Diss. ETH No 11584

CHARACTERIZATION OF THE NEURAL-SPECIFIC N-OCT 3/BRAIN-2 TRANSCRIPTION FACTOR

A dissertation submitted to the
SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZURICH
for the degree of
Doctor of Natural Sciences

presented by
Suzana Atanasoski
Dipl. Natw. ETH
born 29.2.1968
citizen of Frauenfeld (CH)

accepted on the recommendation of

Prof. Dr. M. Schachner, examiner
Prof. Dr. A. Fontana, co-examiner

1996

TO MY FAMILY

ACKNOWLEDGEMENTS

I am very grateful to Dr. A. Fontana and to Dr. W. Herr for their support and scientific input during my time as a graduate student. I appreciate their collaboration on my thesis project which allowed me to learn from the expertise of two successful scientists with different backgrounds.

I would also like to thank Dr. M. Schachner for giving me the opportunity to graduate at the Swiss Federal Institute (ETH) of Zurich while I was working externally in other laboratories.

Many thanks to Dr. E. Schreiber for initiating part of these projects and for his suggestions and comments via e-mail.

I very much appreciate the support from all my colleagues and friends who introduced me into techniques of molecular and cellular biology and who took their time to discuss and interprete interesting and less interesting findings with me.

Finally, I would like to thank my parents and my husband Alain very much for their support and encouragement during the last years.

TABLE OF CONTENTS

1. ZUSAMMENFASSUNG

Der humane N-Oct 3 Transkriptionsfaktor gehört zu einer Familie von eukaryontischen Transkriptionsregulatoren, die in Zelldifferenzierungs- und Entwicklungsprozessen wichtige Funktionen ausüben. Diesen Proteinen ist eine DNS-Bindungsdomäne gemeinsam, die sogenannte POU Domäne, die an die DNS Octamersequenz ATGCAAAT oder an ähnliche Sequenzen bindet, welche in einer Vielzahl von zellulären und viralen Promotoren vorkommen. N-Oct 3 und das entsprechende Mausprotein Brn-2 gehören einer Unterklasse von POU Proteinen an, die vorwiegend im sich entwickelnden oder ausgebildeten Nervensystem exprimiert werden. Es wurde gezeigt, dass Brn-2 für die Etablierung neuronaler Mauszellinien erforderlich ist und dass Mäuse, die kein Brn-2 exprimieren, nicht imstande sind, gewisse Subpopulationen von Neuronen im Hypothalamus auszubilden. Dies verdeutlicht, dass dieser Transkriptionsregulator in der neuronalen Zelldifferenzierung eine entscheidende Rolle spielt.

Ausser im Zentralnervensystem wird N-Oct 3 auch in Melanozyten- und Melanomaextrakten nachgewiesen. Wird die N-Oct 3 Expression in Melanomazellen inhibiert, führt das zum Verlust des malignen Phänotyps dieser Zellen, was zeigt, dass N-Oct 3 für die Differenzierung und Erhaltung des Phänotyps von Melanozytenzellen erforderlich ist. Unerwarteterweise werden in Extrakten von Zellen, die mit N-Oct 3 kodierender cDNA transfektiert wurden, zwei octamerbindende Proteine gefunden: N-Oct 3 und ein zusätzlicher Komplex namens N-Oct 5. Interessanterweise wird N-Oct 5 zusammen mit N-Oct 3 wohl in Melanomaextrakten nachgewiesen, nicht aber in Extrakten von nicht malignen Melanozyten.

Das Ziel der vorliegenden Arbeit war, mehr über die Struktur und Funktion von N-Oct 3 als einen Faktor, der in Entwicklungs- und Differenzierungsprozessen von spezifischen Zelltypen involviert ist, herauszufinden. In einem ersten Ansatz, wurde die humane genomische N-Oct 3 DNS isoliert und das Gen chromosomal lokalisiert, um eine mögliche Verbindung zu einer neurologischen Krankheit im Menschen

herzustellen (Kapitel 4.1). Die Resultate zeigen, dass N-Oct 3 auf Chromosom 6 liegt, in einer Region, die mit dem Locus zweier hereditärer Augenkrankheiten überlappt. Zukünftige Experimente werden zeigen, ob Patienten mit diesen Retinopathien Mutationen im N-Oct 3 Gen tragen.

Im zweiten Projekt wurde die Beziehung zwischen N-Oct 3 und N-Oct 5 untersucht, um eine mögliche Rolle dieser zwei Faktoren in der Melanomabildung zu finden (Kapitel 4.2). Die vorliegenden Ergebnisse zeigten initial, dass N-Oct 5 vom N-Oct 3 Gen kodiert wird und in einem Posttranslationsprozess aus dem N-Oct 3 Protein entsteht. Weitere Untersuchungen hingegen ergeben, dass dieser Prozess der N-Oct 5 Entstehung während der Präparation der Zellextrakte *in vitro* geschieht, womit eine biologische Funktion für N-Oct 5 in der Tumorbildung wahrscheinlich ausgeschlossen werden kann. Da N-Oct 3 in einer Region von Chromosom 6 lokalisiert ist, die in Melanoma Patienten häufig deletiert oder rearrangiert ist, wird es von Interesse sein, eine mögliche Funktion für N-Oct 3 in diesem Tumortyp ausfindig zu machen.

2. SUMMARY

The human transcription factor N-Oct 3 belongs to a family of eukaryotic transcriptional regulators that perform important functions in cell differentiation and development. These proteins share a conserved DNA-binding domain called the POU domain, which binds to the DNA octamer motif ATGCAAAT and related sequences found in a variety of cellular and viral promoters. N-Oct 3 and its mouse homolog Brn-2 are members of a subclass of POU-domain proteins that are predominantly expressed in the developing and adult nervous system. Brn-2 is required for establishing mouse neural cell lineages, and Brn-2-deficient mice fail to generate specific subpopulations of neurons in the hypothalamus, clearly demonstrating a role for this transcriptional regulator in neural cell differentiation.

Besides being expressed in the central nervous system, N-Oct 3 protein has also been found in extracts of melanocytes, which are derivatives of the neural crest, as well as in extracts of their tumorigenic form, the melanoma cells. Studies show that inhibition of N-Oct 3 expression in melanoma cells leads to a loss of the tumorigenic phenotype of melanoma, demonstrating a fundamental role for N-Oct 3 in the differentiation and maintenance of the melanocytic phenotype. An interesting aspect of N-Oct 3 is that cells transfected with N-Oct 3-encoding cDNA yield two DNA-protein complexes: one containing N-Oct 3 and one with a faster mobility containing an octamer-binding protein designated N-Oct 5. Interestingly this N-Oct 5 protein is found in extracts of melanomas along with the N-Oct 3 protein but is undetectable in extracts of melanocytes.

The goal of this thesis was to learn more about the structure and function of N-Oct 3 as a developmental regulator involved in the differentiation process of specific cell types. In a first attempt, the genomic human N-Oct 3 DNA was isolated and mapped within the human genome, as an effort to find a possible link to a nervous-specific disease in humans (chapter 4.1). Results are provided that assign N-Oct 3 to the long arm of chromosome 6, in a region overlapping the locus of two retinal diseases. Future studies will show whether patients with

these congenital disorders carry mutations in the N-Oct 3 gene.

In another approach, the relationship between N-Oct 3 and N-Oct 5 was examined to elucidate a possible involvement of these two factors in tumor progression (chapter 4.2). Results show that N-Oct 5 is encoded by the N-Oct 3 gene and arises by post-translational processing of the N-Oct 3 protein. This processing, however, occurs during extract preparation *in vitro*, suggesting that there may be no biological function for N-Oct 5 in the oncogenic process. However, given the fact that N-Oct 3 has been mapped to a region of chromosome 6, which is frequently found to be deleted or rearranged in patients with melanoma, further studies will be necessary to examine a possible role of N-Oct 3 in this type of tumor.

3. INTRODUCTION

3.1 Brain development: The role of transcriptional regulators

The brain is the most complex of all biological organs and therefore, understanding how it works offers one of the greatest challenges in all of biological sciences. Insight into the development of the nervous system provides information about the functional organization of the mature brain and helps the understanding of what goes wrong in nervous and mental diseases.

One of the key events in the development of the nervous system occurs when precursor cells of the neural ectoderm become committed to form different parts of the nervous system. In subsequent steps, the neural plate, a strip of ectoderm on the surface of the embryo, develops into the neural tube which will eventually give rise to the central nervous system (CNS)(1). Associated with the differentiation of the neural tube are changes in surrounding tissues, such as the neural crest, which gives rise to neurons and glia cells of the peripheral nervous system but also to a variety of non-neural cells such as melanocytes, chondrocytes, and myocytes (2, 3).

