9 EC cells

and the neuroblastoma N18 cell line, while Brn-3.0 expression is not affected (181). In addition, intrinsic differences to regulate gene expression may further specify Brn-3 gene function. It has been shown that Brn-3.0 and Brn-3.1 can activate expression of a CAT reporter gene in transient assays from an enhancer element containing five octamer sequence motifs or one octamer-related TAATGAGAT motif, whereas Brn-3.2 shows a repressing activity (19, 129). In contrast, both Brn-3.0 and Brn-3.2 activate a promoter containing three core motifs from the corticotropin-releasing hormone (CRH) gene promoter (181).

4. Aim of this thesis

The studies reviewed above have shown that POU proteins are important regulators of cell differentiation and development. POU proteins are differentially expressed and can interact with other proteins in a cell-specific fashion to regulate expression of genes which further determine the developmental fate of POU protein expressing cell-types.

The POU class III protein Oct-6 was originally identified as a differentially expressed octamer binding factor in neuroectodermal differentiation of P19 embryonal carcinoma (EC) cells, in undifferentiated embryonal stem (ES) cells, and in the adult testis (65, 115, 171). Furthermore, the rat homolog of Oct-6 was identified as a cAMP inducible protein in cultures of Schwann cells isolated from the sciatic nerve of a newborn rat (124). These studies have indicated that the transcription factor Oct-6 may function both as a developmental regulator in the early stages of mouse embryonal development as well as a regulator of Schwann cell differentiation. The work described in this thesis was concerned with two questions. First, what is the Oct-6 gene function in early development and Schwann cell differentiation? Second, what are the regulatory elements that establish the Oct-6 expression pattern? To analyse the experiments addressing these questions a detailed analysis of the Oct-6 gene expression pattern is required. Therefore, a polyclonal antiserum specific for the Oct-6 protein was generated and used in immunohistochemistry experiments to analyse the precise temporal and spatial distribution of Oct-6 protein in the developing mouse embryo. The results of these experiments are described in chapter 2. In a pregastrulation embryo Oct-6 protein expression is restricted to the primitive ectoderm, while during gastrulation it becomes restricted to the neuroectoderm cell layer (chapter 2.1). During mid- and late gestation periods of development expression is maintained in the developing nervous system, but differential patterns of expression are also observed in the retina of the developing eye and in the ectodermal component of the developing hair follicle (chapter 2.2). In chapter 2.3 the pattern of Oct-6 protein expression in the myelinating Schwann cell lineage is described. Oct-6 protein is already present in the embryonic Schwann cell before the last cell division and precedes the expression of the myelin gene P_0, implicating that Oct-6 is involved in maturation of the Schwann cell lineage. This was further substantiated in the analysis of Oct-6 deficient mice which were generated by homologous recombination. Homozygous mutant pups show a temporary arrest in Schwann cell differentiation at the promyelination stage, arguing that Oct-6 is required for the transition of a promyelinating Schwann cell into a fully differentiated myelin producing Schwann cell (chapter 2.3).

In chapter 3 the experiments are described to identify the regulatory elements which are involved in cell-specific regulation of Oct-6 gene expression. Transgenic mice were generated containing a lacZ reporter gene under the control of 32 kb of Oct-6 genomic sequences. In these mice an Oct-6 like pattern of lacZ transgene expression is observed in Schwann cells and the developing hair follicles, indicating that cell-

specific Oct-6 gene enhancer elements reside within this fragment (chapter 3.1). In contrast, in the developing nervous system the Oct-6 expression pattern is only partially reconstituted, arguing that more distal enhancer elements are involved in the regulation of Oct-6 gene expression in these cell types. Furthermore, a second gene was identified within the Oct-6 locus, which may have important implications with respect to the mechanism of Oct-6 gene regulation (chapter 3.2).

In the last chapter (chapter 4) present and future experiments to further establish the role of Oct-6 in cell differentiation and development are discussed.

Chapter 2

Oct-6 protein expression in ectodermal lineages

2.1 The restricted expression pattern of the POU factor Oct-6 during early development of the mouse nervous system.

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Oct-6 is a POU transcription factor that is thought to play a role in the differentiation of cells of neuroectodermal origin. To investigate whether the Oct-6 protein could play a role in the establishment of neuroectoderm *in vivo* we studied the expression of the Oct-6 protein during early mouse development. Expression is first observed in the primitive ectoderm of the egg cylinder stage embryo. In gastrulating embryos, Oct-6 protein is found in the extra-embryonic ectoderm of the chorion and the anterior ectoderm of the embryo proper. As development proceeds, Oct-6 expression becomes more restricted to the anterior medial part of the embryo until Oct-6 positive cells are observed only in the neural groove of the headfold stage embryo. In the late headfold stage embryo, Oct-6 expression is detected in the neuroepithelium of the entire brain and later is restricted to a more ventral and anterior position. As the anterior neuropore closes, Oct-6 protein is detected in a segment-like pattern in the mid- and forebrain. Thus, the expression pattern of the Oct-6 gene agrees with a role for the Oct-6 protein in the establishment and regional specification of the neuroectoderm *in vivo*. The two waves of widespread induction of the Oct-6 gene, one in the primitive ectoderm and another in the primitive brain, both followed by a progressive restriction in the expression patterns suggest a mechanism for the regulation of the gene.

Introduction

Genes involved in neurogenesis have been the subject of intense study in many different organisms. Many of these genes are members of large families and encode DNA binding proteins that could act as transcriptional regulators. These different gene families are defined by their evolutionary conserved DNA binding domain. One of these families, the POU family, consists of a growing number of tissue specific DNA binding proteins that share the conserved POU DNA binding domain (67, 157, 185). The POU domain is a 150-160 amino acid region containing a 60 amino acid POU-homeodomain (POU_{HD}), highly homologous with the classical homeobox, and a 75-82 amino acid POU-specific domain (POU_{SP}). The amino terminal POU_{SP} domain is separated by a short linker sequence (15-27 amino acids) from the carboxyl terminal POU_{HD} domain. Based on homologies within the POU domain these genes have been grouped in seven classes (class I -VII; 63, 185). More than 13 mammalian POU family members have been identified and POU genes have also been cloned from the genomes of *Drosophila* (81), *C.Elegans* (21), *Bombyx mori* (49), zebrafish (63, 80), chicken (137) and frogs (1). During development most POU genes show a tissue specific expression pattern, suggesting that they are involved in cell type determination or regional specification. They will function

during cell differentiation, regulating the tissue specific expression of downstream genes through interaction with octamer and octamer-like DNA elements (147, 185).

We have been studying the Oct-6 gene, which together with the POU genes Brn-1, Brn-2 and Brn-4, form the mammalian class III POU family. We identified the mouse POU factor Oct-6 as an octamer binding protein that is differentially expressed during neuroectodermal differentiation of P19 embryonal carcinoma cells (115). The rapid induction of this gene in differentiating cells followed by a progressive downregulation and the reappearance of expression in fully differentiated cultures suggest that Oct-6 is involved in different aspects of neuronal differentiation. Furthermore, Oct-6 is expressed in non-differentiated embryonic stem cells that are derived from the inner cell mass (ICM) of blastocyst stage embryos (115, 171). The rat homolog, called SCIP or Tst-1, cloned from rat testis (64), was shown to be a cAMP (cyclic adenosine monophosphate) inducible gene in cultured neonatal Schwann cells isolated from the sciatic nerve (124).

Several studies have described the expression of the Oct-6 gene in the developing and adult central nervous system using RNA *in situ* histochemistry. During mid and late gestation periods of mouse embryonal development, Oct-6 RNA is found in the subventricular zones in the brain. Expression is most abundant in prospective cerebral cortex, olfactory bulb, striatum, thalamus and inferior colliculus (171). In rat a similar embryonic expression pattern was observed (64). Expression in the adult rat cerebral cortex is very prominent in layer 5 pyramidal neurons and is detected as these cells originate in the intermediate zone during embryonal development (47). These RNA *in situ* studies suggest that the Oct-6 gene product could be involved in determination of neural identity. In addition, the immediate induction of Oct-6 gene expression in P19 cell aggregates upon retinoic acid (RA) addition, suggests that Oct-6 could be involved in the differentiation of neuroectodermal derivatives from a multipotent embryonic stem cell. However, very little is known about the expression of the Oct-6 gene during early mouse development.

To investigate whether the expression pattern of Oct-6 protein (rather than RNA) agrees with a role of the Oct-6 gene in the establishment of the neuroectoderm, we performed immunohistochemical staining of early mouse embryos, using a polyclonal antiserum directed against the Oct-6 protein.

Results

Oct-6 polyclonal antiserum

A polyclonal antiserum was raised in rabbits against bacterially produced, denatured Oct-6. First, the reactivity of the antiserum with mouse Oct-6 protein was tested in a band shift assay (fig.1a). A nuclear cell extract of the Mes68 cell line expressing the POU proteins Oct-1 and Oct-6 was incubated with a radiolabeled double stranded oligonucleotide containing the octamer ATTTGCAT POU protein consensus binding site. After protein-DNA complexes had formed the Oct-6 antibody was added to the binding reaction. The mobility of the Oct-1 and Oct-6 protein complexes was found to be unaltered when pre-immune serum was added (fig.1a, compare lanes 1 and 2). However, addition of Oct-6 antibody gives rise to a complete retardation of the Oct-6 protein complex in the top of the gel, whereas the Oct-1 complex was unaffected (fig.1a, lane 3). This experiment shows that the Oct-6 antiserum is able to recognize mouse Oct-6 protein. Furthermore the antiserum is highly specific since it does not cross-react with Oct-1 protein despite the presence of a large excess of Oct-6 antibodies.

To further ensure the specificity of

the antiserum we performed an affinity purification. Because the antiserum was raised against the full length Oct-6 protein we were concerned with the possibility of cross-reaction with other POU proteins through conserved epitopes within the POU domain. In addition to affinity purification we competed the purified Oct-6 antibodies with a cellular lysate of bacteria overexpressing the Oct-6 POU domain (Oct-6/POU) during the antibody incubation period in the immunohistochemistry experiments. To determine the efficiency of this method, a similar competition was performed in a western blot assay (see fig.1b). When whole cell extracts of transiently transfected COS-1 cells overexpressing either the Oct-6 POU domain (lanes 2 and 4) or the full length Oct-6 protein (lanes 1 and 3) were stained with the affinity purified Oct-6 antiserum, both Oct-6 and the Oct-6 POU domain were detected when a lysate of non-induced bacteria was used as a competitor (lanes 1 and 2). However, the Oct-6/POU competitor completely eliminated detection of the POU domain, leaving detection of the full length Oct-6 protein unaffected (lanes 3 and 4). This experiment demonstrates that addition of the POU competitor efficiently blocks the epitopes in the conserved POU domain while other Oct-6 specific epitopes are still recognized by the Oct-6 antiserum. Since the Oct-6 POU DNA binding domain is the only region with significant homology to its most closely related POU family members, this method eliminates any cross reactivity with other POU proteins. That this is also the case in the immunohistochemistry experiments comes from a number of observations not shown here. First, the closely related class III POU proteins Brn-1 and Brn-4 are highly expressed in the developing kidney and otic vesicle respectively (64, 100). No signal is detected with our Oct-6 antibody in these structures. Second, the Brn-3 genes are highly expressed in the sensory ganglia (132). No signal is detected with our antibody. Third, the Oct-3/4 gene is highly expressed in the inner cell mass and trophectoderm (156). Again no signal is detected with our antibody in these structures. These and numerous other observations demonstrate that the antibody specifically recognizes the Oct-6 protein and none of the other known POU proteins.

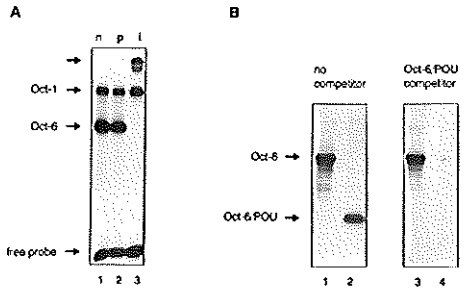


Figure 1. Oct-6 antibodies specifically recognize Oct-6 protein. Panel A shows a band shift experiment of Mes68 nuclear cell extract and a radiolabeled Octamer oligo. Lane 1: no serum was added, lane 2: 1µl preimmune serum, lane 3: 1 µl Oct-6 antiserum. The arrow indicates the retarded Oct-6 protein complex. Panel B shows a western experiment detecting either Oct-6 protein (lanes 1 and 3) or Oct-6/POU (lanes 2 and 4) under competitive conditions of a nonexpressing (lanes 1-2) or Oct-6/POU (lanes 3-4) expressing bacterial lysate.

Oct-6 expression in presomite stage embryos

The Oct-6 gene is expressed in inner cell mass (ICM) derived ES cells (115, 155). To test whether the Oct-6 protein is present in the ICM of pre-implantation blastocyst embryos we performed immunohistochemistry on whole mount blastocyst embryos. Using our polyclonal Oct-6 antiserum, no Oct-6 protein could be detected in these embryos (data not shown). This was not unexpected since a previous paper reported the failure to detect Oct-6 mRNA in these preimplantation embryos

(171). However we could detect the Oct-6 protein in the nuclei of embryonic and extra-embryonic ectodermal cells of a 5.5 dpc egg cylinder stage embryo (fig.2). Thus a major period of onset of expression must occur sometime after or during implantation and subsequent expansion of the epiblast into a cylindrical structure.

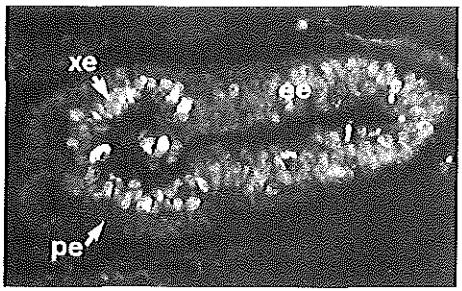


Figure 2. Oct-6 expression in a prestreak embryo. The figure shows an oblique sagittal section of a 5.5 dpc embryo. Oct-6 is expressed in the embryonal and extra-embryonal ectoderm. The bar represents 25 µm. Abbreviations: ee, embryonal ectoderm; pe, proximal endoderm; xe, extra-embryonal ectoderm.

In late streak embryos, Oct-6 protein is found in the anterior half of the embryo. No expression is found in the posterior and lateral-posterior parts of the embryo (fig.3a). Transverse sections of embryos of similar developmental stage revealed that Oct-6 protein is expressed in the cells of the ectodermal layer (data not shown). A sagittal section shows that the posterior boundary of expression extends to the distal tip of the midstreak embryo (fig.3e). This position coincides with the position of the node. In more advanced embryos the lateral expression boundaries progressively restrict to a more anterior position (fig.3b and 3c). Furthermore, a stripe of non-expressing cells becomes visible at the midline of the embryo anterior of the node (fig.3f). This becomes even more evident in the late headfold stage embryo shown in figure 3c as the lateral boundary of expression has regressed to the parallel neural ridges in the headfold. Thus, during gastrulation and subsequent neurulation Oct-6 protein expression is progressively restricted to the walls of the neural groove after initial widespread expression in the primitive ectoderm.

Besides expression in the embryonal ectoderm, Oct-6 protein is also found in the extra-embryonal ectoderm. As shown in figures 3a-f, Oct-6 is expressed in the ectodermal component of the chorion membrane. This extra-embryonal tissue derives from both the ecto- and mesoderm and fuses with the allantois and ectoplacental cone to form the placenta.

Oct-6 expression in 5 to 17 somite-pair stage embryos

Formation of the head folds is initiated by invagination of the foregut and is characterized by exceptionally rapid growth of the neuroepithelial cells (see reference 163). In 5 somite embryos, Oct-6 protein is highly expressed in the head folds (fig.4a). Expression extends to the anterior extremity of the neuroectoderm (fig 4b) and is evenly distributed along the entire medial to lateral (M-L) axis of the headfolds (fig.4a). Proceeding in development, Oct-6 protein expression regresses in the hindbrain region in a ventral and anterior direction (fig.4c and 4d). The posterior boundary of expression is indicated with arrowheads in figure 4c and 4d and is located rostral of rhombomere 6. In the lateral extremities of the head folds the lateral and anterior boundaries of expression are regressing, leading to a more ventral position of Oct-6 expressing cells in the neuroepithelium (fig.4c). When the rostral neuropore has closed, Oct-6 expression becomes restricted to the developing fore- and midbrain.

Oct-6 expression in 9 to 10.5 dpc developing forebrain

At 9.5 dpc Oct-6 protein expression has

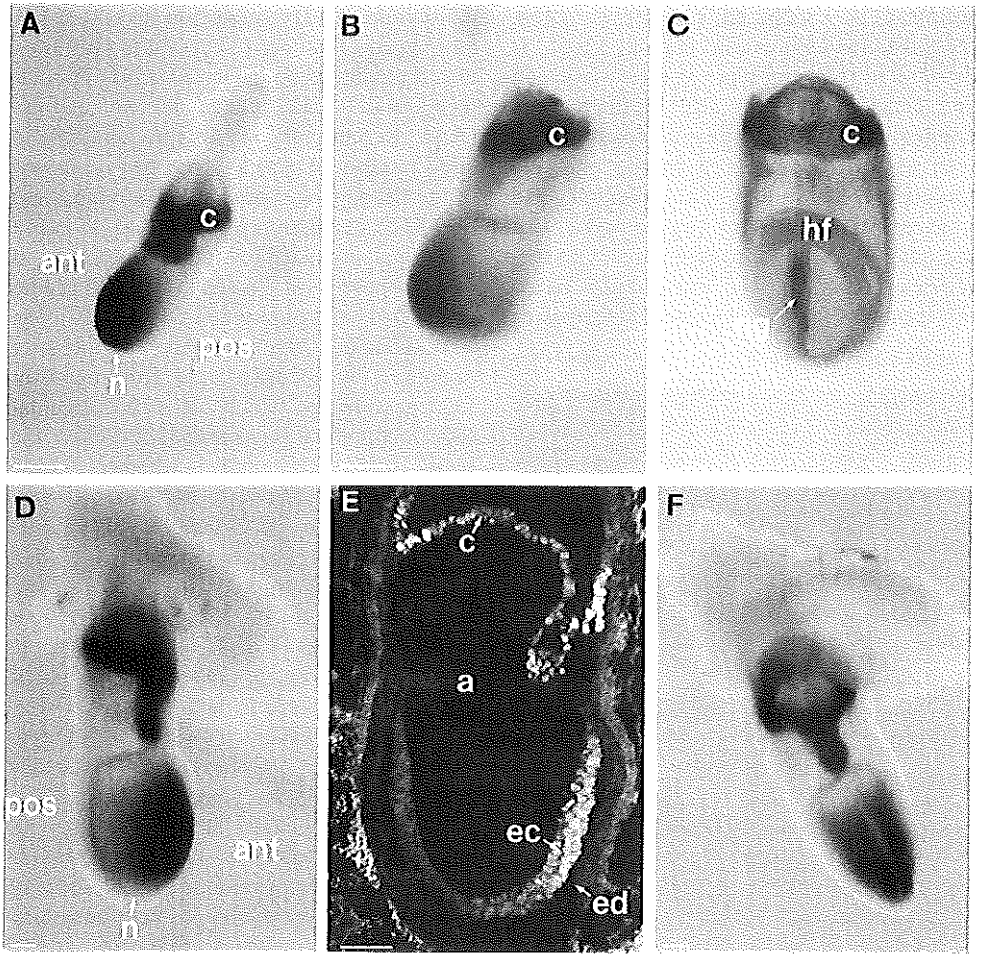


Figure 3. Progressive restriction of Oct-6 in the anterior ectoderm during gastrulation. (A) to (C) show lateral views of whole mount embryos stained for Oct-6 expression. Anterior is to the left. Expression restricts from a rather widespread distribution in the ectoderm to the neurepithial cells of the neural groove in a head fold embryo. (A): late streak, (B) early bud neural plate and (C) advanced head fold stage embryos. The bar represents 400 μm. (D) and (F) respectively show a lateral and anterior view of a no bud neural plate stage embryo. (E) shows a sagittal section of a different embryo of similar stage. In (D) and (E) anterior is to the right. Expression is confined to the embryonal ectoderm and extra-embryonal chorion membrane and is absent from the midsagittal streak. The bar represents 200 μm. Abbreviations: a, amnion; ant, anterior; c, chorion; ec, ectoderm; ed, endoderm; hf, head fold; ng, neural groove; n, node; pos, posterior.

stabilized in two domains within the mid- and forebrain. A posterior domain encompassing the ventral aspect of the rostral mesencephalon and caudal diencephalon. A second, more anterior expression domain is seen at the ventral midline of the secondary prosencephalon which extends at later stages into the telencephalon. (fig 5 a). A frontal

section of a 9.5 dpc embryo shows that in the posterior expression domain Oct-6 is detected in the basal plate of mesencephalon and diencephalon (fig 5b). Furthermore a number of Oct-6 positive neurons can be seen at a dorsal position in the mid and forebrain (see figure 5a). Sagittal and transverse sections show (figure 5c&d) that the anterior expression domain of Oct-6 is restricted rostral of the infundibular recess and caudal of the optic chiasm. Furthermore,

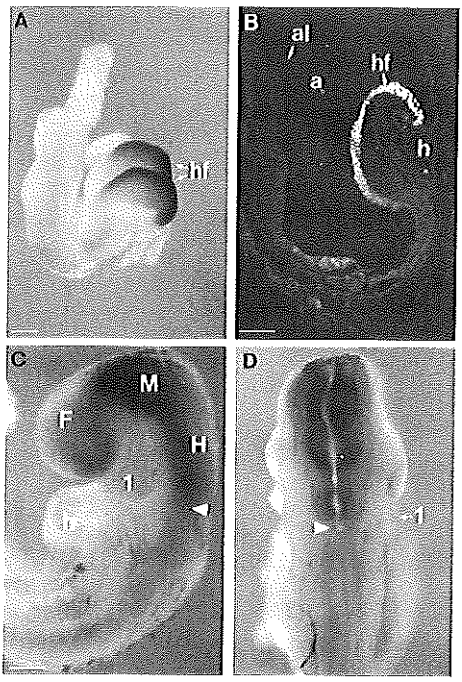


Figure 4. Oct-6 expression in the future brain region in 5 and 17 somites stage embryos. (A) and (B) show Oct-6 expression in a 5 somite embryo. (A) shows a lateral view of a whole mount stained embryo. Anterior is to the right. (B) shows a sagittal section. (C) and (D) show respectively a lateral and dorsal view of a 17 somite embryo. The arrowhead points at the posterior expression boundary. Bar in (A) to (D) represents 500 µm. Abbreviations: a, amnion; al, allantois; F, forebrain; H, hindbrain; h, heart; hf, head fold; M, midbrain; 1, first branchial arch.