Development of the nervous system involves the generation of a large number of heterogeneous neurons capable of forming complex cellular networks between them. This developmental process must be highly regulated to produce the different phenotypes of neurons and their communication systems (4). The differential expression of genes in a distinct spatial and temporal manner provides the molecular basis for these highly regulated developmental events (5).

The mammalian genomes are believed to consist of about 100'000 genes. The complexity of the brain is reflected in the fact that as many as 30'000 genes are thought to be specifically expressed in the brain (6). In eukaryotes, all genes that code for proteins are transcribed by RNA polymerase II (7). Regulatory regions responsible for controlling transcription are positioned either proximal or distal to the gene

sequences coding for proteins (chapter 3.3). These regulatory regions consist of short DNA sequence motifs which serve as specific binding sites for transcription factors (8). Each gene has a particular combination of such sequence motifs that vary in number, type, and spatial array. By binding to these sequence motifs, transcription factors can specifically modulate gene transcription and thus, represent an important class of developmental regulators that can confer cell- and tissue-type specificity.

3.2 Developmental functions of POU-domain proteins

Most transcription factors have two functional domains: the DNA-binding domain, which is responsible for binding to specific DNA motifs in regulatory regions, and the activation domain, which interacts with other proteins of the transcriptional apparatus and regulates the transcriptional machinery (chapter 3.3). The structures of DNA-binding domains in cell-type-specific transcription factors are highly conserved in nature, creating families of transcription factors, such as homeo domain-, leucine zipper- and zinc finger-containing proteins (9-12). The best studied are the homeo domain-containing proteins first identified in *Drosophila* (13). They are believed to encode positional information as evidenced by mutations which affect developmental processes, resulting, for example, in the production of additional legs in the position of the antennae (14). Genes containing sequences homologous to the homeo domain have been isolated from a number of vertebrates, including mammals, implying their potential roles in the development of these higher organisms (15).

More recently, a new family of homeo domain-containing regulatory proteins has been identified. These proteins contain a conserved DNA-binding region known as the POU domain (chapter 3.4). On the basis of the varied degree of similarities in the POU domain, the POU transcription factors have been classified into seven subclasses (Table 1). In contrast to classical homeo domain proteins, which are expressed in the developing hindbrain and different parts of the adult brainstem, the majority of POU-domain proteins are differentially expressed in the developing and mature forebrain, suggesting that they

selectively control differentiation events in specific neuronal phenotypes in this recently evolved brain region (16). The role of some of the described POU-domain factors in determining specific cell phenotypes has been established.

Initially, developmental mutants causing disruption of two POU genes, *unc-86* in nematodes (17, 18) and *pit-1* (19, 20) in mammals provided direct evidence that POU-domain proteins play an important role in defining specific cell phenotypes within an organism. UNC-86 is required for the development of specific sensory neurons in *C. elegans* (17), whereas Pit-1 defines three of five mature endocrine cell phenotypes in the mammalian anterior pituitary gland, the lactotrophs, somatotrophs, and thyrotrophs (21). Two defects in the *pit-1* gene have been described, a chromosomal rearrangement in Jackson dwarf mice and a missense mutation in Snell dwarf mice (22-24), which both lead to a hypoplastic anterior pituitary gland as well as a failure to activate the expression of the pituitary hormones prolactin, growth hormone, and thyroid stimulating hormone (25). Similarly, humans with mutations in the *pit-1* gene suffer from a combined pituitary hormone deficiency, suggesting that Pit-1 is involved in the specification and maintenance of lactotroph, somatotroph and thyrotroph endocrine cells in all mammalian species (26).

Developmental functions of other POU proteins, such as Oct-2 (27-29) and the POU-III transcription factors SCIP/Tst-1/Oct-6 (30-34) and N-Oct 3/Brn-2 (31, 35-37) have been in part established through the creation of null mutant mice (38). Oct-2 is predominantly expressed in B cells but is also found in various parts of the developing and adult nervous system (39). Oct-2-deficient mice contain normal numbers of B-cell precursors, which, however, are impaired in their ability to properly differentiate to mature, immunoglobulin-bearing B cells, indicating that Oct-2 is not required in the early but rather in the late steps of B-cell development (40).

SCIP/Tst-1/Oct-6 has been found to be expressed in a subset of neurons, in testes, in skin and in a transient fashion in glia cells (30-32, 34). SCIP/Tst-1/Oct-6 is up-regulated during a phase of rapid cell division preceding the myelinating phases of Schwann cell

differentiation, suggesting that it plays a role in regulating the correct developmental timing of these myelinating cells. The phenotypes of the SCIP/Tst-1/Oct-6-deficient mice indeed demonstrate that the protein is required for proper development of myelinating Schwann cells (pers. communication). In addition, transgenic mice expressing a truncated form of SCIP/Tst-1/Oct-6, which acts as an antagonist for the actions of the endogenously expressed protein, show pre-mature Schwann cell differentiation and hypermyelination of axons leading to neuropathies in these mice (pers. communication).

An *in vitro* cell differentiation system was used to first demonstrate the role of N-Oct 3/Brn-2 in mammalian neurogenesis (41). P19 cells, a murine embryonic carcinoma cell line, differentiate into neurons and astrocytes when treated with retinoic acid (RA). This differentiation process is associated with an induction of N-Oct 3/Brn-2 expression and can be selectively blocked by antisense RNA-mediated inhibition of N-Oct 3/*brn-2* gene expression, suggesting that N-Oct 3/Brn-2 is essential for establishing mammalian neural cell lineages (41). In another study using an antisense RNA strategy, N-Oct 3/Brn-2 was shown to regulate the melanocytic phenotype (42). Melanocytes, non-neuronal derivatives of the neural crest, as well as their tumorigenic form, the melanoma cells, express N-Oct 3/Brn-2 protein (43). Antisense RNA-mediated loss of N-Oct 3/Brn-2 in melanoma cells leads to a change in morphology and a loss of melanocytic and neural-crest markers in these cells, demonstrating a fundamental role for N-Oct 3/Brn-2 in the maintenance of the melanocytic phenotype and the tumorigenic phenotype of melanoma (42).

Mice carrying a loss of function mutation in the N-Oct 3/*brn-2* gene confirmed previous results obtained from studies with cultured cells which showed that N-Oct 3/Brn-2 is required for determining cell phenotypes (44, 45). In N-Oct 3/Brn-2-deficient mice precursor cells for neurons of the paraventricular (PV) and supraoptic (SO) nuclei of the endocrine hypothalamus die around embryonic day 12. Magnocellular cells of the PV and SO nuclei synthesize the peptide hormones oxytocin (OT) and vasopressin (VP) and release them in the posterior lobe of the pituitary gland. In addition to a subset of magnocellular neurons, the PV nucleus harbors populations of parvocellular cells that synthesize

corticotropin-releasing hormone (CRH). In situ hybridization and immunochemistry studies have shown that the expression patterns of N-Oct 3/Brn-2 overlap with the expression sites of the hormones CRH, VP, and OT, which are synthesized by cells in the PV and SO nuclei. N-Oct 3/Brn-2-deficient mice were capable of normal embryonic development, but die within 10 days after birth. They completely failed to express CRH, VP, and OT transcripts and were depleted of the PV and SO nuclei (44, 45). Thus, N-Oct 3/Brn-2 is not required for initial cell division and migration events, but appears to be critical in events leading to terminally differentiated neuronal phenotypes and the appropriate activation of neuropeptide gene expression.

In a different approach linkage analysis was used to gain more insight into the developmental role of the POU-III transcription factor Brn-4 (46). The *brn-4* gene maps to a region of the X-chromosome (47) that overlaps with the gene locus underlying DNF3, an X-linked hearing impairment resulting from stapes fixation and progressive sensorineural deafness (48). In patients with DNF3 various mutations in the *brn-4* gene were found that resulted in either truncation of the Brn-4 protein or caused non conservative amino acid substitutions, suggesting a role for Brn-4 in the development of the inner ear (48).

Taken together, the varied developmental functions performed by POU-domain proteins in development and differentiation might mirror distinct molecular mechanisms employed by POU proteins in controlling transcription of target genes.

Table 1: Known POU proteins in various species. On the basis of sequence homologies they can be classified in seven subclasses (I–VII).

Class	POU protein	Species	References
I	Pit-1/GHF-1	Ra/Mu/Hu	(19, 20)
II	Oct-1	Mu/Hu	(49)
	Oct-2	Mu/Hu	(27-29)
	Skn-1	Mu	(50)
	dOct-1/dPOU19/pdm-1	Dm	(51, 52)
	dOct-2/dPOU28/pdm-2	Dm	(51, 52)
III	Brn-1	Ra/Mu	(31, 35)
	Brn-2/N-Oct 3	Ra/Mu/Hu	(31, 35, 37, 53)
	Brn-4/RSH2	Ra/Mu	(31, 35, 46, 54)
	SCIP/Tst-1/Oct-6	Ra/Mu/Hu	(30-32, 35)
	cf1a/drifter	Dm	(55, 56)
	CEH-6	Ce	(18)
	xlpou1	Xl	(57)
	xlpou2	Xl	(57)
	zfpou1	Zf	(58)
IV	UNC-86	Ce	(17)
	Brn-3a/Brn-3.0/RDC-1	Ra/Hu	(31, 59)
	Brn-3b/Brn-3.2	Ra	(60-66)
	Brn-3c/Brn-3.1	Ra	(60-66)
	tI-POU	Dm	(67)
V	Oct-3/4	Mu/Hu	(68-70)
	xl p25	Xl	(71)
	xl p60	Xl	(71)
	xl p90	Xl	(71)
VI	Brn-5	Ra	(72)
VII	CEH-18	Ce	(73)

3.3 Gene regulation at the transcriptional level

Gene regulation in higher eukaryotes takes place mainly at the level of transcription. Transcription involves RNA polymerases responsible for incorporating ribonucleotides into an RNA molecule using a DNA template. Since RNA synthesis is the first step in gene expression, its control overrides regulation at all other levels.

In eukaryotes all genes encoding proteins are transcribed by RNA polymerase II (7). Their promoters, the regulatory regions responsible for controlling transcription, consist of two structures: The core promoter, composed of basal and proximal elements, which are positioned near the transcriptional start site, and the enhancer (74, 75). The enhancer can be located far from the transcriptional initiation site either upstream or downstream of a transcription unit and in either orientation relative to the core promoter. The basal promoter elements are essential for the transcription of virtually all genes. They are the assembly sites for RNA polymerase II and its associated factors that together form the basal transcriptional apparatus required for a basal level of gene expression (76, 77). The regulatory elements of both the proximal promoter as well as the enhancer are composed of multiple DNA sequence motifs that serve as binding sites for gene-specific transcription factors and thereby collectively mediate the complex patterns of cell- and tissue-specific gene expression (78).

Most transcription factors contain activation domains and DNA-binding domains (79, 80). Activation domains represent regions that interact with other proteins in the transcriptional machinery and thereby influence the regulation of gene transcription (81). Activating regions identified in various transcription factors do not show strong amino acid sequence homology to each other. However, many members of the POU-domain family as well as other families of transcription factors, share stretches of specific amino acids such as serine/threonine- (Pit-1, Oct-1 and Oct-2), glutamine- (N-Oct 3/Brn-2, Oct-1, and Oct-2), and glycine/alanine- (Tst-1/SCIP/Oct-6) rich activation domains.

DNA-binding domains recognize specific DNA motifs in promoters and thus, attract activation domains to the appropriate regulatory regions of genes (78). There are many distinct classes of DNA-binding domains conserved in nature. One of them is the POU domain whose DNA-binding structure significantly influences the regulatory properties of the large family of POU-domain transcription factors (82).

3.4 DNA sequence recognition by POU-domain proteins

POU-domain transcription factors are characterized by a conserved DNA-binding region, referred to as the POU domain (83). The structure of the POU domain is bipartite in nature, consisting of two structurally independent DNA-binding domains fused together to form a single, functional DNA-binding unit. The highly conserved amino-terminal POU-specific (POU_S) domain and the carboxy-terminal POU-homeo (POU_H) domain are joined by a linker that can vary in length and sequence among POU proteins (82). The POU_H domain is highly related to the classic DNA-binding homeo domain of various developmental regulators first described in *Drosophila* (13), whereas the structure of the POU_S domain is similar to the DNA-binding domains of λ and 434 bacteriophage repressors (84). Contrary to classic homeo domain proteins, the POU_H domain binds only with low affinity to its DNA target sites, and both the POU_S and POU_H are required to permit high-affinity, site-specific binding (85-87).

All the binding sites of POU-domain proteins identified so far are A/T-rich sequences. The best characterized regulatory target for Oct-1 and Oct-2 is the octamer motif ATGCAAAT found in promoters and enhancers of a number of tissue-specific and ubiquitously expressed genes (88-92). The crystal structure of the Oct-1 POU domain bound to the ATGCAAAT octamer site has revealed that the POU domain subunits are positioned on opposite sides of the DNA double helix without contacting one another (93). Each of the globular domains makes specific base contacts to half of the octamer site: The POU_S domain recognizes the 5' portion (ATGC) and the POU_H domain contacts the 3' portion (AAAT) of the octamer recognition motif (82). Due to their conserved DNA-binding structure, POU-domain proteins can bind to related DNA sequences (82, 94). Thus, most POU proteins recognize the ATGCAAAT octamer site, although it does not necessarily represent their preferred binding site.

Recent studies have shown that members of the neuron-specific class III and class IV POU-domain proteins prefer binding to sites where the POU_S binding site ATGC is inverted (94). Thus, the POU_S domain switches its orientation when bound to the variant octamer sequence GCATTAAT (Fig. 1A). In addition, the inverted POU_S domain

preferentially binds to sites with differing nucleotide numbers between the POU_S and POU_H domain-recognition motifs (Fig. 1B).

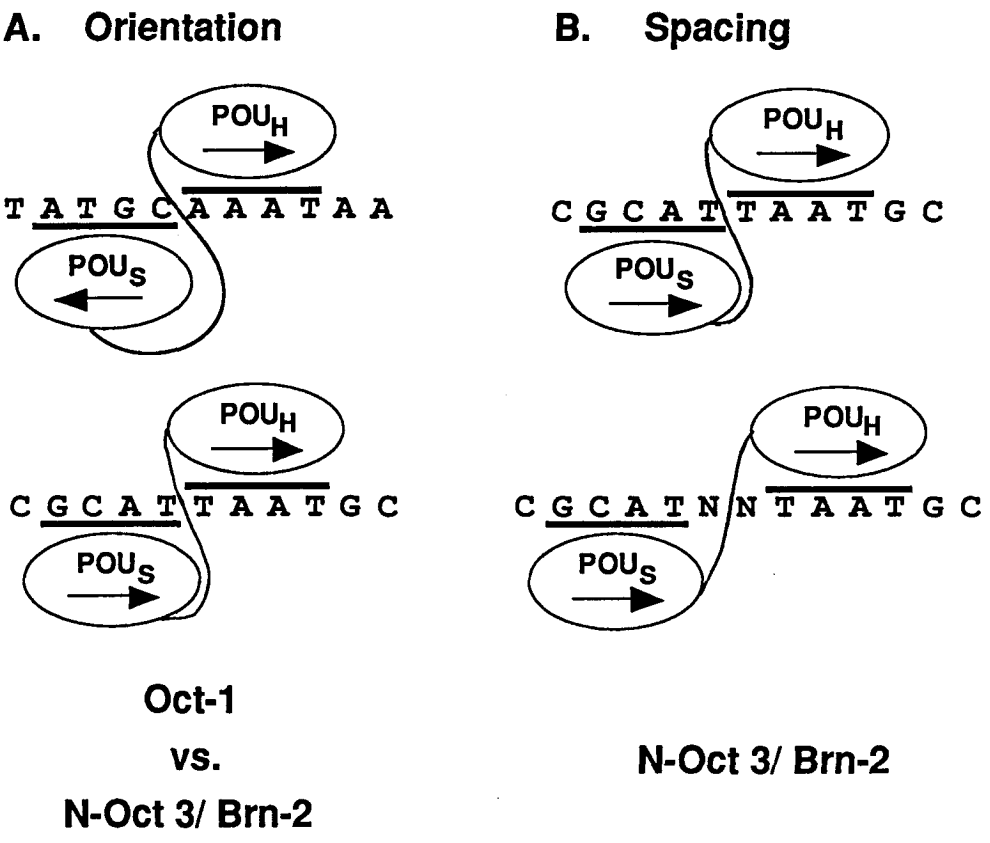


Figure 1. Different arrangement of the Oct-1 and N-Oct 3/Brn-2 POU_S and POU_H domains on DNA. POU_S and POU_H domain-binding sites are underlined and overlined, respectively. Arrows indicate the orientation of the POU_S and POU_H domains. (*A*) Oct-1 bound to an octamer sequence (*top*) and N-Oct 3/Brn-2 bound to a consensus N-Oct 3/Brn-2 DNA-binding site (*bottom*) are shown. (*B*) N-Oct 3/Brn-2 bound to consensus sites with a spacing of 0 (*top*) or 2 (*bottom*) bp between POU_S and POU_H domain-binding sites. (N) Any base. Adopted and modified from Cleary and Herr (1995).