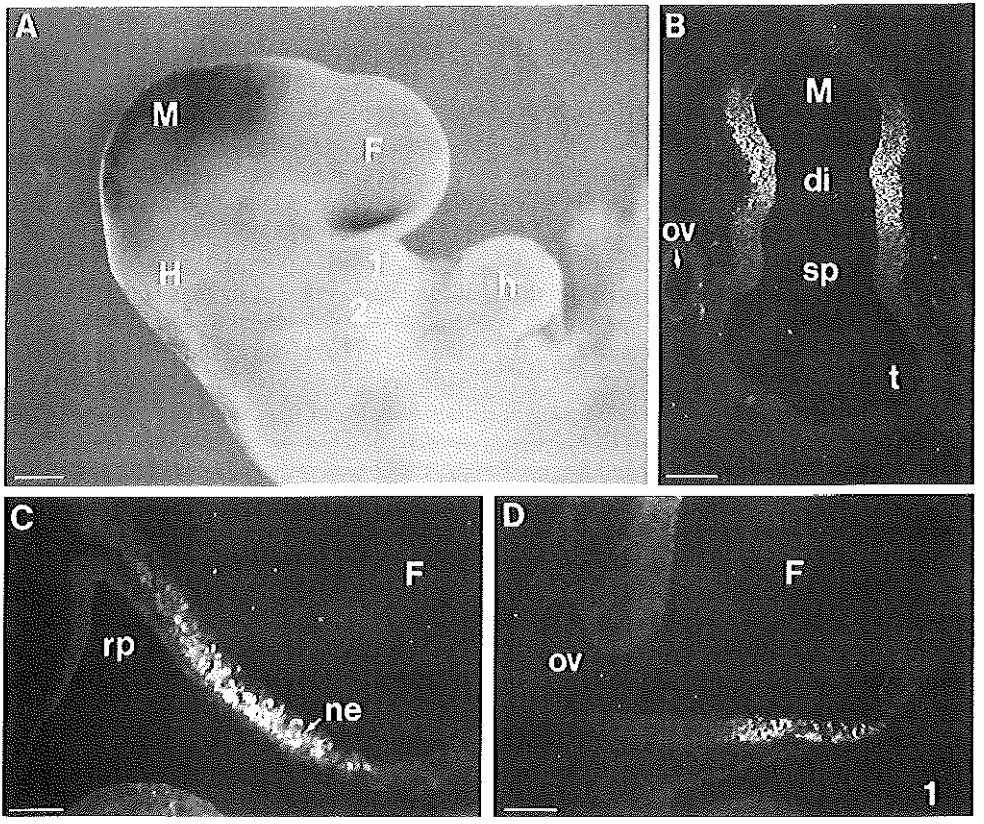
the oblique transverse section shown in fig. 5d indicates that Oct-6 expression is limited by a sharp lateral boundary. The infundibular recess and Rathkes pouch do not express Oct-6. Recently, the expression patterns of a large number of genes have been described in the developing forebrain. The boundaries of mRNA expression would reflect in part a segmental organization of the forebrain as was proposed by Puelles and Rubenstein (1993; 141) and Figdor and Stern (1993; 44). In these models, the forebrain is segmentally organized by six transverse prosomeres or four neuromeres which are subdivided by four longitudinal segments that follow the body axis. To study whether Oct-6 expression is confined to such segments or prosomeres, we performed whole mount RNA in situ hybridization experiments using an Oct-6 and Sonic hedgehog (Shh) probe. We used Shh as its expression has been described in detail (38), and furthermore provides a convenient marker for the border between prosomere 2 and 3 (at the zona limitans intrathalamica (ZLI) = border between domain D1 and D2 in Figdor and Stern, 1993) and the ventral midline of the rostral diencephalon (also called the secondary prosencephalon). Whole mount RNA in situ hybridization of 9.5dpc embryos using Shh, Oct-6 or Shh + Oct-6 anti-sense RNA probes indicates that the rostral boundary of the caudal Oct-6 expression domain coincides with the lateral extensions of Shh expression that mark the boundary between prosomere 2 and 3 (figure 6a ,c and e). Sense RNA probes did not detect any signal (data not shown). Sagittal sections of these embryos show that the rostral Oct-6 expression domain falls within the Shh expression domain at the ventral midline of the rostral diencephalon (figure 6b and d). These results are schematically summarized in figure 6f. Thus Oct-6 expression in the forebrain seems to be restricted to the basal segments of prosomere 1, 2 and 6 according to the scheme of Puelles and Rubenstein. The expression of Oct-6 in the ventral midline of

the rostral diencephalon is maintained at later stages of development but extends rostrally into the telencephalon.

Discussion

Based on *in vitro* cell culture systems and mRNA expression studies in mid- to late gestation embryos, it has been suggested that the POU factor Oct-6 plays a role in various aspects of neuronal differentiation. The rapid induction of Oct-6 mRNA in RA-treated P19 cell aggregates suggests that the gene product could also be involved in the establishment of the neuroectoderm *in vivo*. In this report we have described the expression pattern of the Oct-6 protein during early mouse development, from the pregastrulation stage until midgestation, using highly specific affinity purified antiserum in immunohistochemistry experiments.

Figure 5. Oct-6 expression in the developing forebrain of a 9.5 days pc embryo. (A) shows a ventrolateral view of a 9.5 days pc embryo. In (B) a frontal section is shown through the fore- and midbrain. In (C) a sagittal sections shows expression in the neuroepithelium of the ventral floor of the secondary prosencephalon anterior of the infundibular recess. A transverse section shows that the expression has clear lateral boundaries of expression (D).Bar in (A) and (B) represents 500 μm and in (C) and (D) 150 μm. Abbreviations: di, diencephalon; F, forebrain; H, hindbrain; h, heart; M, midbrain; ne, neurectoderm; ov, optic vesicle; rp, Rathke's pouch; sp, secondary prosencephalon; t, telencephalon; 1, first branchial arch; 2, second branchial arch.



Oct-6 and ectoderm differentiation

During gastrulation and subsequent neurulation, we found that the expression of Oct-6 protein progressively restricts from a rather ubiquitous expression pattern in the primitive ectoderm to the neuroectodermal cells in the neural groove of a head fold stage embryo. It appears that Oct-6 expression stabilizes in cells that are fated to develop into neuroectoderm whereas expression is extinguished in other ectodermal derivatives. This suggests that Oct-6 plays a role in the progression of neuroectodermal differentiation.

The progressive restriction of Oct-6 protein expression to the anterior part of the embryo is reminiscent of the ectodermal expression of the Otx2 gene (6). Otx1 and Otx2 are the mammalian homologs of the Drosophila homeobox gene orthodenticle which is involved in the patterning of anterior head structures. Explant-recombination experiments performed by Ang et al. (1994) indicate that the progressive restriction of Otx2 expression to the anterior part of the mouse embryo is brought about by a negative signal emanating from the posterior mesendoderm (6). This parallel in expression pattern suggests that Oct-6 is similarly regulated and possibly responds to the same signal that downregulates Otx2.

The restriction of Oct-6 protein expression during gastrulation and neurulation is followed by a second round of induction of the gene in the anterior neuroectoderm. As the embryo develops from a late headfold stage to early somite stage, Oct-6 expression is detected in the primitive brain. This induction of high levels of Oct-6 expression coincides with the rapid proliferation of the anterior headfolds (163). Again this widespread expression becomes restricted as the brain further develops and adopts a segment-like pattern in the mid- and forebrain at the time the anterior neuropore closes. Thus, it appears that the early embryonic expression pattern of Oct-6 is the result of two separate waves of induction, both followed by a progressive restriction through negative regulation of the gene. The molecules that execute these functions and the mechanisms by which they act are completely unknown. The identification of the cis-acting DNA elements in the Oct-6 locus that regulate the developmental expression of the gene and the proteins that bind to these elements might give a clue towards the identity of the molecules involved. It is anticipated that negative regulatory elements will play a major role in establishing the expression pattern after the initial widespread activation of the gene.

The high levels of expression of Oct-6 in the ectoderm of the prestreak embryo and in the primitive brain of the advanced headfold stage embryo coincides with a high proliferative activity in these structures. In postnatal Schwann cells high levels of Oct-6 are correlated with proliferation (although this correlation is not a strict one; see Scherer *et al.* 1994 (152) and chapter 2.3 of this thesis). Recently, Faus *et al.* (1994; 40) described the expression of Oct-6 mRNA in the highly proliferative basal layer of the skin. When cells in this layer start to differentiate and migrate out, Oct-6 expression is downregulated. In support of this, we have observed Oct-6 protein expression in the surface ectoderm and in the hair follicles of midgestation embryos. High levels of Oct-6 expression in surface ectoderm were especially evident during the formation of the pinnae of the ear and the vibrissae (see chapter 2.2 of this thesis). It is therefore possible that one function of the Oct-6 gene is in the expansion of undifferentiated ectodermal cells. However, Oct-6 expression has been seen in neurons that have started to differentiate and leave the ventricular zone and in some mature neurons, indicating that the protein might serve an additional function in these cells that is not related to proliferation (47, and our unpublished observations). Ectopic expression

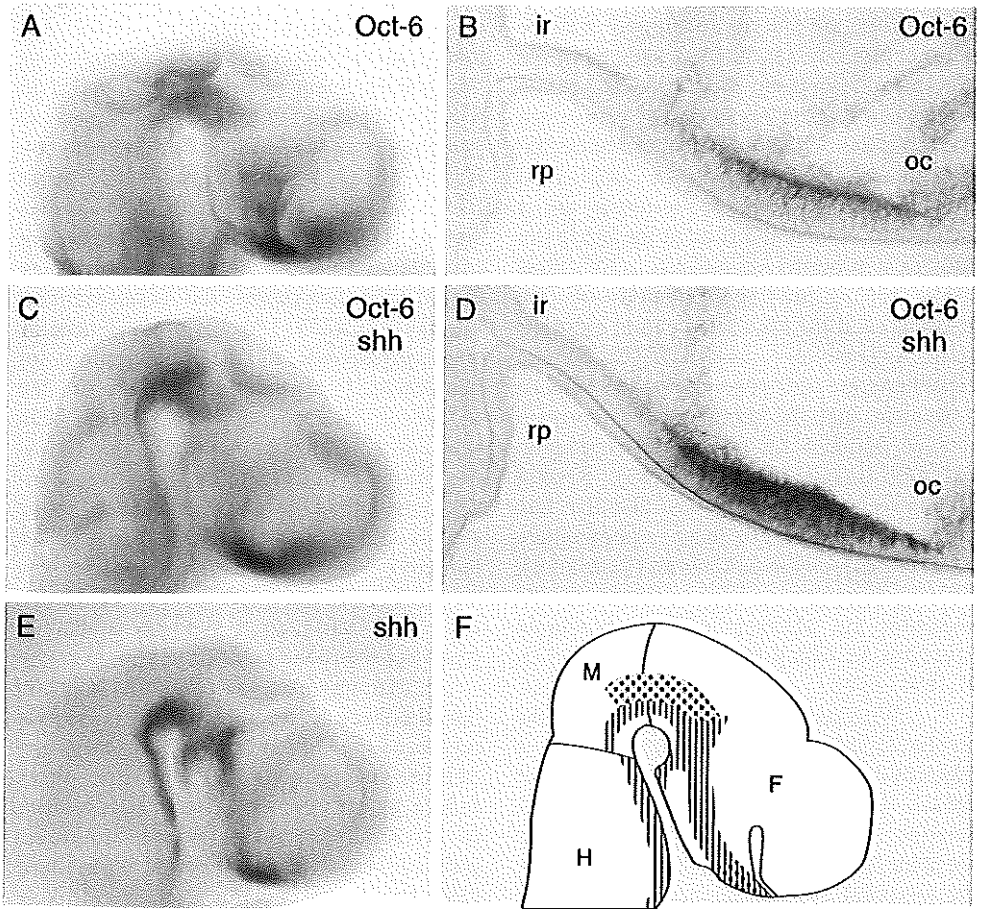


Figure 6. Oct-6 and Shh *in situ* hybridization in the developing forebrain
Lateral views of embryos stained for Oct-6 (A), Shh + Oct-6(C) and Shh (E) are shown. B and D show midsagittal sections of the embryos depicted in A and C, respectively. Note that Oct-6 expression is contained within the Shh expressing domain at the ventral midline of the anteriormost part of the developing forebrain. The diagram in F summarizes the expression patterns of Oct-6 (blue dots) and Shh (red stripes) at the time of rostral neuropore closure. The overlap in expression of Oct-6 and Shh in the rostral diencephalon is indicated with stripes and dots on a red background. Abbreviations are; ir, infundibular recess; rp, Rathke's pouch; oc, optic chiasm.

and gene knock out studies in transgenic mice should reveal if Oct-6 is able to alter the developmental fate of a multi- or totipotent cell type in the neuronal differentiation pathway or influence the differentiation of other ectodermal cells that express Oct-6, like Schwann cells and hair follicle cells.

Segmental expression of Oct-6 in the mid and forebrain

At the time of anterior neuropore closure Oct-6 expression has adopted a segment-like pattern in the mid- and forebrain (see figure 5). The central nervous system of vertebrates overlying the notochord becomes segmented through signalling from the segmented mesoderm (the somites; resulting in a secondary segmentation) or in the hindbrain through a program of intrinsic interactions (107, 166). The segmentation of the hindbrain and the associated neural crest has been extensively analyzed by tissue transplantation and gene expression studies. It is clear that the rhombomeres represent genuine domains for cellular differentiation and cell lineage restriction (83). Several developmental control genes like the hox genes and Krox-20 have rhombomere restricted expression patterns and gene knock out experiments in mice resulted in the alteration or absence of the hindbrain structures in which they are expressed (25, 91, 154, 172).

Whether the mid- and forebrain in the prechordal region also develops through a segmental subdivision is still a matter of debate. Morphological subdivision in the anterior brain region is not as readily apparent as it is in the hindbrain. However, the temporal appearance of furrows in the forebrain has been interpreted in the light of progressive segmentation, defining distinct adult brain structures. Different models have been put forward to describe this subdivision of the anterior brain (for discussion of these different models see Kuhlenbeck (1973; 92) and Puelles and Rubenstein (1993; 141). The recent description of the restricted expression pattern of a large number of regulatory genes in the forebrain support the notion that the prechordal CNS may also develop through segmentation of the anterior neural tube (Puelles and Rubenstein 1993 (141), Figdor and Stern 1993 (44), and references therein). Our immunohistochemistry and RNA in situ data show that Oct-6 expression is also confined to certain domains in the forebrain at the time of rostral neuropore closure. Using Shh as a secondary probe we identified the Oct-6 expression domains as the basal segment of prosomere 1 and 2 in the diencephalon and the ventral part of prosomere 6 of the secondary prosencephalon (figure 6F). A number of regulatory genes like pax-6, BF-2, Wnt-3 and Dlx-1 have a similar boundary (caudal or rostral) of expression at the border between prosomere 2/3 which demarcates the zona limitans intrathalamica (59, 150, 169). This landmark also corresponds to the caudal limit of neuromere 1 of the diencephalon in the model of Figdor and Stern. It has been speculated that this structure functions as an organizer patterning the adjacent neuroepithelium through planar inductive signals (11, 149). It is possible that these inductive signals regulate or restrict the expression of the genes mentioned above, including Oct-6, to a domain caudal or rostral of this boundary.

An organizing or patterning function has also been ascribed to the rostral/ventral region of the forebrain in which Oct-6 and Shh are coexpressed. This region is able to induce, as is COS cell derived SHH, neurons of ventral telencephalic identity in prospective telencephalic neural plate tissue (39). This suggests that a signal, most likely SHH, emanating from the rostral forebrain is able to pattern the ventral part of the telencephalon. Furthermore, experiments in zebrafish have suggested that ventral midline cells in the forebrain also function in patterning the diencephalon (60). Ongoing comparative expression studies for Oct-6 in chick and zebrafish and gene knock out experiments in mice will hopefully shed some light on the possible function of the Oct-6 gene in these anterior brain structures.

Materials and methods

Oct-6 overexpression and purification

For overexpression of Oct-6 protein in E.coli, a BamHI-BglII fragment from pN1Oct-6, containing the full length Oct-6 cDNA plus the ß-globin intron sequences (116), was cloned behind the IPTG inducible T7 promoter in the BamH1 site of the pET11A expression vector (Novagen). This construct was transfected into the BL21(DE3). An overnight culture was diluted 1 in 10 and cultured at RT to an OD_{600}=0.8. Overexpression was induced by adding IPTG to a final concentration of 0.4 mM and the culture was incubated for 4 hrs. Pilot experiments have shown that the Oct-6 protein can be purified by high affinity chromatography on a Ni-NTA matrix (Qiagen). Binding of Oct-6 to this matrix is probably mediated by the polyhistidine stretch present in the carboxyl terminal part of the protein.
For large scale purification, a 500 ml IPTG induced bacteria culture was pelleted, washed once with PBS, resuspended in 10 ml 6M urea/PBS and sonicated. The cell lysate was cleared by centrifugation at 12000 rpm for 5min at 4°C. Imidazole was added to the supernatant to a final concentration of 0.8 mM and incubated overnight at 4°C with 300 µl Ni-NTA beads (Qiagen). The Ni-NTA was washed two times with 1 ml of a 6M urea/ PBS/0.8 mM imidazole solution for 15 min and three times with 6 M/ PBS/ 8 mM imidazole. Oct-6 protein was eluted from the matrix in 500 µl. 6 M urea/ PBS/ 80 mM imidazole. This purification procedure resulted in high yields of pure (>95%) and intact Oct-6 protein as judged by Coomassie stained SDS-PAGE.

Antibodies

The bacterially overexpressed Oct-6 protein was recovered in 6 M urea/ PBS/ 80 mM imidazole. To lower the urea concentration the eluate was dialyzed stepwise against PBS with decreasing concentrations of urea, to a final concentration of 400 mM. This led to the partial precipitation of Oct-6 protein. Antibodies were raised in White New Zealand rabbits by three consecutive injections with 4 wks intervals of 0.5-1.0 mg Oct-6 protein resuspended in Incomplete Freund's Adjuvant.
Oct-6 antibodies were affinity purified by binding to Oct-6 protein immobilized on nitrocellulose. After preincubation with 1%BSA/3%powdered milk/0.05% Tween-20/PBS for 2 hrs at 4°C, the nitrocellulose was incubated overnight with the antiserum that had been precleared with BL21(DE3) cell lysate at RT for 3 hrs. After extensive washing with PBS the Oct-6 antibodies were eluted from the nitrocellulose by a 3 M KSCN/0.1 M $NaPO_4$/500µg/ml BSA solution. To remove the KSCN the antibody solution was passed over a 0.1 M $NaPO_4$ pH 7.5 equilibrated Sephadex G-50 column.

Band shift assay

Band shift assays and preparation of the Mes68 nuclear protein extracts were done as described (115).

Western blot analysis

Cos-1 cells were transiently transfected by the $CaPO_4$ method with 10 µg of the pN1Oct-6 or pN229C52Oct-6 expression vectors encoding the complete Oct-6 protein or the Oct-6 POU domain respectively (116). 72 hrs after transfection whole cell extracts were made. Equal amounts of protein were run on a 10% SDS-PAAGE and blotted via semi-dry electroblotting to nitrocellulose filter. The western blot was blocked by overnight incubation at 4°C in a 5% powdered milk/ PBS solution. Three 10 min washes in washing buffer (PBS/0.5% Tween20) were followed by 2 hrs incubation at 4°C with the affinity purified Oct-6 antiserum in PBS/0.5% BSA/0.05%

Tween20. After six 10 min washes it was incubated for 2 hrs with a 1:1000 dilution of the anti-RabbitIgG alkaline phosphatase secondary antibody (Sigma) in PBS/0.5% BSA. After six washes in washing buffer the blot was washed once more in 0.2 M Tris-HCl, pH 9.0, 10 mM $MgCl_2$. The staining reaction was carried out with the BCIP/NBT substrates.

Immunohistochemistry

Mouse embryos were derived from natural matings of (CBAxB10)F1 mice. The morning of plugging was determined as 0.5 days post coitum (dpc). Litters of the desired developmental stages were isolated, washed in PBS/0.5% Tween20 (PBS-T) and fixed for 2 to 4 hrs in a (35% acetone, 35%methanol, 5% acetic acid and 25% H_2O) solution. After dehydration and paraffin embedding 5µm sections were made. The sections were mounted on a droplet of 10% alcohol on gelatin/chromesulfate coated Klinipath slides and were allowed to stretch by placing the slides on a hot plate at 42°C. For immunohistochemical staining, the sections were dewaxed and rehydrated. All antibody incubations were done in a 10 mM $NaPO_4$ pH7.5 buffer in a horizontally positioned humidified box. The sections were blocked for two hours by 5% goat serum/ 0.05% Tween20 and washed 5 times for 10' in 0.5% Tween20. Incubation with the first antiserum was overnight. To exclude cross reactivity with other closely related class III POU proteins the affinity purified anti-Oct-6 antibodies were preincubated for one hour with a lysate of Oct-6/POU overexpressing bacteria. As a control, adjacent sections were stained with Oct-6 antibodies and competed with a non-expressing or Oct-6 expressing bacterial lysate. After extensive washing the secondary antibody incubation was performed for 2 hrs at roomtemperature (RT). A goat-anti-rabbitIgG secondary antibody (1:80 dilution) coupled to a FITC chromophore (Nordic) was used to visualize Oct-6 protein expression. After five washes in washing buffer the sections were washed with running tapwater followed by distilled water. The sections were air-dried and embedded in a 100 mg/ml DABCO/ PBS solution (Merck). After photographing, the sections were washed in PBS and counterstained with hematoxylin, dehydrated and embedded in entellan (Merck).

In whole mount immunostaining experiments, antibody incubations and washing steps were done in PBSTF (PBS/1% TritonX-100/10% foetal calf serum). Both the primary and secondary antibodies were incubated overnight. As a secondary antibody the alkaline phosphatase conjugated goat-anti-rabbit antibody (Tago) was used. The staining reaction was carried out with the NBT/BCIP substrates and stopped by washing the embryos in a 50 mM EDTA/PBS solution. The embryos were postfixed in 4% paraformaldehyde/PBS and stored in the dark until photographed.

Whole mount in situ hybridization

A digoxygenin labeled Oct-6 in situ probe was generated from a 520 bp Drab template which is located in the 3'UTR of the Oct-6 gene. Hybridization and visualization with alkaline phosphatase (AP) coupled α-DIG antibodies and NBT/BCIP substrates was done as described (27). For double labeling experiments the Shh probe (38) was fluorescein labeled and hybridized together with the Oct-6 probe. After staining of Shh with AP-coupled α-Fluorescein antibodies and Fast Red substrate (Boehringer) the embryos were incubated for 10 minutes in 0.1 M glycine pH2.2, followed by the Oct-6 staining reaction (62). After postfixation in 4% paraformaldehyde/PBS the embryos were embedded in a phosphate buffered 10% gelatin/30%sucrose solution for cryostat sectioning.