Spacing preferences appear to be class specific. Class IV POU-domain proteins are capable of binding with high affinity only to sites containing a spacing of 3 nucleotides between the two binding motifs, whereas class III proteins show more flexibility by binding with high affinity to DNA sites with spacing of 0, 2, or 3 nucleotides (94). Consistent with these findings, several downstream target genes identified for POU-III class factors contain the described high-affinity

binding sites listed in Table 2:

POU-III protein	Potential target		Spacing
cf1a	Ddc:	CATAAAT	0
Tst-1/SCIP/Oct-6	P0:	CAT*TC*TAAT	2
N-Oct 3/Brn-2	CRH:	CAT*AAA*TAAT	3

Table 2: Summary of identified potential target genes for members of the POU-III class according to their spacing preferences. (Ddc) DOPA decarboxylase. (P_0) cell-surface adhesion molecule. (CRH) corticotropin-releasing hormone.

Identification of the preferred DNA sequence targets for different POU- domain proteins is useful for prediction of potential target genes for these transcription regulators and thus, could help explain the observed functional versatility in transcriptional regulation of the different POU-proteins.

3.5 POU-protein interactions with coregulators

The ability of the POU-domain to interact with a range of related DNA motifs is one way for providing regulatory flexibility. Another mechanism used by the POU-domain to differentially regulate gene expression is via specific protein-protein interactions.

Besides being able to bind their recognition elements as monomers, POU-domain proteins also bind DNA cooperatively as homo- or heterodimers. Pit-1, for example, forms homodimeric complexes on its regulatory sites (86) but has also been shown to form a heterodimeric Pit-1/Oct-1 complex on the rat prolactin promoter (95) displaying higher levels of transcriptional activation than on its own (95). DNA-independent interactions between specific POU proteins have been observed in mammals and *Drosophila*. I-POU specifically interacts with Cf1a, inhibiting its ability to bind and activate the neural DOPA

decarboxylase promoter (96). In a similar way, the shorter isoform of Brn-3b interacts with Brn-3a, leading to the formation of an inactive heterodimer that is no longer able to bind DNA (66).

In addition to forming complexes with other family members, POU proteins are able to specifically associate with heterologous proteins. The HSV virion protein VP16 has been shown to selectively interact with Oct-1 but not other POU proteins in activating viral early genes (97-99). Similarly, a B-cell-specific factor OCA-B can selectively associate with both Oct-1 and Oct-2 proteins but not other POU factors and thus, enhance their ability to activate immunoglobulin promoters (100-104). The role of both VP16 and OCA-B is to provide a new activation domain and thus, add to the mechanisms by which POU-domain proteins with similar DNA-binding specificities can differentially activate transcription.

The unique DNA-binding properties of the POU domain as well as the various POU domain – protein interactions refine both the specificity and the diverse functions of the different POU transcription factors. Understanding the diverse mechanisms used by these POU proteins to regulate transcription will help identify their physiological target genes and elucidate their roles in the development of organs, particularly the nervous system.

3.6 Thesis

The aim of the present study was to learn more about the structure and function of the brain-specific, human N-Oct 3 transcription factor. The evidence that this protein is expressed early in the development of the nervous system (16) and that it plays a crucial role in establishing and maintaining neural cell phenotypes (41), suggested that an organism expressing a defective or nonfunctional N-Oct 3 protein would suffer severe consequences.

As an attempt to link a potentially defective N-Oct 3 product to a disease, the gene encoding the human N-Oct 3 was isolated and mapped within the human genome (chapter 4.1). Assignment to the chromosomal region 6q16 made N-Oct 3 an attractive candidate for

two retinal diseases, whose phenotypes are restricted to the central nervous system: North Caroline macular dystrophy (MCDR1) (105) and progressive bifocal chorioretinal atrophy (PBCRA) (106). Both diseases have been mapped to the long arm of chromosome 6, in a region overlapping the genomic locus of N-Oct 3. Screening of the human N-Oct 3 gene for mutations in individuals of families affected by these congenital autosomal disorders will reveal whether N-Oct 3 is implicated in one or both of these diseases. Interestingly, the long arm of chromosome 6 has been shown to be frequently deleted or rearranged in patients with melanomas, malignant tissue, where N-Oct 3 is known to be expressed (107-110).

Comparison of the isolated genomic N-Oct 3 DNA with its previously identified N-Oct 3-encoding cDNA showed that there were no intervening sequences within the open reading frame (chapter 4.1). Thus, N-Oct 3 is an intronless gene like all other POU-III genes (Table 1, page 10) previously characterized, suggesting that they originated by duplication of an ancestral class III POU domain gene. DNA sequencing of the 5' flanking regions of the N-Oct 3 open reading frame revealed multiple AP-2 transcription factor binding sites (111) conserved between human and mouse. AP-2 and N-Oct 3 are co-expressed in neurons and astrocytes (112), suggesting that AP-2 is a major regulator of N-Oct 3 expression.

The second part of this thesis was devoted to the study of a potential regulatory mechanism for N-Oct 3 function. N-Oct 3 has been first described as a member of the human and mouse neural octamer-binding proteins called N-Oct 2, N-Oct 3, N-Oct 4 and N-Oct 5 (36, 113). Unexpectedly, extracts of mammalian cells transfected with the human N-Oct 3-encoding cDNA yielded two octamer motif-binding activities: N-Oct 3 and N-Oct 5, suggesting that the N-Oct 3 gene encodes both these proteins (37). In addition, N-Oct 3 and N-Oct 5 have been shown to be differentially expressed in melanocytes and secondary melanomas, associating them with melanoma progression and metastasis (43, 114). Results are presented demonstrating that the melanoma N-Oct 5 protein is part of the N-Oct 3 protein arising by proteolytic cleavage. Further examination revealed, however, that this processing occurs during extract preparation, suggesting that the N-Oct 5 protein does not

exist *in vivo* and therefore, probably has no biological function in the oncogenic process. However, further studies will be necessary to examine a possible role of N-Oct 3 in melanoma.

4. PUBLICATIONS

4.1 Isolation of the human genomic Brain-2/N-Oct 3 gene (POUF3) and assignment to chromosome 6q16

S. Atanasoski, S. Solinas Toldo, U. Malipiero, E. Schreiber, R. Fries, and A. Fontana (1995). Genomics **26**, 272-280

GENOMICS 26, 272–280 (1995)

Isolation of the Human Genomic Brain-2/N-Oct 3 Gene (POUF3) and Assignment to Chromosome 6q16

S. ATANASOSKI,*,[1] S. SOLINAS TOLDO,† U. MALIPIERO,* E. SCHREIBER,* R. FRIES,† AND A. FONTANA*

*University Hospital of Zurich, Department of Internal Medicine, Division of Clinical Immunology, Haeldeliweg 4, CH-8044 Zurich, Switzerland; and †Department of Animal Science, Federal Institute of Technology, ETH Zentrum, CH-8092 Zurich, Switzerland

Received March 9, 1994; accepted November 25, 1994

N-Oct 3 is a human POU domain transcription factor that binds to the octamer sequence ATGCAAAT. The protein is expressed in the central nervous system during development and in adult brain. We have isolated and characterized genomic clones encoding the human N-Oct 3 gene (HGMW-approved symbol POUF3). Comparison of the structure of these clones with the N-Oct 3 cDNA revealed that POUF3 is an intronless gene. Sequencing of 650 bp of the promoter region showed 84% sequence identity of POUF3 with its murine homologue, the brain-2 (designated *brn-2*) gene. Whereas both POUF3 and *brn-2* lack a TATA box, consensus sequences for AP-2, GCF, and SP1 transcription factors were identified within the highly conserved 5′-flanking region. These sequences may play a crucial role for the tissue-specific transcription activation of the POUF3 gene. Southern blotting and *in situ* hybridization localized the human POUF3 gene to chromosome 6q16. © 1995 Academic Press, Inc.