2.2 Expression of the POU transcription factor Oct-6 in the developing eye and hair follicles

The POU gene Oct-6 is expressed in cells of the developing nervous system and in the Schwann cell lineage. Here we describe the differential patterns of Oct-6 protein expression in the developing eye and hair follicles. In the developing hair follicles Oct-6 is expressed in the epidermal cells that undergo rapid cell division and downgrowth into the dermis. In contrast, expression in the developing eye is limited to differentiating cells in the neural retina. In the adult eye Oct-6 protein is found in the proximal part of the inner nuclear layer. The patterns of expression in both proliferating and differentiating cells suggest that Oct-6 may act via different mechanisms as a regulator of ectodermal cell differentiation.

Introduction

The many different organs present in a vertebrate organism develop from a series of inductive interactions between different cell layers. This results in decisive changes in cellular identity and adaptation of cell-specific function (53). Although the developmental processes are well described, the molecular mechanisms underlying these processes have hardly been resolved.

The hair follicle is an exceptionally suitable model system to study the molecular mechanisms of organ development. The tissue is easily accessible and the possibility to culture epidermal cells and perform grafting experiments has resulted in a detailed description of the temporal order of inducing interactions between the epidermis and underlying dermis (57, 182). A first dermal message derives from condensed mesenchyme tissue and induces thickening and downgrowth of the overlying epidermis. Subsequently, an inducing signal is produced by the epidermal bud. It causes the formation of a dermal papilla, which will form the growth center of the hair. Finally, a second dermal signal from the dermal papilla induces proliferation of epidermal hair matrix cells, followed by differentiation into the mature hair and inner root sheath cell types. Studies on the mRNA distribution of growth factors and cell adhesion molecules have identified a number of molecules that could possibly mediate the inductive signals. Among them are the bone morphogenetic proteins 2 and 4 (BMP-2 and BMP-4), and the cell-adhesion molecules E- and P-cadherin (108). Intriguingly, the signaling molecule sonic hedgehog (Shh) which is expressed in the midline mesoderm and contains the organizing activity providing cells of the neuroectoderm an anterior-posterior (A-P) identity, was shown to be expressed in a similar pattern as BMP-4 in the ectoderm of the developing hairs and whiskers (16). Experiments in which the gene function of the epidermal growth factor receptor (EGF-R) or its ligand transforming growth factor α (TGFα) was disrupted, have provided direct evidence for a role of these molecules in skin development and hair follicle formation (106, 110, 120).

How the inductive signals are transformed into a pattern of hair-specific gene expression is unknown. One possible candidate has recently been identified (198). The Lymfoid Enhancer Factor 1

(LEF-1) protein is induced in the surface ectoderm at the earliest stage of hair follicle formation. LEF-1 is a member of the high-mobility-group (HMG) transcription factor family, and the LEF-1 consensus binding site was found in 13 hair keratin gene promoters. Overexpression of LEF-1 in the ectoderm layer in transgenic mice results in altered patterning and development of hair follicles, indicating that LEF-1 is important for specification of ectodermal development (198).

Development of the eye is initiated by interactions between the optic vesicle and the adjacent surface ectoderm which results in the formation of the lens placode (53). Subsequently, the optic vesicle folds as a bilayered optic cup, and under influence of the lens, these layers will develop into a pigment epithelium and the neural retina. Differentiation of the neural retina from a single, uniform cell-layer of neuroepithelial cells into a stratified layer of different neuronal and glial cell types has served as an convenient model to study neuroepithelial cell differentiation. Turner and Cepko have shown by retro-viral mediated cell-labeling that the progeny of one neuroepithelial precursor cell can consist of multiple neuronal cells and one glia cell type (180). The developmental fate of neuronal precursor cells in the retina appears to be determined by cues from the immediate environment, and by changes in the competence of precursor cells to respond to these signals (26, 114).

We describe here the expression of the POU transcription factor Oct-6 in both the developing eye and hair follicle and discuss the observed patterns of expression with respect to a possible role in neuronal and epidermal cell differentiation.

Results and Discussion

Oct-6 expression in hair follicles

Expression in developing hair follicles is first observed in the dorsal whiskers in 12.5 days pc embryos. At later stages Oct-6 expression is induced in the more ventral whiskers and in the pelage hair follicles. Figures 1a,1b show Oct-6 protein expression in the whiskers of a 14.5 days pc embryo. Expression is confined to cells of the surface ectoderm that overly aggregates of dermal mesenchyme. No expression is observed in adjacent epidermal cells or in the dermis (fig.1a,1b). The pattern of expression is colinear with the temporal pattern of whisker and pelage hair follicle formation, suggesting that Oct-6 is expressed at the earliest stages in hair follicle formation in those cells that respond to the first inducing dermal signal (182). An identical expression pattern has been described for the LEF-1 and BMP-2 genes (108, 198). Furthermore, the expression overlaps with the expression pattern of t4he keratin 5 (K5) gene and the transcription factor AP-2 (22). K5 encodes a type I keratin protein and is specifically expressed in the undifferentiated embryonic ectoderm and in the basal layer cells of the adult skin (89). Based on the observation that Oct-6 overexpression leads to a several-fold reduction in K5 gene promoter activity in transient assays, it has been suggested that Oct-6 may act as a negative regulator of K5 gene expression (40). However, the overlap in Oct-6 and keratin 5 gene expression in the developing hair follicle makes it difficult to envisage a physiological interaction.

Interestingly, both the Oct-6 and K5 gene promoters contain multiple AP-2 binding sites (23, 117). The AP-2 transcription factor has been identified as a mediator of altered gene expression by the cAMP dependent protein kinase A (PKA) signal-transduction pathway (72). In cultured Schwann cells Oct-6 gene expression is strongly increased upon induction of the intracellular cAMP levels, suggesting that AP-2 could act as a

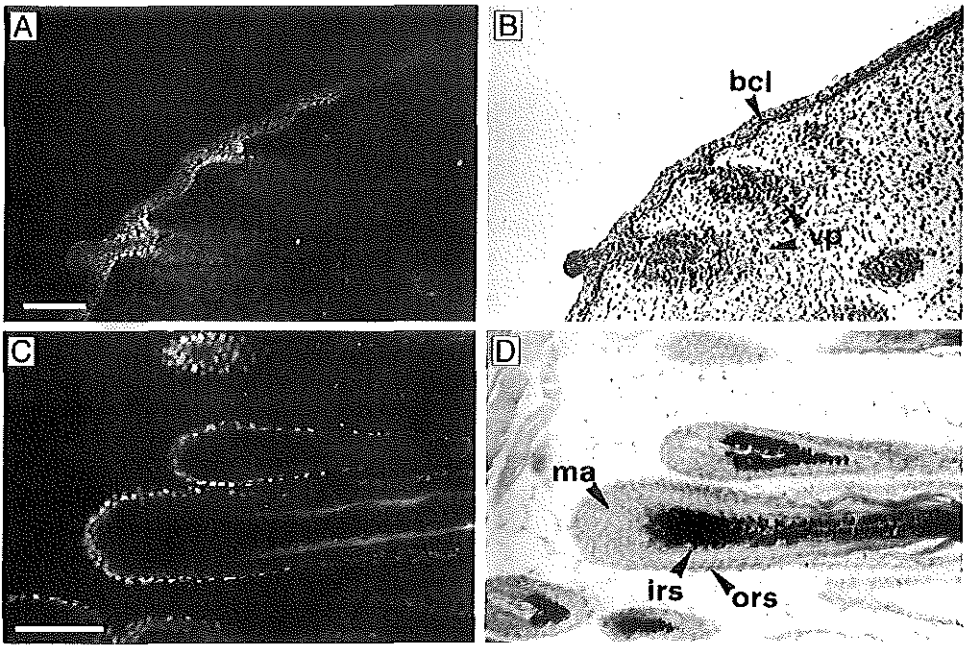


Figure 1. Oct-6 protein expression in the basal layer cells of a 14.5 days pc embryo (A-B) and in the outer root sheath cells of the hair follicles in the skin of an adult mouse (C-D). (B) and (D) show haematoxylin counterstainings of the sections shown in (A) and (C). Abbreviations: bcl, basal cell layer; irs, inner root sheath; ma, matrix; ors, outer root sheath; vp, vibrissa primordium. Bars represent 100μm.

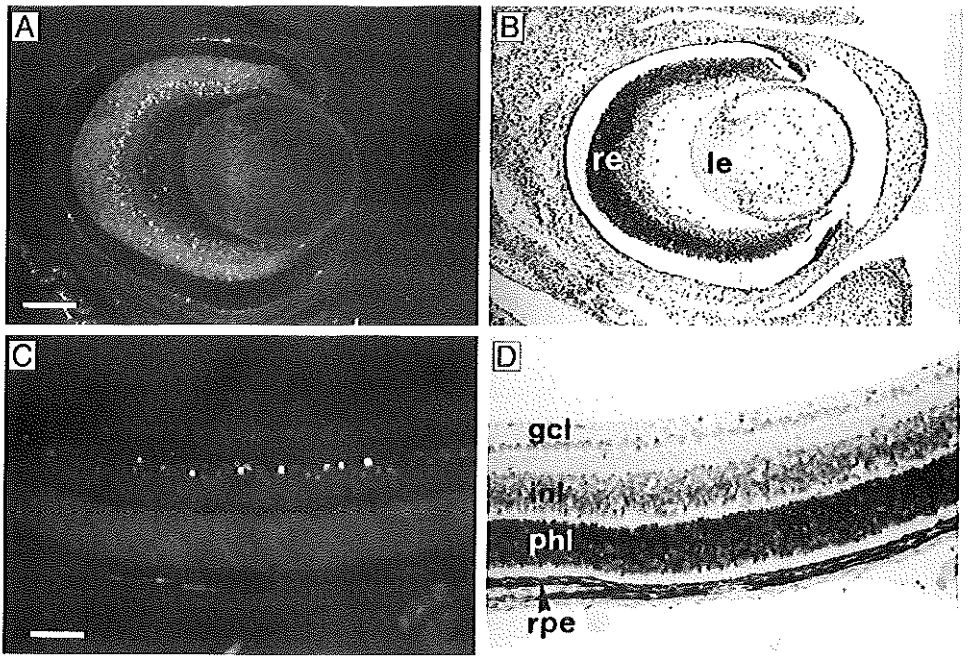
regulator of Oct-6 gene expression in Schwann cells (124). Thus far, transgenic animals carrying a lacZ reporter gene under control of the Oct-6 promoter have not shown any lacZ expression in the Schwann cell lineage. Neither has lacZ expression been observed in the epidermis of these transgenic mice, leaving the role of AP-2 in Oct-6 gene regulation unsolved (chapter 3.1 of this thesis). In contrast, overexpression of the AP-2A splice variant in the HepG2 hepatocyte cell line results in a transactivation of the K5 gene promoter (22). Furthermore, expression of AP-2A in the ectodermal cells of the developing hair follicles and in the basal layer of the skin precedes expression of K5, suggesting that AP-2 is involved in the regulation of K5 gene expression.

In the mature hair follicle Oct-6 protein expression is observed in the basal layer cells of the inner part of the outer root sheath (ORS; fig.1c and 1d). These cells are in a resting phase and could, under the proper conditions, be induced to regenerate a complete hair follicle (48, 57). The pattern of expression in the embryonal and mature hair follicle suggests that Oct-6 could act as a regulator of cell growth. Experiments in which the Oct-6 gene is ectopically overexpressed in the surface ectoderm or ORS cells in transgenic mice could address this question. In contrast, the POU gene Skn-1a/i is expressed in the cortex cells of the hair (3). Skn-1a/i cDNA was isolated from a rat anterior pituitary cDNA library and is expressed at embryonal stages in the epidermis. In transient assays it can activate the keratin 10 (K10) gene promoter. K10 is a molecular marker which is specifically expressed in the suprabasal layer of

differentiating cells, indicating that Skn-1a/i might function as a regulator of terminal differentiation.

Occasionally, we observed Oct-6 protein expression in the embryonic skin (data not shown). At midgestational stages of development this was restricted to parts that were undergoing profound morphological changes, like in the pinnae of the ear of a 13 days pc embryo. In a 18 days pc embryo a higher incidence of expression can be detected in the skin epidermis. BrdU incorporation experiments revealed that only a portion of the Oct-6 expressing cells are present in the proliferative layer of basal cells, whereas others are located in the suprabasal differentiating cell layer (Ronald Zwart, Ludo Broos and Dies Meijer, unpublished observation). RNA *in situ* hybridization

Figure 2. Oct-6 protein expression in the developing mouse eye. (A) and (C) show expression in the neural retina and inner nuclear layer of a 14 days pc embryo and postnatal day 7 pup, respectively. (B) and (D) are bright-field exposures of haematoxylin counterstainings of the sections shown in (A) and (C). Abbreviations: gcl, ganglion cell layer; inl, inner nuclear layer; le, lens; phl, photoreceptor layer; re, retina; rpe, retina pigment epithilium. In (A) and (C) the bars represent 100μm and 50μm, respectively.



experiments with a human Oct-6 cDNA probe, indicated that expression in the basal and suprabasal layers would be a general feature in neonatal human skin epidermis and adult mouse stratified epithilia (40).

Oct-6 expression in the eye

At the earliest stages of eye development no expression is observed in the neuroepithelium that forms the retina or in the surface ectoderm of the lens placode. The earliest timepoint of expression coincides with the occurence of a bilayered structure in the neural retina, of which the inner part is mostly occupied by differentiating cells. Figures 2a,2b show Oct-6 protein expression in the developing eye of a 14 days pc embryo. Although Oct-6 expression is observed over the entire neural retina, expression appears most prominent in the inner part. This result suggests that Oct-6 expression would be restricted to post-mitotic cells in the developing eye. When the retina has fully stratified into its neuronal and synaptic layers, Oct-6 expression can only be detected in the inner nuclear cell layer (INL), which consists of bipolar neurons, amacrine neurons and supporting glia cells. This is shown in figures 2c,2d. No expression is observed in the other cellular or synaptic layers, or in the lens. The Oct-6 expressing cells are concentrated in the proximal part of the INL where also the majority of the amacrine neurons are located.

Recently, expression of the class IV POU proteins Brn-3.0 and Brn-3.2 in the developing and adult eye have been described. At embryonal stages expression of both Brn-3.0 and Brn-3.2 is limited to the inner layer of the neural retina, whereas in the mature eye only a subset of the ganglion cells express Brn-3.2 protein (41, 52, 181, 191). These observations would suggest that expression of different POU factors in distinct populations of differentiating retinal cells plays an important role in the specification of these neuronal phenotypes. In addition, expression of the other class III POU genes and the Oct-2 gene in the retina has been reported (64). Analysis of the expression patterns of these genes in the developing eye would be a first step to test this hypothesis.

Materials and Methods

Embryos isolated from timed pregnancies, and staged pups were processed for immunohistochemistry as previously described (199). After photograhy of the immunofluorescence signal, the sections were washed in PBS and counterstained with haematoxylin, dehydrated and embedded in Entellan (Merck).

2.3 Expression and function of the POU gene Oct-6 in the Schwann cell lineage

Myelin- and non-myelin forming Schwann cells are the glia cell types in the peripheral nervous system (PNS) that ensheath and insulate outgrowing nerve fibers. Both Schwann cell-types differentiate from a common precursor, the embryonic Schwann cell. The rat Oct-6 gene (SCIP) was isolated as a differentially expressed factor in primary Schwann cell cultures from the sciatic nerve of a newborn rat. In these cultures Oct-6 expression preceeds expression of the P_0 myelin gene, a marker for terminal differentiation of myelinating Schwann cells, and in transient assays Oct-6 represses P_0 myelin gene promoter activity. It has therefore been hypothesized that Oct-6 may function as a negative regulator of the final step in the development of myelin producing Schwann cells. To determine if this corresponds with a possible role for Oct-6 in Schwann cell development *in vivo*, we have studied the temporal pattern of Oct-6 protein expression in the myelinating Schwann cells of the mouse sciatic nerve. A major induction of Oct-6 protein expression in the sciatic nerves is observed between 15 and 17 days pc. A subpopulation of the Oct-6 expressing cells are proliferating, indicating that Oct-6 protein is first expressed in the embryonic Schwann cell, and possibly in its precursor. Induction precedes the expression of P_0 protein, and expression of Oct-6 and P_0 is found within a single cell, indicating that Oct-6 protein expression is maintained after the onset of myelination. In addition, functional analysis of the Oct-6 gene function by homologous recombination revealed that in the absence of Oct-6 protein differentiation of myelinating Schwann cells completes, but is severely delayed, due to a temporary arrest at the promyelination stage. These data argue that Oct-6 is required for the progression of Schwann cell differentiation before terminal differentiation into a myelin producing Schwann cell.

Introduction

Schwann cells of the peripheral nervous system (PNS) develop from the neural crest. Migrating neural crest cells that have become associated with the outgrowing axons in the anterior parts of the somites will differentiate in an axon-contact dependent manner into a myelin- or nonmyelin-producing Schwann cell (14, 168). Intermediate cell-types in the development of fully differentiated Schwann cells from the multipotent neural crest have been described in recent years. The Schwann cell precursor was identified in the sciatic nerve of a 14 days pc rat embryo. These cells express the nerve growth factor receptor (NGF-R), and can be distinguished from neural crest cells by expression of the membrane-associated phosphoprotein GAP-43 (79). Survival of these early Schwann cells in culture is dependent on neuron-conditioned medium and the Schwann cell precursors show apoptotic cell death when cultured in non-conditioned medium. In cultures of Schwann cells isolated from the sciatic nerve of 15 to 17 days pc rat embryos, a rapid increase in survival is observed in non-conditioned medium. Furthermore, these cells express the Ca^{2+} binding protein S-100, a molecular marker characteristic for embryonic Schwann cells (77, 79). Thus, a transition from a Schwann cell precursor to an embryonic Schwann cell in the sciatic nerve occurs between 15 and 17

days of rat embryonal development. The dependence of precursor Schwann cell survival on neuron conditioned medium, suggests that *in vivo* axons associated with the precursor Schwann cell might provide a survival signal. In a screen for neuron-derived growth factors containing this activity, basic fibroblast growth factor (bFGF, or FGF2) was shown to prevent apoptotic death of precursor Schwann cells and promote the differentiation into an embryonic Schwann cell (79). However, this activity is dependent on the activation of the type 1 insulin growth factor (IGF) receptor (50). Unlike FGF2, ß forms of the Neu differentiation factor (NDF; ARIA or glial growth factor, GGF) establish long term survival and maturation of the Schwann cell lineage under serum-free and low insulin conditions (36). NDF also promotes the glial fate of neural crest cell differentiation (162). Survival of Schwann cell precursors by neuron conditioned medium is prevented by a dominant negative mutant of the NDF receptor ErbB4 (36). Furthermore, NDF mRNA expression is largely restricted to neuronal cells in the spinal ganglia and spinal motor neurons, adding weight to the assumption that NDF is one of the endogenous neuron-derived signaling molecules promoting precursor Schwann cell survival and progression of Schwann cell development. (119, 162).

Increased numbers of proliferating embryonic Schwann cells will ultimately segregate the nerve in separate axon-Schwann cell units and differentiate into either myelinating or non-myelinating Schwann cells. This depends on the size of the axon individual embryonic Schwann cells ensheath (77). Non-myelinating Schwann cells express many of the molecular markers, which are also expressed in the embryonic Schwann cells, like NGF-receptor, glial fibrillary acidic protein (GFAP), the cell-adhesion molecules L1 and N-CAM, the surface proteins A5E3 and Ran-2 and the paired box transcription factor Pax3 (77, 85). Myelinating Schwann cells start expressing high levels of the major myelin genes P_0 and myelin basic protein (MBP) when these cells have reached a 1:1 ratio with a large caliber axon and have generated a basal lamina. *In vitro* cultures of Schwann cells isolated from the sciatic nerve of newborn rats have been used to study the molecular mechanisms of myelination. Axon-contact dependent induction of myelin gene expression could be mimicked in these cultures by administration of reagents that elevate the intracellular cAMP levels (99). In a search for genes which may act as mediators of cAMP-induced gene expression, the rat Oct-6 gene (SCIP) was isolated (124). Induction of Oct-6 gene expression preceded the induction of the major myelin genes MBP and P_0, and prolonged exposure of Schwann cell cultures to cAMP inducing agents retained both Oct-6 and P_0 gene expression. This led to the hypothesis that Oct-6 may positively regulate myelin gene expression (124). However, the activity of a 1.1 kb 5' promoter fragment of the P_0 gene which is sufficient to drive Schwann cell specific gene expression in transfection assays and transgenic mice, is repressed by overexpression of the Oct-6 gene in transient assays (65, 125, 126). Based on this result, and the notion that inversely correlated levels of Oct-6 and MBP and P_0 mRNA expression are present in the sciatic nerve at postnatal stages of development, it was concluded that Oct-6 acts as a repressor of MBP and P_0 gene activation, and may thus play an important negative regulatory role in the final step of myelinating Schwann cell differentiation.

Expression studies in transected and crushed nerves have shown that in degenerating and regenerating nerves the Oct-6 and myelin genes are expressed in co-linear patterns. After transection of the sciatic nerve in an adult rat, Oct-6 is transiently upregulated within days after transection, but permanently downregulated at later stages. In parallel, the levels of P_0 and MBP myelin gene expression plummet, whereas expression

of the NGFR gene, a molecular marker of non-myelinating Schwann cells, is upregulated (125, 152). After nerve crush, when the axon is able to regenerate, Oct-6 and P_0 are both upregulated in Schwann cells present in the distal nerve stump, and expression is maintained for at least 58 days after the crush (152). Thus, it remains unclear if, and via which mechanism, Oct-6 may regulate P_0 gene expression.

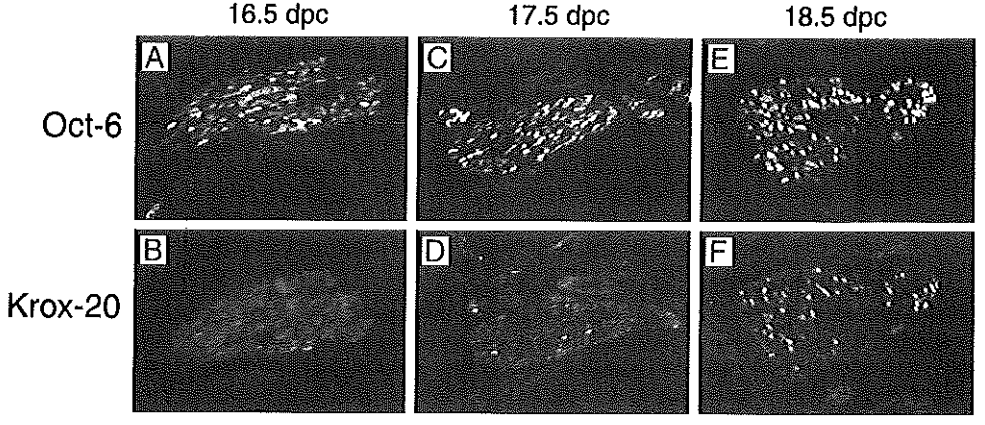
The studies described above have focused on the role of Oct-6 in terminal differentiation of myelinating Schwann cells. However, interpretation of the data is hampered by the absence of a detailed *in situ* expression analysis. Furthermore, Oct-6 expression at prenatal stages of development has not been examined, ignoring a possible role in earlier stages of Schwann cell development. We have therefore performed immunohistochemical experiments using an Oct-6 specific polyclonal antiserum, to analyse the pattern of Oct-6 protein expression in the mouse sciatic nerve at prenatal and postnatal stages of development. In double-labeling experiments Oct-6 expression was compared with the expression of the zinc-finger protein Krox20, the myelin gene P_0, and incorporation of BrdU, a marker for mitotic cell division characteristic for embryonic Schwann cells. These experiments show that Oct-6 protein is first expressed at the embryonic Schwann cell stage and is co-expressed with the myelin protein P_0 at later stages. Inactivation of the Oct-6 gene by homologous recombination reveals a delay in Schwann cell differentiation, arguing that Oct-6 is important for the progression of Schwann cell differentiation at the promyelination stage of development.

Results

Oct-6 protein expression in myelinating Schwann cells of the sciatic nerve

Oct-6 protein expression was analysed in sections of 12 to 15 days pc embryos, and in sections of the hindlimbs of older embryos and postnatal pups. To visualize the outgrowing axons double labeling

Figure 1. Expression of Oct-6 and Krox20 protein in the sciatic nerve of a developing mouse embryo. Adjacent sections of 16.5, 17.5 and 18.5 days pc mouse hindlimbs were stained with Oct-6 (A,C,E) and Krox20 (B,D,F) antibodies. The arrowheads in (E) and (F) indicate cells expressing both Oct-6 and Krox20.



experiments were performed with a monoclonal antibody against the 155 kDa neurofilament-medium (NF-M) protein. In Schwann cells of the sciatic nerve Oct-6 protein expression is first detected between 15 and 16 days of embryonal development. Increasing levels of Oct-6 protein expression are observed at later embryonal stages, as well as an increased number of Oct-6 expressing cells. Adjacent sections of 16.5, 17.5 and 18.5 days pc embryo hindlimbs were stained with antibodies against Oct-6 and Krox20 protein (fig.1). Krox20 is a zinc-finger transcription factor that is involved in hindbrain development, but is also required for completion of myelination in the peripheral nervous system (176). Inactivation of the gene encoding Krox20 does not interfere with the formation of a 1:1 relationship between axon and Schwann cell and expression of the early myelin marker MAG, but prevents MBP and P_0 expression (176). A major increase of Oct-6 protein expression in the Schwann cells of the sciatic nerve is seen between 16.5 and 17.5 days of development, whereas an increase in the levels of Krox20 expression occurs about one day later, between 17.5 and 18.5 days pc. The timepoint of induction, and the comparison with Krox20 protein expression suggest that Oct-6 protein is induced in embryonic Schwann cells during development of the myelinating Schwann cell. To examine this directly, we have performed BrdU incorporation experiments. BrdU incorporation labels those cells that are in S phase of the cell cycle, a property that distinguishes embryonic from myelinating Schwann cells, which have stopped dividing and express the myelin genes P_0 and MBP. Figure 2 shows a cross-section of the sciatic nerve of a 18.5 days pc embryo that has been stained for both Oct-6 (fig.2a) and BrdU (fig.2b). Cells that label positive with both markers are clearly visible (indicated with arrowheads), demonstrating that Oct-6 protein is expressed in embryonic Schwann cells. BrdU-positive cells are also detected that do not express Oct-6 protein (indicated with arrows, figs.2a and 2b). These cells might represent embryonic Schwann cells that have not yet reached the stage at which they start expressing Oct-6. Thus induction of Oct-6 protein expression most likely takes place at a late stage in the embryonic Schwann cell.

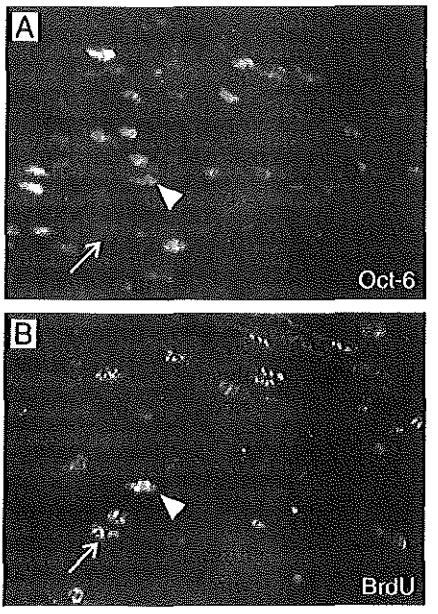


Figure 2. Oct-6 expression (A) and BrdU incorporation (B) in the Schwann cells of the sciatic nerve of a 18.5 days pc embryo. The arrowhead indicates a cell that labels positive for both markers, while the arrow points out a cell that is mitotically active but does not express Oct-6.

Counting the number of Oct-6/BrdU double positive nuclei revealed that these cells constitute only 5 to 10 % of the total number of Oct-6 expressing cells in the sciatic nerve of a 18.5 days pc embryo (data not shown). At comparable stages of embryonal development in the rat it has been shown that embryonic Schwann cells constitute the majority of Schwann cell-types in the sciatic nerve at these developmental

timepoints (167). Furthermore, the S-phase takes about one-third of the 24 hours cell cycle, arguing that BrdU-pulse-labeling visualizes 30% of the total embryonic Schwann cell population (167). Assuming that similar numbers account for Schwann cell division in the mouse, it indicates that Oct-6 protein is not just expressed in dividing embryonic Schwann cells, but also in cells that have left the cell cycle and have started to terminally differentiate into myelinating Schwann cells. To determine this directly, we have performed double-labeling experiments with Oct-6 and P_0 antibodies on sections of embryonic day 18 and postnatal day 14 sciatice nerve sections (fig.3). 18 days pc is the first timepoint that we have observed high levels of P_0 protein expression (data not shown). A confocal microscopy analysis shows that in a subpopulation of Oct-6 positive cells Oct-6 and P_0 protein are localized in a single cell. A representation of this is shown in figure 3a. From these data we conclude that in addition to expression in embryonic Schwann cells Oct-6 protein is also expressed in myelinating Schwann cells. At postnatal stages of development decreasing numbers of Oct-6 positive Schwann cells are observed in the sciatic nerve till at least p14 (fig.3b). This correlates with the Oct-6 mRNA levels that have been reported to decline from the time of birth (152). Probably, downregulation of Oct-6 gene expression is associated with a certain stage in the progression of Schwann cell differentiation. Thus, during development of myelinating Schwann cells in the sciatic nerve Oct-6 protein is expressed in both late embryonic and early myelinating Schwann cells. This expression profile suggests that Oct-6 is involved in maturation and final differentiation of the myelinating Schwann cell lineage.

Individual cells associated with single axons express Oct-6 protein

At 12.5 days pc, and with increasing intensity and numbers at later stages of development, Oct-6 positive cells were observed in muscle anlagen throughout the body plan. Figure 4 shows the expression of Oct-6 in the cervical muscle of a 14.5 days pc embryo. The Oct-6 expressing nuclei (indicated by arrowheads) are clearly arranged in a coordinate alignment and are closely associated with NF-M positive axons (arrows). The identity of these cells has not been determined. The location of the Oct-6 nuclei within the muscle anlagen and the close association with single axons may indicate that Oct-6 is expressed by a telo- or perisynaptic glia cell-type (51). Alternatively, these cells might represent Schwann cells that have already reached a 1:1 relationship with the axon fasicles and are thus most

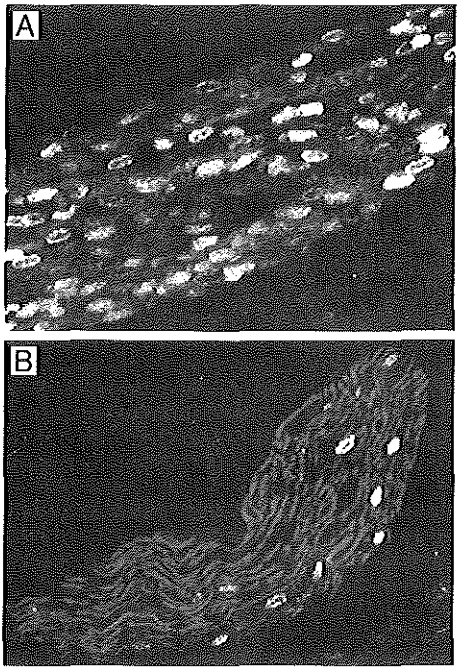


Figure 3. Oct-6 and P_0 expression in the sciatic nerve of a 18.5 days pc embryo (A) and postnatal day 14 pup (B). Oct-6 positive nuclei are visible as bright spots, whereas the P_0 protein signals is shown in gray.

advantageous in their differentiation pathway at midgestation periods of development. No Oct-6 expressing cells were observed in the periphery that were not associated with the nerve fibers, suggesting that expression of Oct-6 protein in these cells is mediated by an axon-contact dependent mechanism.

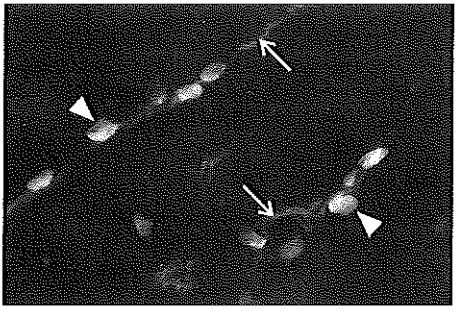


Figure 4. Oct-6 protein expression in cells aligning single axon fasicles. A double labeling is shown with Oct-6 and NF-M antibodies on a section of the cervical muscle of a 14.5 days pc embryo. Arrowheads point at nuclei that label positive for Oct-6 and are in close association with single axon fasicles (indicated with arrows). No Oct-6 expressing cells are found that are not associated with NF-M positive axon fasicles.

Discussion

Analysis of Oct-6 protein expression in the developing myelinating Schwann cells present in the mouse sciatic nerve has shown that Oct-6 protein is induced in embryonic Schwann cells and persists in an early period of terminal differentiation of myelinating Schwann cells. It has been hypothesized that Oct-6 would function in repression of myelin P_0 gene expression and thus negatively regulate the induction of myelination. However, by double-labeling immunohistochemical experiments we have shown that Oct-6 and P_0 expression are not mutually exclusive in one cell, indicating that Oct-6 may not be the only determinant in P_0 gene expression regulation. To study the role of Oct-6 in development of myelinating Schwann cells directly, Oct-6 gene function was inactivated by homologous recombination (75). If Oct-6 would only serve to repress P_0 gene expression, inactivation of the Oct-6 gene should result in a hypermyelinating phenotype due to ectopically induced P_0 gene expression at earlier stages of Schwann cell development. In contrast, homozygous pups that survive beyond 10 days of postnatal development show a clear body tremor. Electron microscopic analysis of the sciatic nerve of pups at the early postnatal postnatal stages of development showed that myelination is delayed and disorganized in Oct-6 homozygous mutant animals (75). No myelin figures are seen until the second postnatal week, whereas a minority of cells form a myelin sheath in the absence of an axon, or before a 1:1 ratio with the axons was established. At P90 the majority of Schwann cells in the sciatic nerve of an Oct-6 homozygous mutant mouse have established a complete myelin sheath which does not show any abnormal features, indicating that myelination is delayed but not blocked. In line with the morphological characteristics, induction of the myelin genes P_0, MAG and MBP is delayed and lower levels of expression are reached (75). The delay in the onset of myelination argues that Oct-6 is involved in the progression of myelinating Schwann cell differentiation. This function is most likely reflected in the pattern of expression in the embryonic Schwann cells.

In a second experiment Weinstein *et al.* (1995; 187) have generated transgenic mice with the Oct-6 POU domain (Oct-6/POU) under transcriptional control of the stage-specific myelin P_0 gene promoter to study the Oct-6 gene function in Schwann cell differentiation. The P_0 promoter directs high levels of Oct-6/POU expression to the final, myelinating stage of Schwann cell development. In transient assays Oct-6/POU was shown to inhibit Oct-6 mediated

repression of the P_0 promoter, indicating that Oct-6/POU acts as an antagonist for Oct-6 protein function. In two independent transgenic lines a premature and hypermyelinating phenotype is observed in newborn animals (187). However, this result is difficult to interpret with respect to Oct-6 gene function. First, overexpression of the Oct-6 POU domain may cause autonomous alterations in gene expression resulting in the observed phenotype. Second, it can not be excluded that Oct-6/POU does not only interfere with Oct-6 itself in DNA binding or protein-protein interactions, but it could also act as a dominant negative for a third protein. Assuming that Oct-6/POU only affects Oct-6 function *in vivo*, it would provide important support for previous conclusions from *in vitro* experiments that Oct-6 functions to repress P_0 gene expression. The Oct-6 inactivation studies however, have shown that this will only occur after Oct-6 has been explored in progression of embryonic Schwann cell differentiation.

Starting at day 12 of embryonal development, Oct-6 positive cells are observed in muscle anlagen that are in close association with single axon fascicles. The location suggests that the single axon-associated Oct-6 expressing cells might be telo-, or perisynaptic glia cells. Teloglia cells cover the neuromuscular junctions and can be recognized by an oval-like cell body, which is oriented parallel to the longitudinal axis of the muscle fiber (51). These cells show an induction of intracellular Ca^{2+} levels in response to neurotransmitter release (76). The morphological characteristic can not be recognized however, in the Oct-6 positive cells. In frogs, nerve transection or blockage of the nerve action potentials by injection of tetrodotoxin (TTX) have been shown to result in a rapid increase of glial fibrillary acidic protein (GFAP) expression in teloglia cells (51). Double-labeling experiments with anti-GFAP antibodies after nerve transection may thus elucidate if the Oct-6 expressing cells have a teloglia identity. Alternatively, the Oct-6 expressing cells may represent a subpopulation of normal Schwann cells that have reached a more advanced stage in their differentiation program than the Schwann cells in the sciatic nerve. This should be confirmed by a temporal expression analysis of other early Schwann cell markers like NGF-R and S-100.

Materials and methods

Antibodies

Rabbit polyclonal antibodies against Oct-6 and Krox20 (gift from N. Becker and P. Charnay) were used 1:200 and immunoreactivity of both antibodies was visualized with a secondary FITC-coupled goat-anti-rabbit antiserum (1:80, Nordic). Mouse monoclonal antibodies against neurofilament-medium protein (NF-M) (1:300,Sigma), myelin protein P_0 ($P_0$7 clone 18, gift from J.J. Archelos), bromodeoxyuridine (BrdU) (1:1000, Sigma) were visualized with TexasRed-coupled goat-anti-mouse antibodies (1:80, ITK).

Immunohistochemistry

Tissue-processing and antibody incubations were done as described (199). For comparative expression analysis of Oct-6 with Krox20 adjacent sections were used, while for the other markers double labeling experiments performed on a single section. To obtain a better exposition of the antigen recognized by the BrdU antibody sections analysed for BrdU incorporation were treated with 2 N HCl for 20 minutes before the first antibody incubation. This did not negatively influence the reactivity of the Oct-6 antibodies.

BrdU incorporation experiments

BCBA females carrying a timed pregnancy

of a cross with a BCBA male were given an intraperitoneal (i.p.) injection with 100 µg BrdU /g body weight dissolved in 0.9% saline/ 0.0014 N NaOH. BrdU incorporation was allowed for 1 hour before the mothers were sacrificed and the litters were isolated for immunohistochemical processing (121).

Chapter 3

Oct-6 gene regulation

3.1 Analysis of the transcriptional regulatory potential of Oct-6 genomic sequences in transgenic mice

The POU gene Oct-6 is differentially expressed during the establishment of a variety of ectodermal cell types. To locate the position of enhancer elements critical for correct expression of the Oct-6 gene we have generated transgenic mice containing the lacZ reporter gene under transcriptional control of Oct-6 genomic sequences. A 32 kb genomic fragment containing the entire Oct-6 gene plus 15 kb of upstream and 14 kb downstream sequences (2coslacZ) is sufficient for expression in the embryonic Schwann cells. An Oct-6 like lacZ expression pattern is also observed in the ectodermal cells of the developing skin and in the developing central nervous system. This construct thus forms a baseline for the isolation of cell-specific regulatory sequences which mediate expression of the Oct-6 gene in ectoderm cell lineages.

Introduction

Mutation analyses in different species have shown that the POU transcription factors function as important regulators of cell differentiation and development (147, 185). It is anticipated that temporal and spatial expression of these factors is required for normal development to occur. Identification of the *cis*-acting elements which regulate the expression of the POU genes itself would thus significantly contribute to our understanding of the molecular mechanisms of cell differentiation.

Information on regulatory elements involved in POU gene expression has remained limited to the Oct-4 and Pit-1 genes. One distal and one proximal regulatory element have been identified in the Oct-4 upstream genomic region (135, 196). They contain independent enhancer activity, driving Oct-4 gene expression in a cell-specific fashion. The distal element is important for expression in the primordial and embryonic germ cells, and for expression in the preimplantation embryo and the embryonic stem (ES) cells, whereas the proximal element is essential for expression in the epiblast (196). In ES cells, Oct-4 gene expression is rapidly down-regulated upon treatment with retinoic acid (RA), which is mediated at the transcriptional level (123). Furthermore, *in vivo* footprinting showed that down-regulation is accompanied with a loss of DNA-binding proteins bound to the promoter and enhancer elements, indicating that binding and release of DNA binding factors is part of the mechanism of Oct-4 gene regulation (123).

Pit-1 gene expression is limited to the lactotroph, somatotroph and thyrotroph cell types in the anterior pituitary and is involved in cell-specific expression of the growth hormone (GH) and prolactin (PRL) genes (74, 101). A 200 bp proximal Pit-1 promoter fragment was identified to confer cell-specific gene expression in the GH-expressing GC and GH3 anterior pituitary tumour cell-lines (28, 111). This element is also sufficient to mediate cAMP induced Pit-1 gene expression (111). Furthermore, two Pit-1 binding sites were identified that overlap with the binding site of a cell-specific factor and the TATA-box. DNA-binding of the cell-specific factor and the TATA-box binding proteins is increased and promoter activity induced upon Pit-1 binding, thus establishing a positive autoregulatory

loop (28, 111, 112). An additional Pit-1 enhancer element critical for pituitary-specific gene expression *in vivo* was identified by transgenic analysis (143). This enhancer element is located 10 kb upstream of the Pit-1 gene transcription start site. It contains multiple core motifs for different DNA binding transcription factors, including five Pit-1 binding sites. The functional organization of this enhancer was extensively analysed by mutation analysis in transient assays (143). In the Pit-1-expressing GC cells three Pit-1 binding sites were shown to be essential for enhancer activation. *In vivo*, Pit-1 autoregulation appears to be required for maintenance of Pit-1 gene expression at postnatal stages of development, as Pit-1 gene expression in the Snell dwarf mouse, which carries an inactivating pointmutation in the Pit-1 DNA binding domain, is affected after birth but not at prenatal stages of development. The distal enhancer element also contains binding sites for the vitamin D and RA receptors, which are important for establishing enhancer activity in GC cells. This activity is synergistically induced upon co-transfection of Pit-1 and the retinoic acid receptor α (RARα) in CV-1 cells. In a band shift assay altered protein complexes are detected upon incubation of both Pit-1 and RARα compared to incubation with either protein alone, suggesting a cooperative interaction between the cell-specific factor Pit-1 and the RARα receptor in Pit-1 gene regulation (143).

The POU protein Oct-6 shows a restricted expression pattern in the neuroepithelium of the developing fore- and midbrain, and is differentially expressed in several ectodermal cell types, among which are the Schwann cells (see chapter 2.3 of this thesis). Based on the pattern of expression in the early embryo it has been postulated that negative regulatory elements may play an important role in Oct-6 gene regulation (199). In cultures of embryonic Schwann cells isolated from the sciatic nerve of a newborn rat Oct-6 protein expression is strongly induced upon elevation of intracellular cAMP levels (124). Elevation of cAMP is generally thought to represent one of the axon-contact dependent signals which drive Schwann cell differentiation *in vivo*, as it induces many of the transcriptional events associated with the transition of an embryonic to a myelin producing Schwann cell (128). Analysis of the critical Oct-6 gene regulatory elements will thus allow to further study the signaling pathways involved in early development and Schwann cell differentiation.

A number of consensus binding sites for different regulatory factors are present in the Oct-6 promoter region (93, 117). Among them are a non-consensus TATA-box, TTTAA, and several CCAAT and Sp1 boxes. Interestingly, two AP-2 binding sites are present in the Oct-6 gene promoter which could possibly mediate cAMP-induced Oct-6 gene expression (72). However, Oct-6 promoter sequences are not sufficient to mediate cell-specific expression of a reporter gene in transient assays (117). Furthermore, only a limited sensitivity to elevated cAMP levels was observed upon transfection of an Oct-6 promoter-CAT reporter fusion gene in the rat B103 neuroblastoma cell line, which shows a more than 60-fold induction of the endogenous Oct-6 gene transcription (Ronald Zwart and Dies Meijer, unpublished results). Therefore, we have isolated large genomic fragments of the Oct-6 locus and analysed the regulatory potential of a 32 kb fragment in transgenic mice to locate the position of cell-specific enhancer elements.