INTRODUCTION

N-Oct 3 is a protein belonging to a large family of transcription factors that bind to the octameric DNA sequence ATGCAAAT. Most of these proteins share a highly homologous region, referred to as the POU domain (Herr *et al.*, 1988), which was discovered in three mammalian transcription factors, the octamer-binding proteins Oct-1 (Sturm *et al.*, 1988) and Oct-2 (Clerc *et al.*, 1988; Ko *et al.*, 1988; Müller *et al.*, 1988; Scheidereit *et al.*, 1988), the pituitary protein Pit-1 (Bodner *et al.*, 1988; Ingraham *et al.*, 1988), and the product of the nematode cell lineage gene unc-86 (Finney *et al.*, 1988; Burglin *et al.*, 1989).

Many of these proteins are transcriptional regulators of development. POU genes can exert critical functions in defining specific cell phenotypes within an organism, as shown by developmental mutations resulting from disruption of two POU genes, unc-86 (Finney *et al.*, 1988) and Pit-1 (Li *et al.*, 1990).

The POU domain is a structural motif that consists of two regions, a 75-amino-acid POU-specific domain and a 60-amino-acid POU homeodomain that are separated by a nonconserved spacer sequence (Herr *et al.*, 1988). Since the molecular cloning of the original POU-domain genes, several additional members of this gene family have been isolated (reviewed in Wegner *et al.*, 1993; Verrijzer and Van der Vliet, 1993; Currie, 1994), including the cDNA for the human POU transcription factor N-Oct 3, which was cloned by our group (Schreiber *et al.*, 1993). These additional POU-domain proteins have allowed a classification of existing POU genes into six subclasses, based on similarities in the sequence of the POU domain, including the linker region, and weaker homologies of the NH_2-terminal domains (He *et al.*, 1989; Hara *et al.*, 1992; Xia *et al.*, 1993).

Class III POU genes are particularly interesting since they are expressed predominantly in the CNS. The genetic program controlling mammalian CNS development is largely unknown. However, it is likely that CNS-specific transcription factors play an important role in mammalian neurogenesis by regulating the diverse patterns of gene expression. Several class III POU genes, *brn-1, brn-2, brn-4,* and *Oct-6* (the latter also known as *Tst-1* and *Scip*), have been cloned and characterized in detail (He *et al.*, 1989; Suzuki *et al.*, 1990; Hara *et al.*, 1992; Le Moine and Young, 1992; Mathis *et al.*, 1992). The human counterpart of the murine *brn-2* was first identified in nuclear extracts from brain and was termed N-Oct 3 (Schreiber *et al.*, 1990, 1993). At embryonic Days 9 and 12, transcripts of the mammalian POU III genes are widely expressed at all levels of the developing rat neural tube (He *et al.*, 1989). During these early stages, expression is widespread and includes the developing telencephalon, diencephalon, brainstem, and spinal cord. During later embryonic stages and in the adult, expression of most of these genes becomes restricted to specific subregions of the brain (He *et al.*, 1989). The mechanisms involving

[1] To whom correspondence should be addressed at present address: Cold Spring Harbor Laboratory, James Laboratory, Cold Spring Harbor, NY 11724. Telephone: (516) 367-8410. Fax: (516) 367-8454.

0888-7543/95 $6.00

the regulation of these genes during development and their physiological target genes await identification. In this report we describe the isolation, characterization, and chromosomal localization of the human genomic POUF3 gene, encoding the N-Oct 3 protein.

MATERIALS AND METHODS

Library screening. Approximately 10^6 plaques of an amplified human genomic library (Stratagene, Cat. No. 9446205) were transferred in duplicates to Plaquescreen filters (DuPont) and then screened with two ^{32}P-labeled N-Oct 3 cDNA probes. One probe comprised a 700-bp *Xba*I–*Nco*I fragment of the 5′ end of the N-Oct 3 cDNA; the other probe included a 1.8-kb *Xho*I fragment of the 3′ untranslated region of the N-Oct 3 cDNA. Phages from plaques that gave rise to identical signals on both filters were further purified by two rounds of plating and screening. To characterize the resulting two positive plaques, λ DNA was isolated (Maniatis *et al.*, 1982) and then subcloned into Bluescript plasmids (Stratagene).

Southern analysis. Recombinant phage DNA containing 7 kb of POUF3 genomic DNA was digested with *Eco*47III and *Xho*I restriction enzymes, electrophoresed on an 0.8% agarose gel, and blotted by alkaline transfer to nylon membranes (Hybond N$^-$, Amersham). The filter was hybridized with a radiolabeled human *Eco*47III–*Xho*I N-Oct 3 cDNA probe. Hybridization was performed according to the standard method (Ausubel *et al.*, 1989). DNA blots were washed to a final stringency of 0.1× SSC, 1% SDS, 65°C and exposed to X-ray film for autoradiography.

Nucleotide sequencing and computer-assisted analysis. Double-stranded DNA sequencing of the subcloned genomic *Not*I fragments was performed in both orientations by the dideoxy chain termination method (Sanger *et al.*, 1977) using POUF3-specific synthetic oligonucleotide primers and Sequenase (U.S. Biochemical Corp., Cleveland, OH). Searches for nucleotide sequence similarities and analysis of the promoter sequence were performed with the GCG program package (Devereux *et al.*, 1984). The nucleotide sequence has been deposited at the EMBL sequence data library under Accession No. Z31606.

Plasmids for riboprobe synthesis and RNase protection assays. A 602-bp *Ava*II–*Eco*47III (−626 to −24 bp) probe of the POUF3 promoter sequence was subcloned into *Hin*dII-cleaved pT3T7*lac*. The riboprobe of N-Oct 3 was prepared by linearizing pT3T7-N-Oct 3 with *Hin*dIII. Transcription with T3 RNA polymerase yielded a 628-nucleotide probe. The probe of the ubiquitously expressed transcription factor Sp1 was a gift of Dr. B. Luescher (Institute of Pharmacology, University of Zurich, Switzerland).

Fifty micrograms of total RNA from human neuroblastoma cell lines SK-N-LE and SK-N-BE and from the human glioblastoma cell line LN-308 and 50 μg of tRNA were hybridized for 16 h at 60°C to [^{32}P]UTP-labeled antisense riboprobes of N-Oct 3 and Sp1 transcription factors. Subsequently, single-stranded, nonhybridized RNA was digested with 4 μg/ml RNase A and 10 U/ml RNase T_1. The RNA–RNA hybrids were separated on a denaturing 6% polyacrylamide/urea gel, which was then exposed to X-ray film with an intensifying screen at −70°C for 14 days. The sizes of the bands were determined by using end-labeled molecular weight markers.

Analysis of hybrid cell panel by Southern blotting. The chromosomal location of the human POUF3 gene was determined by hybridization to *Eco*RI digests of genomic DNA from a panel of human–hamster somatic cell hybrids on a filter purchased from BIOS Laboratories (New Haven, CT). A ^{32}P-labeled 487-bp *Xho*I restriction fragment from a human N-Oct 3 cDNA clone that comprised the 3′ untranslated region of the clone starting at nucleotide 1495 (Schreiber *et al.*, 1993) was used as a probe. DNA blots were washed to a final stringency of 1× SSC, 1% SDS, 65°C.

Chromosomal in situ hybridization with a tritiated probe. Metaphase spreads were prepared from phytohemagglutinin (PHA)-stimulated lymphocytes of a male individual using standard procedures of hypotonic treatment and methanol/acetic acid fixation. QFQ-banding of the chromosomes was performed prior to hybridization by dipping the slides in a solution of 0.005% quinacrine mustard for 30 s. Photographs of selected metaphases were taken with a Leitz Diaplan microscope. Chromosome identification was based on comparison of the QFQ-banding pattern with the international standard (ISCN, 1981).

The Bluescript plasmid containing a 4-kb cDNA of the POUF3 gene was labeled by incorporation of [^{3}H]dATP, [^{3}H]dCTP, and [^{3}H]dTTP to a specific activity of 7.2×10^8 dpm/μg probe DNA using the random priming kit of Boehringer Mannheim. The probe concentration in the hybridization mixture was 2.3 ng/ml. Hybridization was performed as described by Fries *et al.* (1991). Autoradiography was carried out for 6 days after dipping the slides in Ilford K2 emulsion. The slides were developed and stained as described previously (Fries *et al.*, 1991). Silver grains associated with Giemsa-stained chromosomes were marked on the previously QFQ-banded and photographed metaphases. The distribution of the autoradiographic silver grains was evaluated by plotting a histogram over the standard idiogram.

Chromosomal in situ hybridization with a biotinylated probe. Chromosome preparation and identification were as described above for isotopic *in situ* hybridization.