Results

Oct-6/lacZ transgenic mice

A transgenic construct was generated with the cosmid 2cosOct-6 which contains the entire Oct-6 single exon gene plus 15 kb of upstream and 14 kb of downstream sequences (chapter 3.2 of this thesis). The lacZ reporter

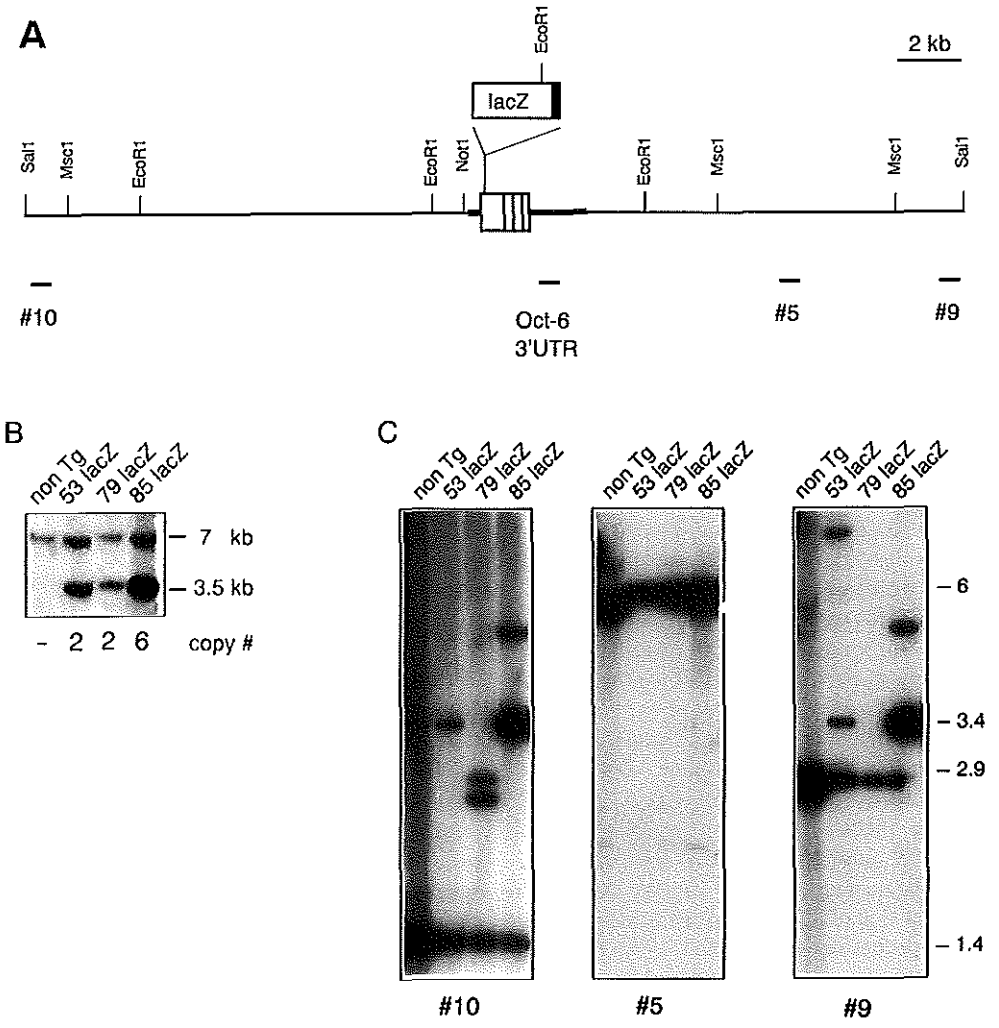


Figure 1. (A) Schematic diagram of the transgenic construct 2coslacZ. The stippled box represents the Oct-6 open reading frame and the position of lacZ insertion is indicated. Also shown are the positions of the relevant restriction enzyme sites and genomic probes. (B) Southern analysis of EcoR1 digested genomic DNA of non-transgenic FVB genomic DNA (-) and the 53lacZ, 79lacZ and 85lacZ homozygous transgenic mice. The blot is hybridized with probe 3'UTR Oct-6 to determine the transgenic copy number, which is indicated at the bottom. (C) Hybridization of Msc1 digested non-transgenic genomic DNA (-) and DNA of the different transgenic lines with the Oct-6 genomic probes #5, #9 and #10. The sizes of the different fragments are indicated at the right.

gene was fused in frame with the Oct-6 open reading frame, resulting in a Oct-6/lacZ fusion gene (fig.1a; 2coslacZ). Transcription of the fusion gene is controled by the endogenous Oct-6 transcriptional start and termination sequences, whereas translation of the Oct-6 coding sequence is prevented by introduction of the lacZ stopcodons. Five

transgenic founders were obtained after injection of this transgenic construct. Three founders transmitted the transgene to their offspring and could be used to establish transgenic lines. No phenotypic deleterious effects were observed when these lines were bred to homozygosity, indicating that integration of the transgenic copies into the mouse genome did not interfere with any essential gene function. Genomic DNA of transgenic and non-transgenic mice was digested with EcoR1 and hybridized with a 520 bp Dra1 fragment from the Oct-6 3'UTR to determine the copy number of the transgene in each line (fig.1b). The probe detects both a 6.5 kb endogenous EcoR1 fragment, as well as a 3.5 kb transgenic fragment due to the introduction of an additional EcoR1 site present in the lacZ gene (fig.1a). From the ratio of the intensities of both fragments it was calculated that two to six transgenic copies had integrated in the genome of each transgenic line (fig.1b).

To check the integrity of the transgenic copies different genomic digests of both transgenic and non-transgenic DNA were hybridized with several Oct-6 genomic probes. Probes #10 and #9 are located in the most 5' and 3' ends of the transgenic construct, respectively (fig.1a; see also chapter 3.2 of this thesis). In a Msc1 digest these probes detect a 1.4 and 2.9 kb fragment respectively, of the endogenous alleles. Furthermore, a predicted 3.4 kb head-to-tail fragment is detected with both probe #10 and probe #9 in lines 53lacZ and 85lacZ (fig.1c). In addition, end fragments of variable lengths are visible with each probe. However, in line 79lacZ two fragments smaller than 3.4 kb are detected with probe #10, whereas probe #9 does not hybridize to any fragment except for the endogenous 2.9 kb fragment (fig.1c). These observations suggest that a deletion of the most 3' end of the transgenic construct is present in both copies in line 79lacZ. Hybridization with probe #5 shows that no other than the 6 kb fragment is detected, arguing that the transgenic copies in all transgenic lines do contain the 3' 6 kb Msc1 fragment. Identical results were obtained with different restriction enzyme digests (data not shown). The sizes of the aberrant fragments detected with probe #10 in a Msc1 digest suggest that this deletion covers 500 to 800 bp of the most 3' end of 2coslacZ. To determine if any additional internal deletions or rearrangements had occured different genomic digests were hybridized with sheared DNA of the entire cosmid 2cosOct-6 under competitive conditions. No aberrant fragments were detected in any of the transgenic lines, except for the head-to-tail fragments in line 79lacZ, which were smaller than those found in lines 53lacZ and 85lacZ (data not shown).

lacZ expression in early development

In the developing embryo two phases of Oct-6 induction take place in the primitive ectoderm and in the anterior headfolds followed by progressive regression of Oct-6 protein expression (199). To determine whether the transgene contains the *cis*-acting elements that confer a similar mode of regulation we performed whole mount lacZ staining on presomite and 5 to 30 somite stage embryos. At the earliest stages lacZ expression could only be detected in line 85lacZ. At the late streak stage lacZ is expressed in the embryonic ectoderm, but not in the posterior-most region, resembling the Oct-6 expression pattern (data not shown). Interestingly, lacZ is not expressed in the extra-embryonic portion which expresses high levels of Oct-6 protein, indicating that expression in the extra-embryonic ectoderm is regulated by DNA elements which are not present in 2coslacZ.

At the early somite stage readily detectable levels of lacZ expression are present in the neuroectoderm of the developing head folds. A stripe of lacZ positive cells over the entire medial-lateral axis is seen in the region of the future midbrain, but not in the most anterior portion

of the head folds (fig.2d). In line 79lacZ a similar expression pattern is observed, although the lacZ expression extends to a more posterior position in the developing hindbrain region (data not shown). Low levels of expression can also be detected in 53lacZ at this stage of development, but only after staining for 40 hrs (data not shown).

In 25-30 somites stage embryos, lacZ expression in lines 53lacZ and 85lacZ is restricted to the basal region of the posterior part of the forebrain and anterior midbrain (figs.2a and 2c). These results show that the induction of expression in the more posterior region of the head folds followed by regression to a limited pattern in the basal region of the posterior forebrain and midbrain is correctly mediated by this

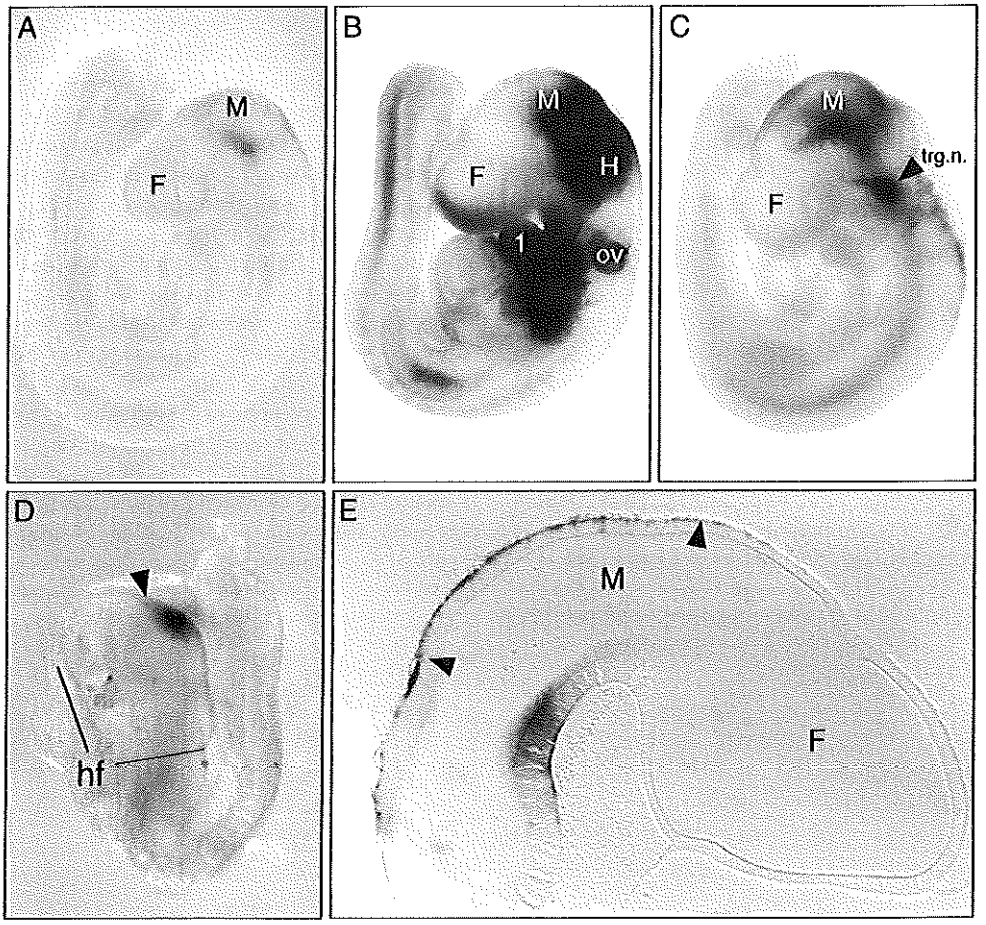


Figure 2. LacZ transgene expression in the developing brain. (A-D) show whole mount stainings of an early somite stage embryo of line 85lacZ (D) and 9.5 days pc embryos of the lines 53lacZ (A), 79lacZ (B) and 85lacZ (C). In (D) the arrowhead points at the anteriormost position of lacZ expression. In (E) a midsagittal section is shown of the embryo depicted in (C). The arrowheads point at lacZ positive cells in the distal portion of the midbrain neuroepithilium. Abbreviations: F, forebrain; H, hindbrain; hf, head folds; M, midbrain; ov, otic vesicle; trg.n., trigeminal nerve; 1, first branchial arch.

transgenic construct. In contrast, no lacZ expression is observed in these lines in the ventral midline of the anterior forebrain as shown in a midsagittal section of a 25 somite stage embryo of line 85lacZ (fig.2e). The Oct-6 gene is highly expressed in this region indicating that sequences regulating Oct-6 gene expression in the most anterior part of the head folds and developing forebrain are missing. At this stage of development, lacZ expression in lines 53lacZ and 85lacZ is also detected in cells that are in a distal position of the midbrain neuroectoderm (figs.2a and 2c). In fig.2e these cells are indicated by arrowheads. An identical location was found for Oct-6 expressing cells at this stage of development, which most likely represent the first differentiating neurons in the entire neuroepithelium (199). This result indicates that the sequences within the transgenic construct are sufficient to confer induction of Oct-6 gene expression in this population of differentiating neuronal cells.

No ectopic expression is observed in line 53lacZ, whereas in line 85lacZ the only sites of ectopic expression are found in the trigeminal nerve and in a single band in the developing hindbrain, which could match with a rhombomere segment (fig.2e). In contrast, line 79lacZ shows high levels of ectopic expression in a multiplicity of cell types and tissues, like the somites, the branchial arches, posterior midbrain and hindbrain and limb buds (fig.2b). It was therefore impossible to determine if the transgene is expressed in an Oct-6 like pattern at this stage of development.

lacZ is expressed in Schwann cells

To examine the transgene expression in the developing Schwann cells whole mount lacZ staining was performed on the hindlimbs of developing mouse embryos. At 14 and 16 days of gestation no lacZ expression was observed in either of the three transgenic lines (data not shown). Schwann cell specific lacZ expression in all three lines could first be detected in the sciatic nerves of new born animals. This implicates that transgene expression is induced between 16 days of gestation and new born stage. Oct-6 protein expression in the mouse sciatic nerve is first observed between 15 and 16 days of embryonal development, followed by increased levels at later embryonal stages (chapter 2.3 of this thesis). The timepoint of induction of lacZ transgene expression thus correlates well with the induction of high levels of Oct-6 protein expression in the

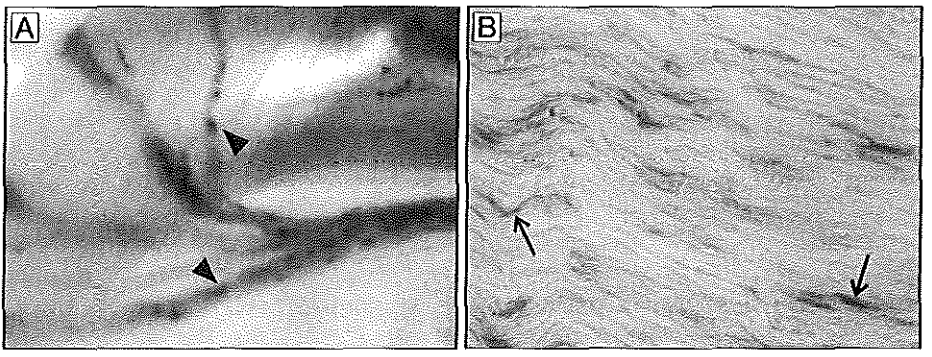


Figure 3. LacZ transgene expression in the Schwann cell lineage of line 85lacZ. (A) whole mount staining of a partially dissected postnatal day 3 sciatic nerve. The arrowheads indicate the lacZ expressing Schwann cells aligning the nerve fibers. (B) shows a cross-section of a postnatal day 10 sciatic nerve. lacZ expressing Schwann cells have adapted a curved shape at this stage (arrows). The data shown in this figure are representative for all three transgenic lines that have been analysed.

developing Schwann cells. Fig.3a shows a whole mount lacZ staining of a sciatic nerve isolated from the hindlimb of a 3 days old pup. LacZ expressing Schwann cells which are coordinately positioned along the nerve are indicated with arrowheads (fig.3a). Expression in the developing Schwann cell is maintained until at least postnatal day 10 of development. Fig.3b shows a cryostat section of a day 10 sciatic nerve. LacZ expressing cells appear in a curved shape as visualized by the lacZ staining pattern. This shape is indicative for Schwann cells that are elaborating a myelin sheath. In summary, these data suggest that all elements necessary for induction and maintenance of Oct-6 expression in the Schwann cells are present within the 32 kb fragment.

lacZ expression in the developing skin

The Oct-6 gene is expressed in the basal layer cells of the developing skin that are involved in hair follicle formation (chapter 2.2 of this thesis). In the mature hair follicle expression is limited to the cells of the inner part of the outer root sheath. We examined if the 32 kb fragment is able to mediate hair follicle-specific lacZ expression in the developing skin. In all three transgenic lines lacZ expression is first observed at 14 days pc in the basal layer of the most dorsal whiskers (data not shown). At 16 days pc lacZ expression has extended to the more ventral whiskers and the pelage hair follicles. Fig.4a shows the lacZ expression in the whiskers of a 16.5 days pc embryo. Strongest expression is observed in the hair follicles of the vertical row (vert) and in the most dorsal follicles of the five horizontal rows (I-V). LacZ is not expressed in the basal layer cells that are in a position adjacent of the condensed mesenchymal buds. Furthermore, no expression is seen in the hair matrix or in the developing root sheath (fig.4b). The timepoint of induction and restriction of expression to the basal layer cells that overly the mesenchymal condensates is identical to the Oct-6 expression pattern. In the mature hair follicle, lacZ expression is found in the outer root sheath cells, but also in the matrix cells (data not shown). Thus the 32 kb genomic fragment contains all the regulatory elements which are necessary for an Oct-6 like temporal and spatial expression pattern in the developing hair follicles.

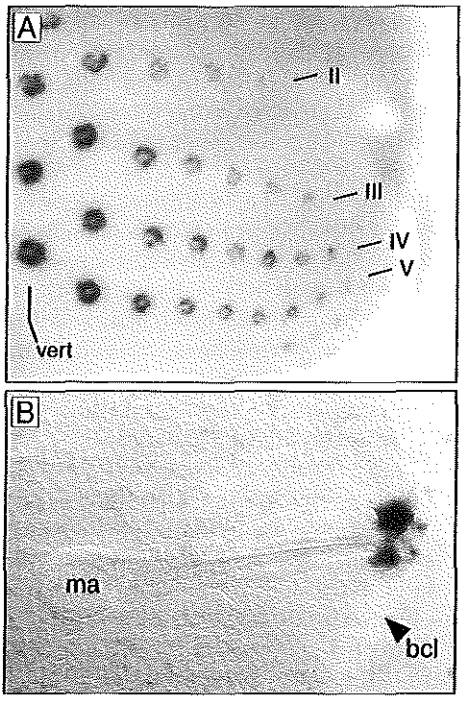


Figure 4. LacZ transgene expression in the developing hair follicles. (A) whole mount staining of the snout of a 16.5 days pc embryo of line 85lacZ. LacZ staining follows the pattern of whisker development. The vertical (vert) and horizontal rows (II-V) are indicated. In (B) a cross-section is shown of a developing hair follicle in the vertical row. Strong staining is limited to those cells of the basal cell layer that overly the ingrowing hair follicle. Abbreviations: bcl, basal cell layer; ma, matrix.

Discussion

Three transgenic lines were generated containing a lacZ reporter gene under control of 32 kb of Oct-6 genomic sequences. In lines 53lacZ and 85lacZ the lacZ transgene is expressed in a pattern which strongly resembles the pattern of Oct-6 in the midbrain neuroectoderm of an early embryo. This indicates that the critical regulatory elements necessary for the correct patterns of expression in these cell types are present within the 32 kb genomic fragment. No expression is observed in the ventral midline of the developing forebrain, or in the extra-embryonic ectoderm in line 85lacZ, suggesting that the regulatory elements necessary for Oct-6 gene expression in those tissues are located in a more distal position of the Oct-6 gene. It also shows that Oct-6 gene expression in different tissues at the early stages of development is regulated by distinct sets of regulatory elements, suggesting that different mechanisms are involved in Oct-6 gene regulation.

In line 79lacZ we observed high levels of ectopic expression in many different cell types at the early somite stages of embryonal development (fig.2c). Analysis of the integrity of the transgenic copies present in this line showed that both copies carry a deletion of 500 to 800 bp at their 3' end (fig.1c). From the Oct-6 expression pattern during early stages of development we hypothesized that transcriptional repression could be an important mechanism of Oct-6 gene regulation (199). The observed deletion might therefore have eliminated a negative regulatory element, which causes a severe deregulation of the lacZ transgene expression. This element could for example bind a protein that directly acts in a negative manner on the activity of the transcription machinery. To further explore the presence of a negative regulatory function additional transgenic lines should be generated with a construct lacking the same DNA fragment that is absent from the transgenic copies present in line 79lacZ.

In all three transgenic lines lacZ is expressed in an Oct-6 like pattern in the Schwann cell lineage and in the epidermal cells that are involved in hair follicle formation. Schwann cell specific lacZ expression in the sciatic nerves can first be detected between 16 days of gestation and newborn stage. This timepoint of induction corresponds well with the onset of Oct-6 expression in the Schwann cells of the sciatic nerve. However, the two methods that have been used to detect Oct-6 and lacZ protein expression, may introduce differences in the sensitivity of detection due to differences in threshold levels of detection and differences in Oct-6 and lacZ protein stability. Homologous recombination of a lacZ gene into the Oct-6 gene has now provided a tool which allows us to directly compare the temporal patterns of lacZ expression in the transgenic mice and in heterozygous animals (75).

Hair follicle formation consists of a cascade of epithelium-mesenchyme interactions, which provides an attractive model system to study the molecular nature of inductive tissue interactions (57). Based on gene expression and loss-of-function studies different growth factors, cell adhesion molecules and transcription factors have been identified to play a possible role in hair follicle development (see chapter 2.2 of this thesis). Progressive deletion analysis of the 2coslacZ construct will reveal the regulatory sequences that are important for epidermis-specific Oct-6 gene expression, and allows to substantiate the role of epidermally expressed growth factors in Oct-6 gene regulation and hair follicle development. These experiments will also identify the critical Schwann-cell specific regulatory sequences. Furthermore, this will elucidate if Oct-6 gene expression in the developing Schwann cells and epidermal cells of the developing hair follicle is regulated by overlapping or distinct enhancer sequences.

In parallel with the transgenic

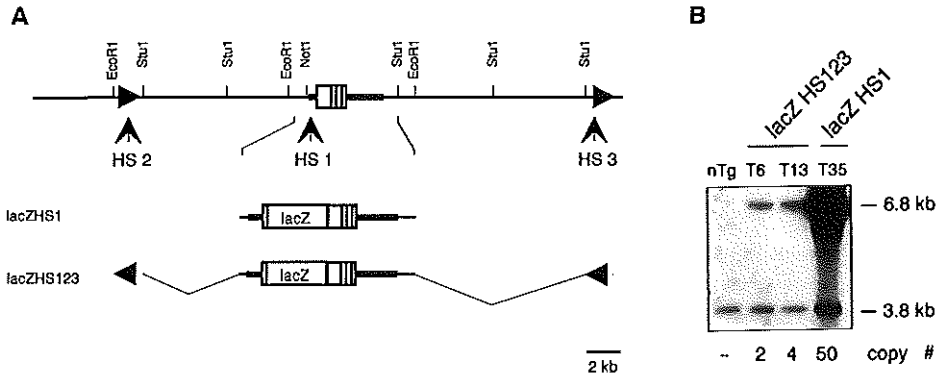


Figure 5. (A) Upper part: schematic diagram depicting the position of DNaseI hypersensitive sites in the Oct-6 genomic locus. The open box represents the Oct-6 open reading frame. Below are shown the lacZ-HS1 and lacZ-HS123 transgenic constructs that have been used to test the regulatory potential of the DNaseI hypersenstive sites. (B) Southern blot analysis to determine the copy number present in transgenic mice that have sofar been generated with these constructs. Genomic DNA of wild type and heterozygous transgenic mice was digested with EcoR1 and hybridized with probe 3'UTR Oct-6. The number of integrated copies is indicated at the bottom.

experiments the Oct-6 genomic region has been analysed for the presence of DNaseI hypersensitive sites, which can be indicative for the position of regulatory sequences. Three sites have been identified, one that overlaps with the Oct-6 promoter region (HS1), and two others (HS2 and HS3) which are located at more distal positions in the Oct-6 genomic locus (117; Wim Mandemakers and Dies Meijer, unpublished results). To analyse the potential enhancer function in transgenic mice we have fused these sites with the lacZ reporter gene in different combinations (fig.5). At present, only a limited number of transgenic lines have been generated, one that contains the HS1lacZ construct and two that contain HS123lacZ. The Oct-6 promoter present in both constructs contains two binding sites for the transcription factor AP-2, which was identified to mediate cAMP induced gene expression (72). The rat homolog of Oct-6 was isolated as a cAMP induced gene in Schwann cell cultures, which may implicate a possible role for AP-2 in Oct-6 gene expression regulation. Furthermore, Oct-6 and AP-2 show overlapping patterns of expression in the developing hair follicles, indicating that AP-2 might have a more general role in Oct-6 gen regulation (23). However, neither of the transgenic lines do express lacZ in the Schwann cell lineage or in the developing hair follicles (Ronald Zwart, Wim Mandemakers and Dies Meijer, unpublished results). Although the number of transgenic lines that have been analysed is low, the results obtained sofar do not establish a function for the Oct-6 promoter sequence as an independent Schwann cell-specific regulator of Oct-6 gene expression. Furthermore, addition of HS2 and HS3 to the Oct-6 promoter in HS123lacZ does not result in tissue-specific transgene expression in any of the Oct-6 expressing cell types. This would suggest that the Oct-6 promoter alone, or in combination with the two distal DNA elements, does not contain a cell-specific enhancer activity *in vivo*. However, several reservations have to be made. First, only three independent transgenic lines have been analysed sofar. These lines may suffer from silencing activity by the site of integration

which obscures the regulatory potential of the transgenic construct. Second, due to the cloning procedure HS2 and HS3 are positioned in an antisense orientation. This might have a negative effect on a possible enhancer activity. Third, HS2 and HS3 may contain important enhancer activity, but in combination with other regulatory elements. Therefore, to establish a possible enhancer activity present in the DNaseI hypersensitive sites generation and analysis of additional transgenic lines will be required.

Materials and methods

lacZ transgenic constructs

The lacZ reporter gene was isolated as a 3.4 kb BamH1 fragment from the p610ZA vector, in which a DraI site 60 bp downstream of the lacZ stopcodons had been converted into a BamH1 site by introduction of a BamH1 linker (5' CGGATCCCG 3'). This fragment was fused in frame with the coding region of the Oct-6 gene. For this, a 1.65 kb BamH1Xho1 Oct6 BCBA genomic fragment was subcloned into a Bgl2 and Xho1 digested pSP71 vector. It contains the first 1100 bp of the Oct-6 encoding region plus 550 bp of upstream sequences. At position +69 with respect to the Oct-6 translation start site a unique Nsi1 site was blunt-ended by T4 DNA polymerase to introduce a BamH1 linker, which was used for insertion of the lacZ reporter gene. This resulted in a fusion gene encoding the first 22 N-terminal amino acids of Oct-6 followed by a threonin, which was introduced by the cloning procedure, and the lacZ open reading frame that lacks the first 68 amino acids. The fusion gene was isolated as a Not1Xho1 fragment and inserted into a Not1 and Xho1 digested 6.3 kb EcoR1 Oct-6 BCBA genomic subclone in pTZ19 (p6.3lacZ).

The fusion gene was isolated from p6.3lacZ as a 8 kb Not1-EcoR1 fragment and inserted into cosmid 2cosOct-6, which contains the complete Oct-6 gene plus 15 kb of upstream and 14 kb of downstream sequences (see chapter 3.2 of this thesis). Since 2cosOct6 contains six EcoR1 sites, a RecA-AC (Achilles cleavage) reaction was performed to facilitate cloning (88). After linearization of 2cosOct-6 with Not1 the 3'EcoR1 site was protected from EcoR1 methylase activity by triple helix formation with RecA and a 41-mer oligonucleotide, which is complementary to the 3'EcoR1 site and its surrounding sequences. After methylation and deprotection, EcoR1 digestion resulted in the deletion of an expected 5 kb fragment. The Not1 and EcoR1 digested 2cosOct6 was extensively dephosphorylated and used in a ligation with the 8 kb Not1-EcoR1 Oct6lacZ fragment. The ligation mixture was packaged (Stratagene GoldPack) and transformed into the HB101 bacteria strain. Colonies positive for cosmids containing the insertion of the fusion gene (2coslacZ) were identified by hybridization with a 1.4 kb Ava1 lacZ fragment as radioactive probe and further characterized by diagnostic restriction enzyme digests and sequencing of the 5' Oct6lacZ fusion.

The 35 kb genomic fragment containing the fusion gene was isolated from 2coslacZ by Sal1 digestion. The digest was run on a low melt agarose (BioRad) gel and the gel slice containing the fragment was isolated. The fragment was purified by electroelution. After ethanol precipitation the fragment was washed three times with 70% ethanol and dissolved in 10mM Tris, 0.1mM EDTA. The DNA concentration was estimated from an ethidium bromide stained agarose gel with lambda DNA as a reference and diluted to 2 ng/ml. The DNA solution was centrifuged for 15' at 10,000 rpm and the supernatant was used for injections.

LacZ-HS1 was generated by insertion of a Sal1 linker in the unique Stu1 site present in the 3' genomic sequence in p6.3lacZ (p6.3lacZ-Sal1). Subsequently, a 1kb Xba1 fragment was deleted from the 5' genomic

sequence by making use of the XbaI site in the pTZ19 vector and an XbaI site at position -580 of the Oct6 transcription start site. This resulted in a minimal promoter construct containing the lacZ/Oct-6 fusion gene under transcriptional control of 1 kb 3' genomic sequence and 580 bp 5' genomic sequence. This construct contains DNaseI hypersensitive site 1 (HS1) which was mapped at the Oct-6 transcriptional start site.

DNaseI hypersensitive site 2 (HS2), identified 10 kb 5' of the Oct-6 transcription start site, was cloned as a EcoR1Stu1 fragment in the EcoR1 and EcoR5 sites of the pBluescript polylinker. An XbaI linker was inserted in the unique Hind3 site enabling isolation of HS2 as an XbaI fragment. This was inserted in the anti-sense orientation in XbaI digested p6.3lacZ-SalI replacing the 5' genomic XbaI fragment to generate lacZ-HS12.
The DNaseI hypersensitive site 3 (HS3) located 16 kb downstream of the Oct-6 gene was cloned as a 2 kb Hind3Stu1 fragment in the Hind3 and EcoR5 sites of pBl. XhoI digestion was followed by treatment with Klenow and SmaI digestion to ligate HS3 into the StuI site of p6.3lacZ (p6.3lacZ3'HS). Antisense insertion resulted in correct positioning of a SalI site that was introduced with the fragment.

LacZ-HS13 and LacZ-HS123 were generated by fusion of a 13 kb ScaINotI fragment isolated from p6.3lacZ3'HS and containing the fusion gene plus HS3, with 1.5 kb and 2.5 kb ScaINotI fragments isolated from lacZ-HS1 and lacZ-HS12 respectively.

SalI digestion could be applied to isolate all four transgenic constructs from their vectors. Digests were run on a low melt agarose gel, the gel slices containing the fragments were cut from the gel, melted at 65°C for 60 minutes in the presence of one volume TE and 0.2 M NaAc, extracted twice with acidic phenol and once with chloroform followed by ethanol precipitation. After dissolving in TE the DNA was purified by Elu-Tip chromatography and ethanol precipitated. DNA was dissolved in injection buffer (10 mM Tris pH7.5, 0.08 mM EDTA) and the concentration was adjusted to 2 ng/ml. The solution was centrifuged for 20 minutes and the supernatant was taken for injection.

LacZ enzymatic staining

To perform whole mount lacZ enzymatic staining the isolated embryos were washed in PBS/0.02% NP40 and fixed in a (1% formaldehyde/0.2% glutaraldehyde) solution for 1 to 3 hrs. After washing in PBS/0.02% NP40 embryos were incubated in a 1 mg Xgal/ml Fe^{2+}/Fe^{3+} staining solution at room temperature. The reaction was stopped at different time points by extensive washing in PBS/0.02% NP40. The embryos were postfixed in a 4% paraformaldehyde/PBS solution for 2 to 15 hrs and washed in PBS/0.02% NP40.

Whole mount photographing

LacZ stained material was placed in a petridish on a layer of 1% normal agarose, which contained small wells to facilitate positioning. To prevent reflection of light and dehydration of the embryos they were submerged in PBS which was filtered through a 45 µm filter to minimize contamination of dust particles. The photos were made with a Tungsten 64 ASA artificial light film on a Leica Wild M10 microscope using bright field conditions with maximal illumination from above.

Sectioning of lacZ stained embryos

LacZ stained material was embedded in gelatin to generate 30 µm cryosections. After staining the embryos were incubated overnight in a 30% sucrose solution in 10 mM phosphate buffer. Subsequently they were incubated in a 10% gelatin/30% sucrose

solution at 40°C for 1 to 3 hrs before embedding in the same solution at room temperature. The gelatin was allowed to harden by putting the samples at 4°C for 1-2 hrs. Gelatin embedded tissue was fixed overnight at room temperature in a 10% formalin/30% sucrose solution. After washing in 30% sucrose, it was stored in 30% sucrose at 4°C for several days. To generate cryosections the gelatin was frozen in solid CO_2. Sections were collected in phosphate buffer and soaked in chromesulphate solution before positioning on the object glass. Sections were allowed to dry on the air for 24 hrs before embedding in Permount. To perform counterstaining, the dried sections were incubated for 30 seconds in neutral red staining solution, rinsed in running tapwater for 1 minute and washed in 50% alcohol for 1 minute. The sections were incubated in 70 % alcohol until the counterstaining had fully differentiated. The sections were further dehydrated through alcohol and xylene incubations, followed by Permount embedding.

Transgenics

Fertilized oocytes were isolated from superovulated three weeks old females of the FVB inbred strain that had been mated with male FVB studs. After injection, the eggs were reimplanted in pseudopregnant BCBA foster mothers. Transmission of the transgenic construct was kept in a FVB genetic background.

Southern blot analysis

Southern blot analysis with cosmid 2cosOct-6 was done under competitive conditions of total mouse genomic DNA. Competitor DNA, 20 mg/ml FVB mouse genomic DNA, was sonicated to an average size of 300 to 500 bp. After phenol extraction and ethanol precipitation, it was dissolved in 300 µl TE. 100 µl (2 mg) competitor DNA was added to 20 ng radiolabeled 500 bp fragments of cosmid 2cosOct-6 in 100 µl TES and boiled together for 5 minutes. The mixture was immediately placed to 68°C for 20 minutes before it was added to the hybridization mixture. Hybridization was performed at 68°C for 20 hrs. The blots were washed 0.3xSSC.

3.2 Identification of a gene closely linked with the POU gene Oct-6 that is expressed in the mouse brain

Analysis of the POU gene Oct-6 genomic locus revealed the presence of a single copy fragment, called #7a, located 1 kb downstream of the 3' end of the Oct-6 gene. It contains a nucleotide sequence which is highly conserved in the human genome and has maintained a close linkage with the Oct-6 gene. On Northern blots the DNA fragment #7a detects a 4 kb transcript in adult mouse brain, as well as a 7 kb messenger RNA in mouse embryonic stem (ES) cells. We therefore hypothesize that fragment #7a contains sequences of a gene that is expressed in tissues and cells that also express Oct-6. The presence of an additional gene within the Oct-6 locus might have important implications with respect to the mechanism of Oct-6 gene regulation.

Introduction

Identification of the critical regulatory sequences of differentially expressed genes provide a tool to study the signals that regulate cell-specific gene expression and mediate cell differentiation and development. Oct-6 protein expression correlates with different developmental processes in a variety of ectodermal cell lineages (chapter 2 of this thesis). To analyse the signals that mediate Oct-6 gene expression a large 32 kb fragment of the Oct-6 genomic region has been isolated. Characterization of the isolated genomic sequences revealed the presence of a second gene within the Oct-6 locus, which is conserved in man.

Results

A 520 bp DraI fragment of the Oct-6 3'UTR was used to screen a mouse CCE genomic phage library. Two overlapping phages were isolated and reconstituted into a cosmid construct called 2cosOct-6 (Fig.1). This cosmid contains the entire intronless gene plus 15 kb upstream and 14 kb downstream sequences. A restriction enzyme map of 2cosOct-6 was generated and is shown in fig.1. In addition, we searched for small fragments within 2cosOct-6 that were devoid of repetitive sequences by hybridization of mouse genomic DNA against a panel of restriction enzyme digests of 2cosOct-6. Six fragments were isolated and their position was determined (fig.1).

To verify whether these fragments did contain any low copy repeat sequences, they were used as probes in a southern hybridization of mouse genomic DNA digested with different restriction enzymes. In these experiments we also included human genomic DNA to analyse whether the mouse genomic fragments contained sequences that are conserved between mouse and man. Fig 2. shows a southern blot hybridized with fragment #7a. #7a is a 790 bp StuI-EcoRI fragment located 1 kb downstream of the 3' end of the Oct-6 gene. It recognizes single restriction fragments in the mouse genomic digests, as well as in human DNA. Hybridization and washing procedures were performed at high stringency, indicating that #7a contains a DNA sequence that is highly conserved between mouse and man. It is unlikely that this result reflects the presence of conserved DNA sequences that function as *cis*-regulatory elements, since these sequences in general occupy only a short stretch of DNA and are very degenerate. Therefore, the strong cross-hybridization of #7a with human

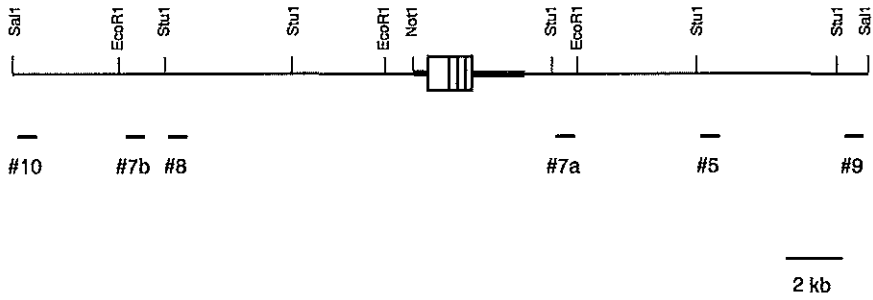


Figure 1. Restriction enzyme map of 2cosOct-6, a cosmid containing 32 kb of the mouse Oct-6 genomic region, and the position of single copy fragments. The Oct-6 gene is indicated by a thich black bar, whereas the coding region is indicated by a shaded box. The arrow represents the Oct-6 transcriptional start site. Single copy fragments are numbered with a # and indicated by horizontal bars.

genomic DNA might indicate the presence of a coding sequence within the #7a fragment, of a gene which is conserved between the two species. To analyse this possibility we performed northern blot analyses. Fig.3 shows a panel of total RNA isolated from different mouse tissues hybridized with fragment #7a. A 4 kb messenger RNA is detected in the major compartments of the adult brain, as well as a larger transcript of about 7 kb in embryonic stem cells (ES). No transcript is detected in the liver or in the thymus (fig.3 and data not shown). This expression pattern is similar to the expression of Oct-6 in these cells and tissues (115). However, the gene represented by #7a is not expressed in embryonal stages of development or in the P19-15 EC cell-line, which express high levels of Oct-6 (data not shown). This result shows that #7a contains a nucleotide sequence, which is part of a gene that is expressed in a pattern partially overlapping with the Oct-6 gene. Although the fragments #7b and #9 also crosshybridized with human DNA, they did not detect any messenger RNA in the mouse tissues and cell lines that have been analysed (data not shown).

The nucleotide sequence of fragment #7a was determined and compared with the sequence databases (fig.4). This failed to detect any homology with known DNA sequences. To allow a direct comparison of the homologous mouse and human #7a sequences a human cosmid library was screened with the mouse #7a fragment as a probe. Two overlapping cosmids were isolated and a restriction enzyme map was generated (fig.5). Cytogenetic analysis mapped the human #7a cosmids to

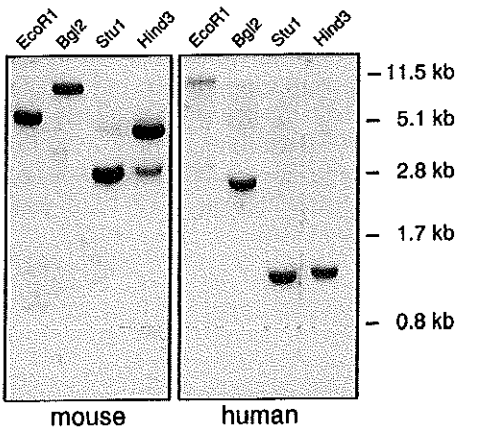


Figure 2. Fragment #7a is a single copy fragment with a strong sequence conservation in the human genome. Two panels of mouse and human genomic DNA digested with four different restriction enzymes are hybridized under stringent conditions (65 °C, 1xSSC) with fragment #7a as a probe. 10 µg of mouse BCBA and 10 µg of human genomic DNA was used per lane.

chromosome #4 to which the mouse Oct-6 gene has been mapped (146). Furthermore, the human #7a and Oct-6 3'UTR sequences were mapped within a 5 kb chromosome 1#p34 (B. Smit and A. Hagemeijer, data not shown). This region is syntenic with the distal end of mouse distance of each other (fig.5.), showing that the Oct-6 gene and the #7a fragment have maintained a close linkage in the human genome. A human 1.2 kb StuI fragment that hybridized with #7a was subcloned and sequenced. Comparison with the mouse #7a fragment showed that both fragments shared a highly homologous sequence of 150 nucleotides that includes a consensus splice acceptor sequences at the 5' end of the sequence homology, and is abrogated at the 3' end by the StuI cloning site of both the mouse and human #7a fragment (fig.6). The nucleotide sequence is conserved for more than 90%, while no significant homology is present 5' of the splice acceptor sequence. The presence of a consensus splice acceptor site and the limitation of sequence conservation beyond this site argue that the conserved nucleotide sequence represents an exon. The orientation of this exon is opposite with respect to the direction of Oct-6 gene transcription. Whereas a possible open reading frame is present in the mouse sequence, a nucleotide change in the human sequence results in the interruption of this open reading frame (fig.6).

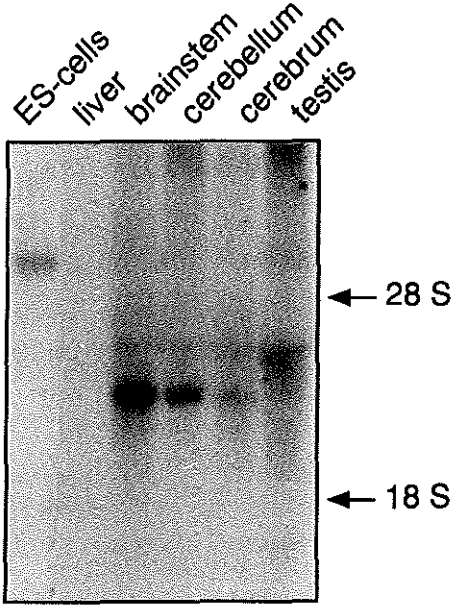


Figure 3. Fragment #7a detects a 4 kb messenger RNA in brain and and a 7 kb mRNA in embryonic stem (ES) cells. Equal amounts (20 µg) of total RNA samples from the different tissues were loaded in each lane. The position of the ribosomal RNAs are indicated by the arrows.

Discussion

We have identified a single copy fragment called #7a, in the mouse genome, which is located 1 kb downstream of the 3' end of the Oct-6 gene. It contains a possible exon sequence, which is highly conserved in man and detects a 4kb mRNA in the mouse brain. Therefore, we hypothesize that #7a is part of a second gene present in the mouse Oct-6 genomic region.