A recombinant phage containing 10 kb of POUF3 genomic DNA was labeled with biotin-16–dUTP (Boehringer Mannheim) by nick-translation. The efficiency of the labeling was determined by dot blot assay (BluGene Kit, BRL). Hybridization, including competitive *in situ* suppression and signal detection, was carried out as described by Solinas Toldo *et al.* (1993) with small modifications. Briefly, 60 ng of probe DNA was combined with 4 μg of Cot-1 DNA (Gibco BRL) and hybridized under an 18 × 18-mm coverslip. One round of amplification (a layer of biotinylated anti-avidin antibody followed by a layer of avidin–FITC) was necessary for detection of a distinct hybridization signal. The chromosomes were counterstained for 5 min in DAPI (4,6-diamidino-2-phenylindole) at a concentration of 200 ng/ml in 2× SSC (0.3 *M* NaCl and 30 m*M* sodium citrate, pH 6.8). Antifade solution was used to cover each slide. FITC and DAPI were excited separately at 450–490 and 340–380 nm, respectively. Image acquisition was performed using a computer-controlled (Macintosh Quadra 800) CCD camera system (Photometrics, Tucson, AZ) coupled to the Leitz microscope.

The site of hybridization was assigned to a chromosome band by determining the FLpter value (Lichter *et al.*, 1990) and by applying this value to the corresponding idiogrammatic chromosome of the standard.

RESULTS

Isolation of Human POUF3 Genomic Clones

To isolate POUF3 genomic DNAs, a human genomic library was screened with radiolabeled probes derived from the N-Oct 3 cDNA. Phage DNA from two strongly positive plaques was purified. The two phages carried a 7- and 10-kb genomic POUF3 DNA insert, respectively. The 7-kb insert was cut into a 2-kb and a 5-kb *Not*I fragment and was subcloned into Bluescript plasmid. The 2-kb subcloned fragment, which comprised the longest region 5′ to the open reading frame, was chosen for sequencing and further analysis.

Genomic Structure Analysis of the Human POUF3 Gene

To elucidate the genomic structure of the human POUF3 gene, Southern blots were performed using the

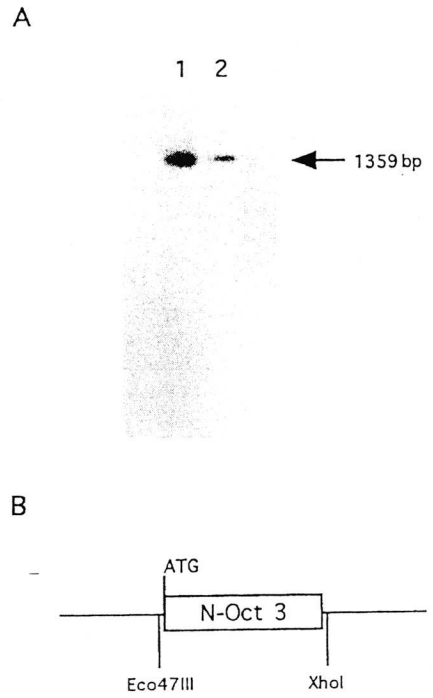


FIG. 1. Southern blot analysis of human genomic POUF3 DNA. **(A)** N-Oct 3 cDNA was digested with *Eco*47III and *Xho*I as a positive control (lane **1**). In lane **2** phage DNA with a human genomic POUF3 DNA insert of 7 kb was digested with *Eco*47III and *Xho*I. ((**B**) Digestion of the N-Oct 3 cDNA (open bar) with the *Eco*47III and *Xho*I restriction enzymes yields the 1359-bp probe used in this experiment. The translational start is indicated by the ATG codon.

7-kb genomic POUF3 DNA, which was cut with the restriction enzymes *Eco*47III and *Xho*I. Hybridization with an *Eco*47III–*Xho*I N-Oct 3 cDNA probe (Fig. 1B) showed the same band of 1359 bp in length as that of the cDNA clone of the POUF3 gene that was hybridized with the same probe (Fig. 1A). Thus, the portion of POUF3 genomic DNA that corresponded to the open reading frame of the N-Oct 3 cDNA did not contain an intron.

Search for Potential Regulatory Sequences

A total of 650 bp of DNA sequence containing 5′ untranslated regions of the POUF3 gene was determined from both strands. The sequence is shown in Fig. 2. Comparison of the human POUF3 and mouse *brn-2* promoter regions revealed an identity of 84% (Fig. 2). Both the human and the mouse gene lack a TATA box. Analysis of putative transcription regulating DNA motifs (Faisst and Meyer, 1992) showed that within the sequenced 5′ untranslated region AP-2 consensus sites were the most abundant of all putative regulatory sequences identified. AP-2 sites common to both the human POUF3 and the murine *brn-2* promoter were found in the POUF3 gene at the following positions relative to the translation start site: −350/−343 bp, −364/−357 bp, −575/−568 bp, and −583/−576 bp. Additionally, there were two GCF sites (−581/−575 bp, −584/−578 bp), one SP1 site (−584/−576 bp), one CF-1 site (−590/−585 bp), one E2A site (−592/−586 bp), one TCF-1 site (−382/−378 bp), one LyF-1 site (−367/−359 bp), and two CCAAT boxes (−369/−365 bp, −430/−426 bp). These regulatory elements, which were shared by human and mouse, are shown schematically in Fig. 3. Regulatory sequences that were found in human but not in mouse were eight AP-2 sites (−342/−335 bp, −416/−409 bp, −453/−446 bp, −458/−451 bp, −459/−452 bp, −532/−525 bp, −533/−526 bp, −619/−612 bp), five GCF sites (−348/−342 bp, −351/−345 bp, −420/−414 bp, −551/−545 bp, −624/−618 bp), one PuF site (−378/−372 bp), two SP1 sites (−351/−343 bp,

```
hu .....GTCTGTGGCCGATAAGAGCACCGGGACCGCCCCC....CTG -614
mu CTTCCAAGCCTCTGTAGTCCACCAGAGCGCCTGGACCGACCGCCCGGCTG 1349

   CCGGTCTTCTCTGCCTGGGAGACAGATGGGGGGCGGGGCCCATCCGAGAG -564
   CTGGGTTTCTCAGCCTAGGAGACAGATGGGGGGCGGGGCCCATCCGAGTG 1399

   AGGGCGGAGGAGGCCCCGGGTGAGGAAGAAGCGGGGGGGTGAGAACTGAG -514
   .GGGCGGTGGAGGCCGGGGACAGAAGAGA....GGGGGGTGAGAACTAAG 1444

   AGAATCTGAATCGGGAGGCGAAGGGGACGGGGA.....GGAGGGCTAGG -470
   AGAATCTGAATCGCTGAGCTAGGGGTGGGGGTACCAGAGGAAGGACTGAG 1494

   AGGACTCCGAGCCCGGGGGAGGGGGAGGGAG.....TAGCTCTCCCAATC -425
   AAGACTGGGCGCCCGAGGGAAGAAGAGGGGGGTACAGCTCTGCACCAATC 1544

   AGTGGCGCCGG.CTGGGAGGTTGCTAGCGGTATCCACGTAAATCAAAGGG -376
   ACTGGCTCCGGTCTGGGAGGTTGCTAGCGGTATCCACGTAAATCAAAGGG 1594

   TG.GGAGCCAATGGGAGGGGGTGGAGGGGCGGGGCCCAGGC.CGTGCCGC -328
   CGCAGAGCCAATGGGAGGGGGTGGAGGGGGCGGGGCCAGGCGCGTGCCGC 1644

   TGCGAGCCGCGCTGCCAAGAGAGCGGGAGAGAGCTGGAGAGAGCAGGGAG -278
   TGCGAGCGGCTCTGCCAAGAGAGCGGGAGAGAGCTTGAGAGCGCGGGGAG 1694

   AGGGGGGAGCGCCGAGCTAGTCAGAGAGTGAGCGAGAGCGAGAAGGAGGG -228
   AGG.GGGAGCGCCCAGCGAGTCAGAGAGAGTGAGCGAGAGCG.AGGAGGG 1742

   AGAGGAGGAGAAAGAGAGCGAGGGCGGGCGGGAGGCGGCGGCGGCGGCAG -178
   AGAGGAGGAGAAAGAGCGAGGGCGGGCGGGCGGGCGGGAGGCAGC.GCGG 1791

   CAGCAGCAGTAATAGCAGGAGCAGCAACAGAAGGCGTCGGAGCGGGCGTC -128
   CAGCAGCAGTAATAGCAAGAGCAGCAACAGAAGGCGTCGGAGCGGGCGTC 1841

   GGAGCTGCCCGCTGTGGGAGAGAGAG...GAGACAGAAAGAGCGAGCGAG -81
   GGAGCTGCCCGCTAGGGGAGAGAGAGAGAGAGAGAGAGTGAGAGAG 1891

   .....GAGAGGGAGCCCGAGGCGAAA -60
   AGAGAGTGGGAGAAGCGGGCGAGCGAGGAGAGAGAGAGCCCAAGGCAGAA 1941

   AAGTAACTGTCAAATGCGCGGCTCCTTTAACCGGAGCGCTCAGTCCGGCT -10
   AAGTAACTGTCAAATGCGCGGCTCCTTTAACCAGAGCGCCCAGTCCGGCT 1991

   CCGAGAGTCATG 1
   CCGAGAGTCATG 2000
```

FIG. 2. Comparison of the human POUF3 and mouse *brn-2* gene 5′-flanking regions (upper sequence, human; lower sequence, murine). In human, numbering starts from the ATG translation start (+1). In the mouse, nucleotides are numbered in 5′ to 3′ directions, according to the mouse *brn-2* sequence deposited in the GenBank database (Accession No. M88300). The sequences from mouse and human were aligned using the FASTA algorithm, allowing introduction of gaps (dots). Nucleotide identities are indicated by vertical lines. The initial methionine codons and the CCAAT boxes are typed in boldface. The putative transcription start is located about 300 bp upstream of the translational start site.

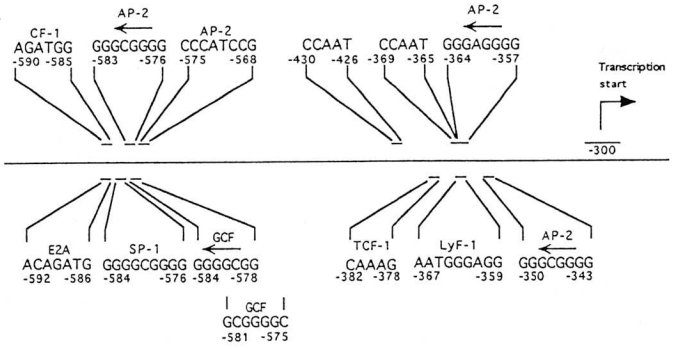


FIG. 3. Schematic summary of the potential regulatory elements of the 5′-flanking region of the human POUF3 gene that are also found in the mouse homologue *brn-2*. Arrows indicate transcription factor consensus sequences in the reverse orientation. Bent arrow indicates putative transcription start region. Numbering is relative to the ATG translation start (+1).

−499/−491 bp), one PEA3 site (−541/−536 bp), one SIF site (−488/−483 bp), and two H4TF-1 sites (−449/−441 bp, −455/−447 bp).

Mapping the Transcription Start Site of the Human POUF3 Gene

The transcription start of the human POUF3 gene was identified by RNase protection analysis. A probe for mRNA of the ubiquitously expressed transcription factor Sp1 was used as an internal control and yielded a major protected fragment at 108 bp and a minor protected fragment at about 190 bp. A single band between 270 and 280 bp in length was obtained in human neuroblastoma cell lines SK-N-LE and SK-N-BE, which are known to express N-Oct 3 (Schreiber *et al.*, 1990), corresponding to a putative transcription initiation site at about 300 bp upstream of the translational initiation codon (Fig. 4). No band was obtained with the glioblastoma cell line LN-308, a cell line that is known not to express N-Oct 3 (Schreiber *et al.*, 1990) and the tRNA. In experiments where no Sp1 riboprobe was added to the hybridization mix, only the protected fragment of the POUF3 between 270 and 280 bp was detected in the neuroblastoma cell lines (data not shown).

Chromosomal Localization of Human POUF3

Hybrid panel mapping. To assign the human POUF3 gene to a particular human chromosome, Southern blot hybridization analysis was carried out using a panel of human–hamster hybrid cells containing varying complements of human chromosomes. We detected unique human POUF3 bands in five *Eco*RI-digested hybrid cell line DNA samples (Table 1). No human-specific band was detectable in cell lines that did not contain chromosome 6. The level of sensitivity was probably not sufficient for detecting the human-specific band in one cell line (756), in which only 65% of the cells contained the human 6 chromosome. Based on these data it can be concluded that the human POUF3 gene is located on chromosome 6.

Radioactive in situ hybridization. A total of 47 metaphase spreads were analyzed and 720 autoradiographic silver grains were counted on chromosomes (Fig. 5A); identification was based on photographs of QFQ-banded chromosomes taken prior to *in situ* hybridization. Eighty-eight of these grains (12%) were associated with chromosome 6. The majority of grains on chromosome 6, 43 of 88, were above band q16 (Fig. 5B).

Fluorescence in situ hybridization. All of 15 metaphases examined exhibited fluorescent double spots (resulting from hybridization on both chromatids) on at least one of the two homologous chromosomes 6 (Fig. 5C). Measurements on 12 chromosomes 6 yielded an average FLpter value of 0.56 ± 0.089, locating the hybridization site of the POUF3 probe to band q16. The identification of chromosome 6 was also based on photo-

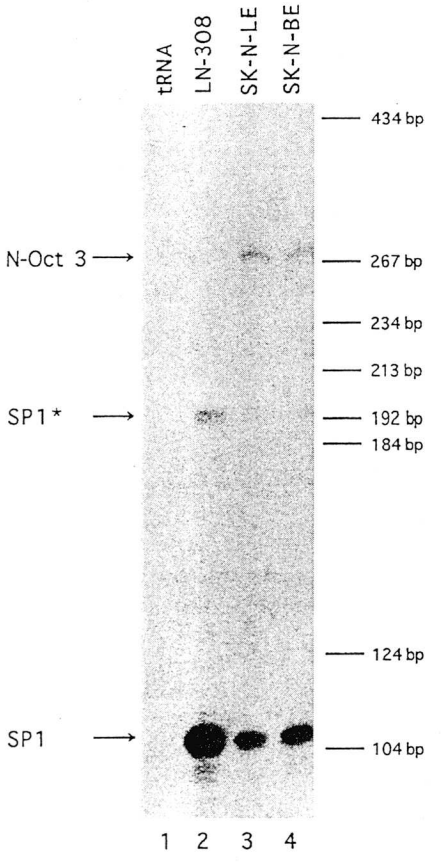


FIG. 4. RNase protection analysis of the transcription start site of human N-Oct 3. RNase protection assays were carried out as described under Materials and Methods. Total RNA from human neuroblastoma cell lines SK-N-LE and SK-N-BE and from the human glioblastoma cell line LN-308 and 50 μg of tRNA were hybridized to [^{32}P]UTP-labeled antisense riboprobes of N-Oct 3 and Sp1. Lane **1,** tRNA; lane **2,** LN-308; lane **3,** SK-N-LE; lane **4,** SK-N-BE. The band between 270 and 280 bp shows a protected fragment with N-Oct 3; the bands at 108 bp (Sp1) and about 190 bp (SP1*) show the major and minor protected fragments of the human Sp1, respectively. Numbers to the right refer to a DNA molecular weight marker (Boehringer Mannheim).

TABLE 1

Segregation of Human POUF3 in Human–Rodent Hybrids

Hybridization/ chromosome	Human chromosome																						
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y
+/+	0	0	2	0	5	5	0	2	0	2	0	1	0	2	0	1	0	0	2	0	3	0	2	1
−/−	27	30	29	28	4	30	28	25	27	29	29	26	22	21	26	28	29	25	21	27	24	25	29	27
+/−	5	5	3	5	0	0	5	3	5	3	5	4	5	3	5	4	5	5	3	5	2	5	3	4
−/+	4	1	4	3	27	1	3	6	4	2	2	5	9	10	5	3	2	6	10	4	7	6	2	4
% Discordance	25	20	19	22	75	3	22	25	25	14	19	25	39	36	28	19	19	31	36	25	25	31	14	22

Note. + or − indicates the presence or absence of the human POUF3 fragment in relation to the presence (+) or absence (−) of a particular human chromosome.

graphs of QFQ-banded chromosomes taken prior to *in situ* hybridization. Based on the grain distribution after radioactive *in situ* hybridization and on the position of the signal after fluorescence *in situ* hybridization, the POUF3 gene can be assigned to 6q16.