The limited sequence information prevents conclusions on the identity and possible function of this gene. Attempts to extend the length of coding sequence by amplification of cDNA ends from the conserved exon have been unsuccesful. An exon trap analysis on the mouse #7a fragment did identify a second possible exon (fig.4). RT-PCR and Northern blot analysis with single stranded exon-specific probes should reveal if this exon is indeed part of a mRNA transcribed from the gene present on the opposite strand of the Oct-6 locus.
The DNA sequence of the trapped exon is not, or only poorly conserved in man. In a StuI digest of human genomic (fig.2) or human #7a cosmid DNA (data not shown), only the 1.2 kb fragment containing the conserved exon sequence, is detected with

```
1                                        gaa ttctagggac aaaagggtga cggcttggac
34   ttcataccgc gcanacagaa gcccgtataa cctgcggcag gagatgaggg agaagggctt
94   ggtgctcaag acagggtgcc ttctgggttt gccttaacga ccacaatggc tacettcctt
154  ccttcttttc cagcgctaag aggcccttac tcctgtaaat actctagatc agattcccag
214  taataagatg gattcccccg taccatgtgg ctctggacca gtcatggatt cccacacaca
274  cagcagaaga cacaaatatc agcaggtatg ttttctcacg caaccagtca agttctactg
334  acctgctctg tgtacaggaa ataactttga gtgtcaaggg tgagagtggc gctcagaagt
394  tccaagtttg caatggaagg atgcacttct caggccgaag tctgaggaac tctggcacag
454  gcaggtagac ctacatccaa gaaacccaga agnccacaac tgatgtgcct ttcttcaact
514  tattctgtgg aagggttagg actttgggtt cctaggttgc ctgangcagg gtagagatcc
574  caggctggta tctcataagc caaacccttg cagtgcagga tcctctccct ccccctctt
634  tctttttgtt ctatccctca gCTGCTGCTG GCTCTTGGGG GGCTGCATTG TTTTGCTATT
694  TTTGAGTTGA CTTGCTTCAA TAATAAGCCC ACCCTCTTGT ACAAGATTAA GCAGACAATT
754  AGGAAATCAA TCAATCAATT GGTGTCAACT GGAGAGAAGA GGCCT
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Figure 4. Nucleotide sequence of the 790 bp Stu1-Sal1 mouse #7a fragment. The conserved exon sequence is indicated in bold uppercase lettering, whereas the exon identified with an exon trap assay is shown in bold lowercase lettering.

the mouse #7a sequence. The absence of additional fragments indicate that no sequences homologous to the trapped exon are present elsewhere in the human genome. It is possible that this exon was isolated as a false positive in the exon trap procedure, because of the presence of sequences resembling the splice acceptor and donor consensus sequences. Alternatively, it may in fact represent a second exon of the gene on the opposite strand. This may either be a part of the gene that has maintained a poor sequence conservation in man, or could provide evidence for a possible loss of gene function in man. A similar situation has been described for the genes encoding the α and ß chains of the complement C4b binding protein (C4bBPα and C4bBPß) (145). Both genes have maintained a close linkage in the mouse and human genome. However, only the C4bBPα protein is expressed in both species. Two out of six exons encoding the human C4bBPß protein have been found in the mouse genome which contain two stopcodons introduced by nucleotide substitutions. A loss of C4bBPß function in mouse is explained by the fact that in mouse only one C4b isotype is present whereas man has two, which might require a different composition of C4b binding protein. The absence of a conserved open reading frame in the mouse and human #7a sequences may provide additional evidence for a loss of gene function in man. Alternatively, it may indicate that the conserved exon is located in an untranslated region of the gene, or that the gene, like the Xist gene, produces a transcript that is not translated into protein. The Xist gene is transcribed as a 15kb mRNA from the inactive X chromosome, but does not contain a conserved open reading frame in the mouse and human Xist transcripts. Therefore, it has been proposed that transcription itself or the transcription product are involved in reorganization of the chromatin structure resulting in X chromosome inactivation (18). Discrimination between these possibilities awaits the isolation of additional gene sequences.

The identification of a second gene within the Oct-6 locus raises two important questions. First, does the loss-of-function

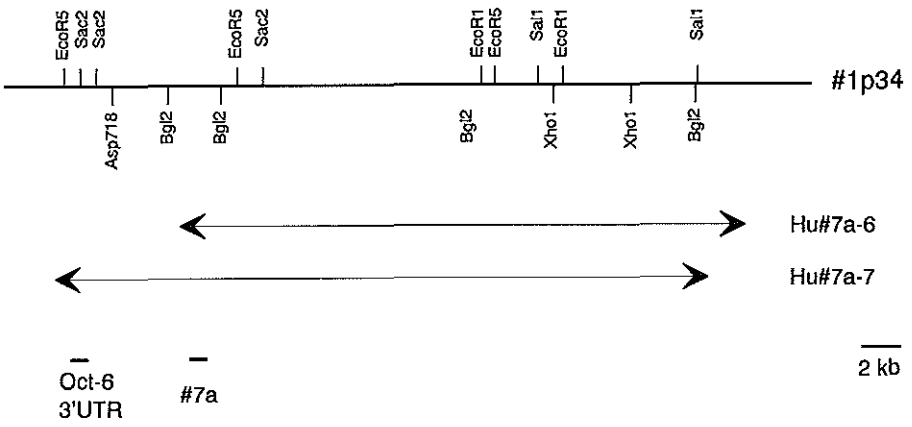

Figure 5. Schematic diagram of the human Oct-6/#7a locus and the position of the two human #7a cosmids. The bar represents a genomic distance of 2 kb.

mutation of the Oct-6 gene generated by homologous recombination affect the expression of this gene, and thus generate an alteration in the function of both genes, and second, does a functional interaction in the transcriptional regulation of both genes exist? The possibility that homologous recombination of the Oct-6 gene affects #7a gene expression should be carefully examined. Recently, altered levels of Oct-1 mRNA and ectopic Oct-1 mRNA products were found in mice in which the CD3 T cell receptor (TCR) subunit was disrupted by homologous recombination (90, 133). The CD3 gene is located in the Oct-1 genomic locus on the opposite strand and recombination of the *neo* gene in the CD3 5' flanking and coding region, also disrupts the Oct-1 3'UTR (133). The increased mortality of neonates observed in the CD3 TCR negative strain of mice is thus most likely caused by functional disruption of both CD3 TCR and Oct-1. Recently, Oct-6 gene function was studied by an identical approach (75). Homologous recombination of the Oct-6 gene did not delete any genomic sequences. However, it is possible that insertion of the *neo* selectable marker and lacZ reporter gene interferes with transcription of the anti-sense gene. To determine if altered anti-sense gene transcription is possibly involved in the observed phenotype of an Oct-6 deficient mouse, northern blot analysis should be performed on wild-type and Oct-6 deficient mouse tissues.

The conservation of a second gene in the Oct-6 locus creates the possibility that expression of both genes is regulated by a common regulatory mechanism. Several mechanisms have been described which are involved in the regulation of clustered gene expression. The human ß-globin genes e.g. are organized in a single cluster in the genome and have differential patterns of expression during fetal, embryonic and adult life. Expression of each gene is regulated by a locus control region (LCR), which interacts in a dynamic and competitive fashion with the globin gene promoters and enhancer sequences (188). Competitive interaction might also explain the mechanism of *H19* and *Insulin-like growth factor 2* (*Igf2*) gene expression. *H19* and *Igf2* lie within a 100 kb distance on the distal end of mouse chromosome 7 and expression of both genes is regulated by a single set of endoderm-specific *cis*-acting elements located proximal to the *H19* gene. This provides the *H19* gene

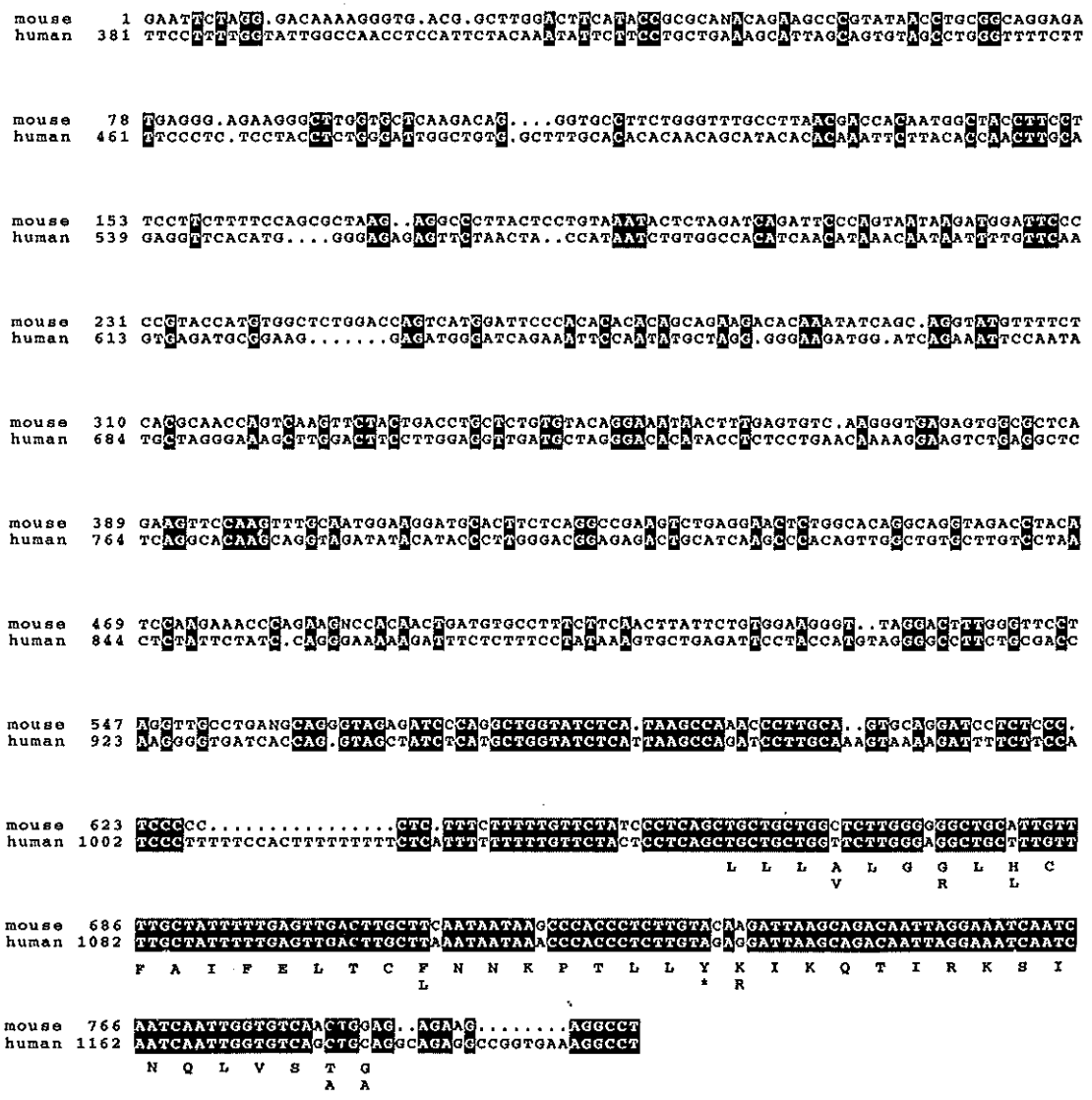

Figure 6. Sequence comparison between the mouse and human #7a fragments. Conserved nucleotides are boxed. The amino acid sequence of a possible open reading frame in the mouse sequence indicated by a single letter amino acid code. Changes in amino acid codons in the human sequence are given below. The stopcodon present in the human sequence is indicated by an asterix.

promoter with an advantage over the *Igf2* gene promoter for interaction with the enhancer element. However, methylation-mediated inactivation of the *H19* promoter permits expression of the *Igf2* gene (98). cDNA cloning and an *in situ* expression analysis of the gene present on the opposite strand of the Oct-6 locus, as well as identification of cell-specific Oct-6 regulatory sequences will allow us to examine if an intergenic mechanism of regulation is possibly involved in the transcriptional control of the Oct-6 genomic locus.

Materials and methods

Southern blot analysis

10µg genomic DNA was digested for 5-6 hrs and run overnight on a 0.8% agarose gel. DNA was transferred to a polarized nylon membrane by semi-dry blotting and hybridized overnight at 65°C in Hyb$^+$-hybridization mix (90g dextransulphate in 1l of 10xDenhardts/ 3xSSC/ 0.1%SDS /50mg ssDNA).

Northern blot analysis

Total RNA of different mouse tissues and cultured cells was isolated with .the lithiumchloride extraction method (8). Gel preparation and electrophoresis was done as described (46). A minimal amount of ethidium bromide was added to the gel to visualize the RNA under UV-light. RNA was blotted to polarized nylon membrane in 10xSSC and hybridized in Hyb$^+$-hybridization mix at 52°C overnight.

DNA sequencing

DNA fragments were subcloned in the pBluescript vector. DNA was isolated from a 1ml bacteria culture by alkaline lysis and T7 and T3 primers were used in a sequence reaction with the ^{T7}Sequencing^{TM} Kit (Pharmacia).

Library screening

A mouse CCE genomic phage library was hybridized with a 520bp 3'UTR Oct-6 probe. Two overlapping mouse genomic phages were digested with SalI, to isolate genomic insert from the phage, and NotI, which is a unique restriction site present in the Oct-6 promoter region. Those fragments of each phage, containing the most 5' and 3' extended sequences, were used in a 3-point ligation with a SalI digested pTCF cosmid vector.

A human lymphocyte cosmid library in pWE15 (Stratagene) was hybridized at 58°C with the mouse #7a fragment and 300bp Pvu2 Oct-6 cDNA probe. Positive clones were rescreened in duplicate for each probe. Two #7a positive clones were identified. In addition, one clone scored positive with the Oct-6 probe, but was determined to contain an insert of the human Brn-1 locus. Cosmid containing bacteria were grown in LB-medium with low ampicillin concentrations at 25°C and cosmid DNA was isolated with a standard alkaline lysis protocol.

Chapter 4

General discussion

Concluding remarks and future prospects

1. Introduction

The Oct-6 gene product is a member of the POU transcription factor family, which constitutes a group of important regulators of cell differentiation and embryonal development. To examine the role of Oct-6, and the signaling pathways that establish the Oct-6 expression pattern, we first analysed the pattern of Oct-6 protein expression during mouse embryonal development. The pattern of Oct-6 protein expression during mouse embryonal development is described in chapter 2. Differential patterns of expression were found in embryonal ectoderm, in cells of the developing nervous system, in the Schwann cell lineage and in the ectodermal portion of the developing eye and hair follicles. No expression was observed in other ectoderm cell lineages like neural crest derived melanocytes or neurons in the dorsal root ganglia, nor in any non-ectoderm cell types. The spatial and temporal distribution of Oct-6 protein correlates with different inductive developmental processes. We therefore hypothesize that Oct-6 is an ectoderm-specific transcription factor that may act via different mechanisms in the transcriptional regulation of differentially expressed genes, which further determine the developmental fate of ectodermal cell types. This is further emphasized by the temporary arrest of Schwann cell differentiation in Oct-6 deficient mice. In this chapter possible mechanisms of Oct-6 protein action are discussed, as well as possible experiments to further delineate the Oct-6 function in cell differentiation and development.

2. Genetic analysis of Oct-6 gene function

2.1 Oct-6 and neurogenesis

Deletion of the Oct-6 gene from the mouse genome by homologous recombination results in lethality of nearly all homozygous offspring at the time of birth (75). No phenotypic differences were observed between wild type, heterozygous and homozygous embryos up to the stage of birth, indicating that Oct-6 is not essential for embryonal development. However, the widespread expression in the developing nervous system may point at a developmental disorder of the nervous system as the cause of premature death. A particularly important function for Oct-6 may reside in the development of the hypothalamus. The hypothalamus functions to maintain a homeostatic condition in the internal environment of the body. It acts on the endocrine system, the enteric nervous system and parts of the central nervous system (94). High levels of Oct-6 protein expression are present in the ventral floor of the secondary prosencephalon, from which the hypothalamus develops (199). In addition, overlapping patterns of expression for the other mammalian class III POU proteins, Brn-1, Brn-2 and Brn-4, were observed in the developing hypothalamic region, leading to the hypothesis that the regulatory events which mediate development of the hypothalamus do involve the action of the class III POU proteins (158). This is supported by the observation that mice carrying a deletion of the Brn-2 gene are impaired in maturation of the magnocellular neurons of the supraoptic (SO) and paraventricular (PV) nuclei of the endocrine hypothalamus, which project their axons into

the posterior pituitary gland (130, 158). Recently, a cDNA encoding the chicken homolog of the mouse Oct-6 gene was isolated (Francoise Levavasseur and Dies Meijer, unplubished results). Antibodies were raised against the chicken Oct-6 protein and in immunohistochemistry experiments it was shown that Oct-6 protein expression in the ventral floor of the anterior-most portion of the forebrain is a conserved feature of the Oct-6 gene in mouse and chicken (Ludo Broos and Dies Meijer, unpublished results). This strongly argues that an evolutionary conserved function is contained within Oct-6 in the development of neuronal cell types in the hypothalamic region. Disruption of Oct-6 gene expression may thus interfere with an indispensable function in hypothalamus development.

In the mouse, anterior and posterior boundaries of Oct-6 protein expression in the neuroepithelium of the ventral floor of the developing forebrain correlate with the expression boundaries of the sonic hedgehog (Shh) gene (199). Furthermore, the expression of both proteins in the ventral floor is maintained till at least 13.5 days pc and is restricted to the ventricular layer of neural precursor cells (Ronald Zwart, Ludo Broos and Dies Meijer, unplublished results). Shh encodes a secreted signaling protein that is involved in dorsal-ventral (D-V) patterning of the neural tube along its entire anterior-posterior (A-P) axis. In explant studies, Shh expressing COS cells, or cells from the ventral floor of the forebrain induced differentiation of ventral neuronal cell types in tissue from different prospective forebrain regions (39). This implicates that Shh expressing cells in the ventral midline are an endogenous source for ventral patterning of the forebrain. The co-localization of Shh and Oct-6 may thus implicate a more general function of Oct-6 in establishment of neuronal cell identity in the developing forebrain. Oct-6 could either be involved in the regulation of Shh gene expression or function as a downstream mediator of the Shh signaling pathway. Analysis of the expression patterns in Oct-6 homozygous mutant mice of Shh, and of the SC1, Nkx-2.1, Lim-1 and Isl-1 transcription factors, which have been used as molecular markers for different ventral neuronal cell types in the developing forebrain (39), will provide a better insight in the possible role of Oct-6 in ventral forebrain patterning.

2.2 Oct-6 and Schwann cell differentiation

During Schwann cell development Oct-6 protein expression has a transient appearance (chapter 2.3 of this thesis). A major onset of induction is observed in the embryonic Schwann cells before their last cell-division and expression is maintained in the first stages of terminal differentiation into a myelin producing Schwann cell. Oct-6 has been implicated in the control of myelination, in particular in the negative regulation of the major myelin genes P_0 and MBP (65, 125, 126). Inactivation of Oct-6 gene function however, did result in a delay of myelination, rather than a premature hypermyelination (75). Although the numbers of Schwann cells observed in the sciatic nerve of a 18.5 days pc embryo are normal, major induction of P_0 myelin gene expression is only observed between postnatal day 8 and 11. This suggests that Oct-6 is essential for progression of embryonic Schwann cell differentiation, but is not directly involved in regulation of major myelin gene expression (75). It has therefore been postulated that Oct-6 functions in the regulation genes which are required to establish axon-Schwann cell interactions. These contacts have been shown in *in vivo* and *in vitro* assays to be essential for progression of an embryonic Schwann cell into a mature myelinating Schwann cell (reviewed in 77, 153). Axons of the peripheral nerves are in part derived from the spinal motor neurons which express high levels of Oct-6 protein (Ronald Zwart, Ludo Broos, Casper Hoogenraad and Dies Meijer, unpublished results). Thus a first important

issue to be adressed in the further characterization of Oct-6 gene function in Schwann cell differentiation, is to establish if the delay in myelination is caused by an intrinsic impairment of the Schwann cell to respond to external cues, or if the axonal signaling itself is affected which results in an altered Schwann cell differentiation program. Examination of Oct-6 deficient Schwann cell differentiation in *in vitro* cultures will enable to determine which of these possibilities is the cause of the delay in myelin formation, or if it is caused by a combination of both. Culture conditions required for the survival and progression of differentiation of Schwann cell precursors and embryonic Schwann cells have been established (79, 99). Furthermore, a great number of differentiation-specific molecular markers have been described, which can be used to follow the differentiation of Oct-6 mutant cells in comparison with wild type Schwann cells (77).

To determine if a Schwann cell defect is caused by the disruption of Oct-6 gene function, a Schwann cell-specific rescue of Oct-6 gene expression has to be established. In a transgenic approach the Oct-6 knock-out strain is crossed with a transgenic line carrying an extra copy of the Oct-6 gene under control of a Schwann cell-specific enhancer element. To obtain the best possible levels of transgene expression in the correct temporal fashion the transgenic construct is preferably driven by the Schwann cell-specific enhancer elements of the Oct-6 gene itself. Cosmid 2cosOct-6 which contains the entire Oct-6 gene plus 29 kb of genomic sequences, drives Schwann cell-specific lacZ expression in transgenic mice and could thus be used for this experiment (chapter 3.1 of this thesis). The P_0 myelin gene promoter which also drives Schwann cell-specific transgene expression, is less suitable due to its late onset of expression and the high and constitutive levels of expression during terminal differentiation of myelinating Schwann cells (118). Alternatively, a rescue of Oct-6 expression can be carried out in *in vitro* cell cultures of Oct-6 deficient Schwann cells. Transfection of expression vectors and retroviral infection have been used to stably introduce a gene of interest in cultures of primary Schwann cells (24, 136). Both methods however, suffer from a low efficiency and require several weeks of culture before Schwann cell colonies can be picked and analysed. This might hamper a proper analysis of the Schwann cell differentiation program and the effect of reconstitution of the Oct-6 gene function.

3. Oct-6 protein actions

3.1 Oct-6 and protein-protein interactions

Interaction of POU proteins with cell-specific proteins have been shown to have dramatic effects on DNA binding affinity and cell-specific activity (reviewed in chapter 1 of this thesis). Protein-protein interactions could thus be one particularly important determinant in establishing the cell-specific function of the Oct-6 protein. Two proteins have been identified to interact with Oct-6. The high mobility group protein HMG-I/Y, and the JC viral largeT antigen interact with Oct-6 to synergistically activate the expression of the papovavirus JC early and late genes in transient assays (97, 142). The tropism of the JC virus for myelinating glia cells, which express Oct-6 protein, renders Oct-6 an important cell-specific factor in progression of papovavirus infection.

To isolate factors that interact with Oct-6 in Schwann cells and other cell types to establish its cell-specific function, different approaches can be followed. A modified form of the one-hybrid assay has been used succesfully to isolate a B-cell specific factor that interacts with the POU proteins Oct-1 and Oct-2 (54, 170). This protein, in association with Oct-1, confers a B-cell specific activity to the transcriptional potential of Oct-1. When this technique is

applied to find dimerization partners for Oct-6, the reporter gene has to be placed under control of an enhancer element that binds Oct-6. But in contrast with the immunoglobulin enhancer and promoter sequences to which the Oct-1 and Oct-2 proteins bind, no enhancer elements have been isolated through which Oct-6 acts under physiological conditions. Considering the observation that cooperative DNA binding might dramatically alter the DNA binding site preferences, this presents a major problem in the choice for a certain DNA sequence in the enhancer element, and the use of this assay.

A second approach is to identify proteins in analogy with reported interactions between POU proteins and members of other protein families. In *C.elegans* a functional interaction has been described between the POU protein Unc-86 and the LIM homeodomain protein Mec-3. Cooperative interaction of both proteins is required for the differentiation of touch cell receptor neurons (45, 192, 193). Based on the sequence conservation encoding the LIM homeodomains a pituitary-specific LIM homeodomain gene was isolated by RT-PCR with degenerate primers against the conserved sequence (10). This gene encodes the P-LIM protein that interacts with Pit-1 to synergistically activate the prolactin and TSHß gene promoters in transient assays. This technique could be applied to identify cell-specific LIM homeodomain proteins, e.g. in Schwann cells, that may functionally interact with Oct-6.

Oct-6 expression studies in the developing neural tube revealed strong evidence that Oct-6 may interact with cell-specific LIM homeodomain proteins in differentiating motor neurons. Oct-6 protein expression is induced in cells that have left the cell cycle and have migrated out to find a position in the mantle layer of differentiating neuronal and glia cell types (Ronald Zwart, Ludo Broos, Casper Hoogenraad and Dies Meijer, unpublished observations). Although induction of Oct-6 protein expression appears a rather general feature in the mantle layer of the neural tube, particularly strong expression is observed in the lateral part of the lateral motor columns. Recently, the expression of a number of LIM homeodomain proteins has been analysed in the longitudinal motor neuron columns present in the ventral half of the neural tube of a developing chicken embryo (179). These experiments show that a distinct set of expressed LIM homeodomain proteins mark each motor neuron column. To determine if Oct-6 expression represents a specific subpopulation of motor neurons, double labeling experiments with LIM homeodomain protein antibodies have been performed. It appears that the columnar organization and expression of LIM homeodomain proteins is conserved in mouse. Double-labeling experiments with Oct-6 and Isl-1/2 antibodies have confirmed the position of Oct-6 expressing cells in the lateral motor column, and have shown that Oct-6 and Isl-1/2 are co-expressed in a single motor neuron. This raises the possibility that Oct-6 and Isl-1 or Isl-2 could functionally interact in the differentiation of a subset of motor neurons. DNA binding assays should reveal if Oct-6 and Isl-1/2 or a third LIM protein show a synergistic DNA binding activity, in analogy with the synergistic interaction observed between Unc-86 and Mec-3. Interestingly, expression of the other POU class III genes in the developing neural tube has been reported (64). Possibly, these genes are expressed in different parts of the motor neuron columns and may thus form different POU-LIM protein complexes with distinct transcriptional regulatory properties.

3.2 Oct-6 and its downstream targets

The widespread and differential expression pattern in ectodermal cell types suggests that Oct-6 may function in the regulation of a variety of different genes. In transient assays Oct-6 overexpression represses the myelin P_0

and the epidermis-specific keratin 5 and 14 (K5 and K14) gene promoters (40, 65, 125, 126). However, it is unlikely that Oct-6 plays a critical role in the regulation of these genes. In the sciatic nerves of Oct-6 deficient mice P_0 gene expression reaches almost wild type levels and the induction of expression is delayed, rather than premature (75). A role for Oct-6 in keratin gene expression regulation is also questionable, as morphological examination of the pelage hairs in Oct-6 mutant mice did not show any abnormalities.

The genes that are transcriptionally regulated by Oct-6 thus remain elusive. Possible candidate genes might be identified based on an overlap in expression with the Oct-6 gene. Those genes should be examined in an Oct-6 negative background to determine if their expression is affected by the absence of functional Oct-6 protein. A more direct approach is to compare the phenotype in Oct-6 deficient mouse with the phenotypes described for naturally occuring mutant mouse strains and engineered mouse mutants to identify genes that act in the same pathway as Oct-6. An autosomal recessive mutation has been described (claw paw; *cl/p*) which shows a delay and abnormal myelination in the peripheral nervous system (66). This phenotype shows a strong similarity with the phenotype observed in the Oct-6 mutant mice and may thus carry a mutation in a gene that is possibly regulated by Oct-6. The mutation is localized on mouse chromosome 7, but the gene that is affected has not yet been identified.

Recently, different molecular approaches have been developed to systematically search for downstream target genes. These techniques aim at exposing those genes that are differentially expressed in wild type tissue versus tissue that is deficient for the gene of interest. A conventional method is to perform subtractive hybridization of wild type and Oct-6 mutant cDNA libraries (58). Other techniques are based on the polymerase chain reaction (PCR). In a differential display assay gene-specific PCR tags are produced for each gene that is expressed in wild type and mutant tissue by amplification with a short 5' primer of 10 to 13 nucleotides (103). Tags that show a difference in intensity between both tissues represent candidate downstream genes. Disadvantagous of this approach is that each expressed gene is amplified and requires an identical efficiency in both tissues, which is hampered by the use of short primers. In the representational difference analysis (RDA) assay only cDNAs representing genes with differential expression levels in both tissues are amplified (71). This would yield a much higher efficiency in the isolation of differentially expressed genes. The RDA assay subtracts one cDNA population (tester) by hybridization with excess amounts of a second cDNA population (driver), followed by PCR amplification of the non-hybridized cDNAs. When these techniques are applied to find downstream target genes of Oct-6 in the developing Schwann cell lineage it is important to avoid the preferential isolation of genes that are only delayed in their expression, like the major myelin genes P_0 and MBP. This requires that analysis of differentially expressed genes is carried out with Schwann cell populations at a stage when Oct-6 is just expressed, i.e. 16 days pc, but do not yet contain a large number of terminally differentiating Schwann cells. In the RDA assay the driver cDNA population can be saturated with cDNAs of the myelin genes to further reduce the chances of amplifying these genes (71). Genes isolated by one of these methods will have to be analysed for their expression in the Schwann cell lineage to obtain a first indication if they may indeed be regulated by Oct-6.

Analysis of the expression pattern during mouse embryonal development has provided important information concerning the different cellular processes in which the Oct-6 protein is involved. Identification of the signals regulating Oct-6 gene expression

and the genes that are regulated by Oct-6 will gain further insight in the function of the Oct-6 gene in cell differentiation and embryonal development.

Chapter 5

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Summary

Development of a mammalian organism from a single fertilized egg into a collection of specialized cell types consists of a complex balance between cell division, cell differentiation and cell death.These cellular processes are regulated at the molecular level by a great number of different molecules which have a specific role in the establishment and function of different cell types. Temporal and cell-specific expression of these molecules is regulated by DNA binding transcription factors which are therefore thought to play a particularly important role as developmental regulators. The POU family constitute a family of transcription factors which share a highly conserved 160 amino acid POU DNA binding domain. Mutation analyses in the fruitfly *Drosophila melanogaster*, the worm *Caenorhabditis elegans* and in mouse and man have shown that POU proteins are important regulators of cell differentiation. These studies and the biochemical aspects of POU protein functioning are reviewed in chapter 1.

The mouse POU protein Oct-6 was identified as a differentially expressed factor in neuroectodermal differentiation of the P19 embryonal carcinoma cell line. The transient expression shortly after initiation of differentiation and the reappearance of expression when the cells are committed to a neuroectodermal cell type indicated that Oct-6 may be an important determinant in establishment and function of neuroectodermal cell types. Furthermore, the isolation of Oct-6 as a cAMP inducible protein during terminal differentiation of myelinating Schwann cells in *in vitro* cultures have indicated that Oct-6 may fulfill multiple roles in the control of ectodermal cell differentiation. The work described in this thesis was concerned with two questions. First, what is the role of the POU factor Oct-6 in ectodermal cell differentiation and development,and second, what are the critical regulatory elements which mediate cell-specific Oct-6 gene expression. To address these questions the temporal and spatial expression pattern during mouse embryonal development has first been analysed. For this, an Oct-6-specific polyclonal antiserum was generated and used in immunohistochemistry experiments. These experiments are described in chapter 2. Expression in the earliest stages of embryonal development was studied to determine if the expression pattern in differentiating P19 cells agrees with a role in early development. Two rounds of widespread expression are observed, followed by progressive regression. Oct-6 protein is first expressed in the embryonic ectoderm of an egg cylinder embryo. During gastrulation Oct-6 expression restricts to the cells in the walls of the neural groove that are committed to the neuroectodermal lineage. Subsequently, strong induction of expression is seen in the anterior head folds which regresses to a restricted expression in the basal part of the neuroepithelium after neural tube closure. mRNA *in situ* double-labeling analysis with probes for the Oct-6 and Shh genes showed that the pattern of Oct-6 expression at this stage has adapted a pattern that correlates with the prosomere model of segmented forebrain development (chapter 2.1). These studies suggest that Oct-6 is involved in establishment of the neuroectodermal lineage *in vivo* and in patterning of the developing forebrain.

During mid- and late gestation periods differential patterns of Oct-6 protein expression was found in the developing hair follicles and in the developing retina in the eye. These are described in chapter 2.2. Expression in the developing hair follicle is restricted to those regions in the ectodermally derived epidermis or basal cell layer that

overly dermal condensates that stimulate cell-division and ingrowth of these cells. In contrast, expression in the developing retina is limited to cells that have left the cell cycle and becomes restricted to the inner nuclear layer in the mature retina. Thus, the Oct-6 gene is differentially expressed in a variety of ectodermal cell types, implying that Oct-6 is involved in the regulation of different cell-specific genes.

In chapter 2.3 the pattern of Oct-6 protein expression in the myelinating Schwann cell lineage of the sciatic nerve is described. A major induction of Oct-6 protein expression is observed between 15 and 17 days of embryonal development and precedes the induction of the zinc-finger protein Krox20 by 1 to 2 days. In BrdU incorporation experiments Oct-6 protein expression was observed in mitotically active cells in the sciatic nerve of a 18.5 days pc embryo. These data demonstrate that Oct-6 protein is first expressed in the embryonic Schwann cells during Schwann cell development and imply a role for Oct-6 in the progression of embryonic Schwann cell differentiation, preceding a possible involvement in regulation of myelination. This is confirmed by the analysis of Schwann cell differentiation in Oct-6 deficient mice that have been generated by homologous recombination. The myelinating Schwann cell lineage in homozygous mutant mice shows a temporary arrest at the promyelination stage, arguing that Oct-6 is required for the transition of developing Schwann cells into a myelin producing cell.

Regulation of Oct-6 gene expression is likely to be tightly regulated to achieve normal regulation of the Oct-6 target genes. Identification of the cell-specific enhancer elements which regulate Oct-6 gene expression will thus allow to study the signals that regulate Oct-6 gene expression and control cell differentiation and development in general. A cosmid spanning 32 kb of the Oct-6 genomic locus was isolated and the regulatory potential of these sequences were analysed in transgenic mice. These experiments are described in chapter 3.1. A transgenic construct containing a lacZ reporter gene under control of the 32 kb fragment was used to generate three transgenic lines. In all these lines an Oct-6 like pattern of lacZ expression is observed in the Schwann cells of the sciatic nerve and in the developing hair follicles. Progressive deletion analysis of this construct should thus lead to the identification of Schwann cell- and hair follicle-specific enhancer elements. However, in the developing nervous system the Oct-6 expression pattern is only partially reconstituted. Whereas the lacZ gene is expressed in the basal portion of the posterior part of the developing forebrain, lacZ expression is absent from the ventral midline of the anterior-most region. The partial reconstitution of the Oct-6 expression pattern in these transgenic lines argue that Oct-6 gene expression in different tissues is regulated by at least partially distinct enhancer elements.

In chapter 3.2 the identification of a second gene present in the Oct-6 genomic locus is reported. A 140 nucleotides sequence located 1 kb downstream of the 3' end of the Oct-6 gene, is part of a gene that is expressed in the adult mouse brain. The nucleotide sequence is highly conserved in the human genome and has maintained its linkage with the Oct-6 gene. This may be indicative for a certain mode of interaction in the regulation of both genes.

In the last chapter present and future studies to further elucidate the function of Oct-6 in cell differentiation and development are discussed.

Samenvatting in het Nederlands

De vorming (ontwikkeling) van een organisme zoals de muis of de mens vanuit één enkele bevruchte eicel vereist vier verschillende processen. Dat zijn celdeling (vermenigvuldiging van één cel in twee identieke nakomelingen), celdifferentiatie (verandering van een cel tot één met een karakteristieke functie), celmigratie (het verplaatsen van een cel naar de juiste positie in het embryo) en celdood of apoptose (het doodgaan van cellen die de ontwikkeling van het embryo kunnen verstoren). Dit leidt tot de ontwikkeling van een organisme dat bestaat uit vele verschillende celtypen (zoals levercellen, spiercellen en huidcellen bijvoorbeeld), die elk hun eigen functie hebben. Binnen vrijwel elke cel van een organisme bevindt zich hetzelfde genoom dat bestaat uit DNA dat is overgeërfd van de ouders. Het genoom bevat de genen die coderen voor eiwitten die nodig zijn voor de uitvoering van bovengenoemde processen en voor het functioneren van ieder celtype. Niet in iedere cel wordt echter een zelfde set genen vertaald tot hun eiwitten, maar is voor de ontwikkeling en het functioneren van ieder celtype een specifieke combinatie van eiwitten noodzakelijk.

Elk celtype heeft een eigen controlemechanisme voor de vertaling van de juiste set genen op het juiste ogenblik. Dit mechanisme wordt gedeeltelijk verzorgd door zogenaamde transcriptie-factoren. Transcriptie-factoren zijn een bepaald type eiwitten die een speciale DNA-volgorde (sequentie) kunnen herkennen in het genoom en zich daaraan binden. Dit geeft ze de mogelijkheid om de vertaling van een gen, via een boodschapper molekuul (mRNA), tot een eiwit te beïnvloeden. Transcriptie-factoren spelen dus een zeer belangrijke rol bij de ontwikkeling en het functioneren van een organisme.

Het onderzoek dat in dit proefschrift beschreven wordt, is gericht op de rol van een transcriptie-factor die Oct-6 genoemd wordt. Dat deel van het Oct-6 eiwit dat zorgt voor binding aan DNA (DNA bindend domein), lijkt heel veel op (heeft homologie met) het DNA-bindend domein zoals dat in een aantal andere transcriptie-factoren is gevonden. Deze eiwitten zijn daarom samen onder een familienaam (POU) gerangschikt. In het eerste hoofdstuk wordt een overzicht gegeven van de POU-eiwitten die tot op heden bekend zijn en worden studies besproken waaruit blijkt dat POU-eiwitten een essentiële rol spelen in de regulatie van celdifferentiatie.

Het was reeds bekend dat Oct-6 aanwezig is (tot expressie komt) in cellen van het zich ontwikkelende zenuwstelsel van de muis en de rat. Tevens is het aanwezig in de cellen (de Schwann cellen) die een isolerende vetlaag (myeline schede) vormen rond de zenuwbanen van sommige zenuwcellen. Oct-6 is echter alleen aanwezig voordat deze myeline schede volledig is gevormd. Om de rol van Oct-6 in de ontwikkeling van het zenuwstelsel en de Schwann cellen beter te kunnen begrijpen, zijn verschillende experimenten uitgevoerd. Daarin heeft de muis als proefdiermodel gediend. Allereerst is nauwkeurig geanalyseerd in welke cellen en op welk moment in de ontwikkeling Oct-6 tot expressie komt. Hiertoe is een anti-lichaam gemaakt dat gericht is tegen het Oct-6 eiwit en dit is gebruikt in zogenaamde immunohistochemische experimenten. Deze experimenten staan beschreven in hoofdstuk twee. Het vroegste stadium in de embryonale ontwikkeling waarop Oct-6 tot expressie komt, is wanneer het embryo zich juist heeft geïmplanteerd en een cylindervorm heeft

aangenomen. Het eiwit is dan aanwezig in de cellen van de ectodermale cellaag, waaruit zich de cellen van het centrale zenuwstelsel, de huidcellen en cellen van de zogenaamde neurale lijst ontwikkelen. Gedurende de ontwikkeling blijft Oct-6 aanwezig in het centrale zenuwstelsel, maar niet in alle celtypen. In de vroege ontwikkelingsstadia is het aanwezig in de cellen binnen een regio die een specifiek deel van de zich ontwikkelende voorhersenen definieert. Op latere tijdstippen is het aanwezig in een beperkt aantal zenuwcellen die zich aan het vormen zijn (differentiëren). Dit duidt erop dat Oct-6 betrokken is bij zowel de aanleg van het zenuwstelsel als bij de karakterisering van individuele zenuwcellen (hoofdstuk 2.1).

Op laat embryonale tijdstippen is het Oct-6 gedetecteerd in de retina van het oog en in de haarzakjes in de huid. In beide organen komt Oct-6 alleen tot expressie in ectodermale celtypen. In de haarzakjes is de expressie beperkt tot die subpopulatie van cellen die aan het delen zijn, terwijl in de retina Oct-6 expressie karakteristiek is voor een subpopulatie van differentiërende zenuwcellen (hoofdstuk 2.2).

De ontwikkeling van gespecialiseerde Schwann cellen vanuit een uniforme neurale lijst is een proces dat verloopt langs een aantal ontwikkelingsstadia. Tot nu toe werd aangenomen dat Oct-6 alleen een rol speelt in de laatste stap van de myeline producerende Schwann cel ontwikkeling. Daarin is het mogelijk betrokken bij de negatieve regulatie van de expressie van myelin-specifieke genen die nodig zijn voor de aanleg van de myeline schede. Immunohistochemische analyse heeft echter laten zien dat het eiwit al op een vroegtijdig ontwikkelingsstadium van de Schwann cel aanwezig is. Het is daarom zeer wel mogelijk dat Oct-6 niet alleen een rol speelt bij de productie van myeline, zoals tot nu toe werd aangenomen, maar ook betrokken is bij de vorming van de Schwann cel zelf. Deze hypothese wordt bevestigd door de analyse van de Schwann cel ontwikkeling in muizen die niet langer een functioneel Oct-6 eiwit kunnen produceren. Deze muizen vertonen namelijk een vertraging in de aanleg van de myeline schede (hoofdstuk 2.3).

Het specifieke expressiepatroon dat is waargenomen in een verscheidenheid aan celtypen, suggereert dat voor een normale ontwikkeling van een organisme het gen dat codeert voor het Oct-6 eiwit in de correcte celtypen en in de juiste hoeveelheden vertaald moet worden (genregulatie). In het derde hoofdstuk staan de experimenten beschreven die zijn uitgevoerd om het mechanisme dat zorgt voor de juiste expressie van het Oct-6 gen, te achterhalen. Daarbij is beoogd om DNA sequenties te isoleren uit het genoom die daar een belangrijke rol in spelen (zogenaamde 'enhancer elementen'). Het localiseren van enhancer elementen die de expressie van het Oct-6 gen reguleren, is gebeurd met behulp van transgene muizen. In deze experimenten is het bacteriële lacZ markerings gen (reporter gen), tesamen met een stuk DNA uit de Oct-6 genomische regio, door middel van microinjectie toegevoegd aan het genoom van een muis. Het eiwit dat gecodeerd wordt door het lacZ gen, is in staat een oplosbare stof (het X-gal substraat) om te zetten tot een onoplosbaar blauw neerslag. Daarmee is het mogelijk om op relatief eenvoudige wijze te analyseren waar en wanneer het lacZ gen tot expressie wordt gebracht. Wanneer een zeer groot stuk DNA van de Oct-6 genomische regio (meer dan 32000 DNA-bouwstenen of baseparen) met het lacZ gen wordt geïnjecteerd, wordt het reporter gen vertaald in Schwann cellen op een wijze die zeer veel lijkt op dat van het Oct-6 gen. Tevens wordt het lacZ gen in deze muizen vertaald in de Oct-6 expresserende cellen van het zenuwstelsel en in de huid. DNA sequenties die belangrijk zijn voor de regulatie van Oct-6 gen expressie in

Schwann cellen, huid en zenuwcellen moeten dus binnen dit fragment van 32000 baseparen aanwezig zijn. Hiermee is een basis gelegd voor vervolgexperimenten waarin met behulp van deleties van dit grote fragment de positie van belangrijke regulatie elementen in het DNA bepaald kunnen worden. In de zich ontwikkelende voorhersenen is echter geen blauw neerslag is zichtbaar. Dat duidt erop dat de DNA sequenties die de vertaling van Oct-6 in deze cellen reguleren, ontbreken (hoofdstuk 3.1).

Tijdens de analyse van de Oct-6 genomische regio is een stukje DNA geïdentificeerd, dat vlakbij het Oct-6 gen ligt. Dit stukje DNA bleek geconserveerd in het genoom van de mens en ook de positie ten opzichte van het Oct-6 gen was behouden gebleven. Tevens detecteert het een transcriptieproduct in de hersenen van de volwassen muis, wat erop duidt dat in de nabijheid van het Oct-6 gen een tweede gen in het genoom aanwezig is. Dit zou belangrijke consequenties kunnen hebben met betrekking tot de regulatie van het Oct-6 gen (hoofdstuk 3.2).

In het laatste hoofdstuk volgt een algemene discussie over de verkregen resultaten. Dezen worden met name besproken met betrekking tot het fenotype (een verzameling van uiterlijke en functionele kenmerken) dat is waargenomen in een muis die geen functioneel Oct-6 gen in zijn genoom heeft. Tevens worden de mogelijkheden voor toekomstig onderzoek besproken (hoofdstuk 4).

Curriculum Vitae

De auteur van dit proefschrift is geboren op 06 mei 1968 te Haarlem. In mei 1986 werd in diezelfde plaats aan het Mendel College het Gymnasium ß diploma behaald. In september 1986 werd begonnen met de studie Scheikunde aan de Rijksuniversiteit Leiden. Tijdens deze studie werd een hoofdvakstage van zestien maanden gelopen bij de afdeling Medische Biochemie van Prof. van der Eb onder leiding van Dr. Rienk Offringa. Tevens zijn er vijf maanden doorgebracht bij de afdeling Biologie van de University of California San Diego in de Verenigde Staten, onder leiding van Dr. Cees Murre. Voor en na het verblijf in San Diego werd tweemaal drie maanden een functie vervuld als wetenschappelijk medewerker bij de afdeling Cancer Research van het biotechnologisch bedrijf Centocor Europe B.V.. In september 1991 werd het doctoraal diploma behaald.

Vanaf october 1991 tot april 1996 is er een aanstelling geweest bij afdeling Celbiologie en Genetica van de Erasmus Universiteit Rotterdam, voor het doen van een promotieonderzoek. Dit onderzoek werd uitgevoerd onder leiding van Dr. Dies Meijer en de professoren Dick Bootsma en Frank Grosveld. Het proefschrift dat hieruit is voortgekomen, getiteld 'Studies on the POU gene Oct-6 in ectoderm cell differentiation', zal op 9 oktober 1996 verdedigd worden. Vanaf 1 juni 1996 is hij aangesteld als postdoc op het Nederlands Kanker Instituut in Amsterdam en werken aan een door de Nederlandse Kankerbestrijding (NKB) gefinancierd project getiteld 'Molecular control of embryonic implantation' onder leiding van Dr. Denise Barlow.

De auteur is tevens co-auteur op de volgende wetenschappelijke publikaties:

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Nawoord

Het werk dat in dit proefschift beschreven staat is mede het resultaat van de onmisbare medewerking van een groot aantal mensen. Niet alleen de 'Oct-zessers', de deelnemers aan het lange dinsdagmiddag werkoverleg, en alle mensen die met mij op 734 en 791 gewerkt en muziek geluisterd hebben, maar ook al diegenen die op welke manier dan ook binnen en buiten het lab een bijdrage geleverd hebben, wil ik daarvoor enorm bedanken.