DISCUSSION

The human genomic POUF3 gene has been cloned and its promoter region sequenced (Fig. 2). The gene is intronless, as are the mouse homologue *brn-2* and

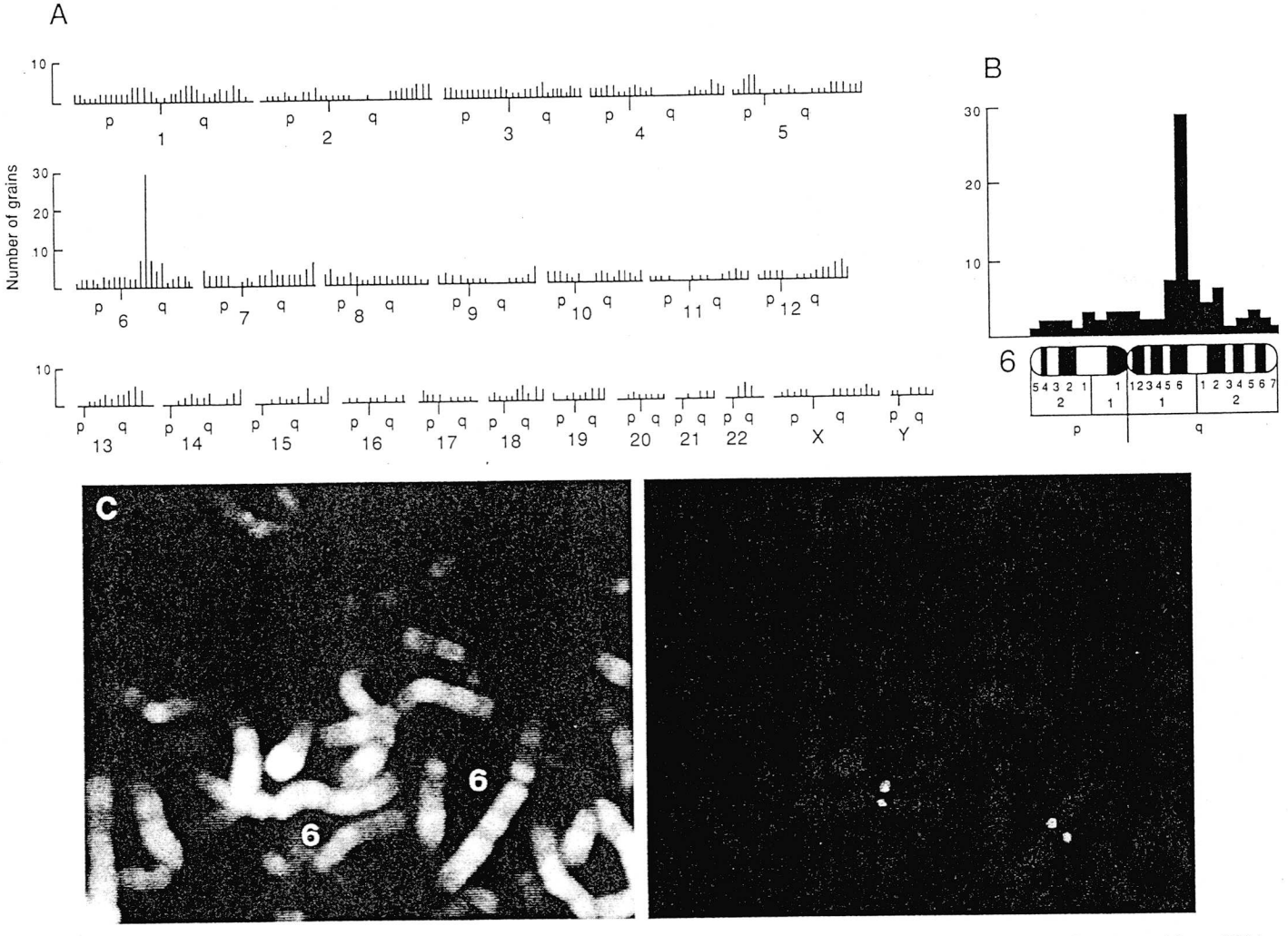


FIG. 5. **(A)** Histogram showing the distribution of autoradiographic silver grains in 47 metaphases after hybridization with a cDNA probe for the POUF3 gene. **(B)** Histogram showing the distribution of autoradiographic silver grains on a standard idiogram of chromosome 6. **(C)** Partial metaphase spreads before and after fluorescence *in situ* hybridization with a POUF3-specific probe. **(Left)** QFQ-banded chromosomes prior to *in situ* hybridization. **(Right)** Specific FITC double spots on chromosome 6q16 after *in situ* hybridization.

other members of the class III POU-domain genes, *brn-1, brn-4,* and *Tst-1* (Hara *et al.,* 1992). The ancestral class III POU-domain gene may therefore have originated by reverse transcription of a molecule of POU-domain mRNA followed by insertion of the cDNA into germ cell genomic DNA. In consequence, the class III POU factors originated by duplication of an ancestral class III POU-domain gene.

Comparison of the 5′ untranslated regions of the human POUF3 gene with the mouse genomic *brn-2* promoter sequence reveals a striking similarity of 84% (Fig. 2). However, the 5′ and 3′ noncoding regions of *brn-1, brn-2, brn-4,* and *Tst-1,* all of which belong to the class III POU genes (Treacy and Rosenfeld, 1992), show no obvious sequence similarities except for the presence of similar di- and trinucleotide repeats (Hara *et al.,* 1992). The high homology between the *brn-2* and human POUF3 predicts similar regulatory mechanisms for these two genes. Both lack the consensus TATA-box sequence but contain two conserved CCAAT boxes (Figs. 2 and 3) preceding the transcription start site of the gene by approximately 60 and 120 nucleotides, respectively. CCAAT consensus sequences are normally positioned at −70 to −80 from the transcriptional start (Breathnach and Chambon, 1981). We searched the region of the transcriptional initiation site for potential transcriptional initiator (Inr) sequences. Inrs can be defined as DNA sequence elements overlapping a transcription start site and recruiting initiator binding protein(s), which enable(s) the transcription machinery to determine the start site location in a promoter that lacks a TATA box (Smale and Baltimore, 1989; Javahery *et al.,* 1994). None of the functional Inr sequences that have been described to date was found in the region of the transcription start of the POUF3 gene. The POUF3 (*brn-2*) promoter is unusual since it lacks a TATA box as well as typical initiator sequences. The dinucleotide CG is present nearly as often as GC (48 versus 57) in the sequenced promoter region. This C+G-rich region may represent a CpG island that contains high levels of the otherwise underrepresented dinucleotide CG, a target for DNA methylation (Bird, 1986). It will be of interest to determine the methylation status of the POUF3 promoter in expressing and nonexpressing cells.

Using RNase protection assays with an Sp1 mRNA internal control probe, we have mapped a single transcriptional initiation site in the POUF3 promoter to a region about 300 bases upstream of the translation initiation site (Fig. 4). The positive signal seen in neuroblastoma cell lines SK-N-LE and SK-N-BE compared to the glioblastoma cell line LN-308 is consistent with previous observations that N-Oct 3 is expressed in the two neuroblastoma cell lines but absent in the glioblastoma cell line LN-308 (Schreiber *et al.,* 1990). In contrast to the glioblastoma cell line LN-308, freshly isolated human primary glioblastomas express N-Oct 3 protein (Schreiber *et al.,* 1994), indicating that the loss of N-Oct 3 expression seen in some tumor cell lines occurs at the transcriptional or posttranscriptional level.

Among the more interesting potential regulatory sequences found in this promoter is the DNA consensus sequence for the developmentally regulated AP-2 transcription factor (Williams *et al.,* 1988; Mitchell *et al.,* 1991). The AP-2 motif is found at 12 sites within the 5′-flanking region of the POUF3 gene. Four of these sites have also been found to be conserved in the mouse *brn-2* promoter (Fig. 3). Recently, we have shown that AP-2 RNA is strongly induced during the retinoic acid (RA)-mediated differentiation of P19 cells into neuroectodermal cell types such as neurons and astrocytes (Philipp *et al.,* 1994). Others have recently demonstrated an induction of the transcription factor *brn-2* mRNA when P19 cells differentiated to neural cells upon RA treatment (Fujii and Hamada, 1993). These findings strongly suggest that AP-2 could be a major regulator for the N-Oct 3 transcription factor which is thought to play a crucial role in establishing mammalian neural cell lineages (Fujii and Hamada, 1993). The identification of numerous consensus sequences of the AP-2 transcription factor in the POUF3 promoter region should prove useful in further experiments aimed at elucidating the function and regulation of the N-Oct 3 transcription factor and its putative involvement in neural cell differentiation.

To date very few data on the target genes of octamer-binding proteins are available, and therefore their precise biological role remains largely unknown. In the case of rat *brn-2,* transcripts are known to be expressed in the paraventricular hypothalamus, which synthesizes and secretes corticotrophin-releasing hormone (CRH) (Li *et al.,* 1993). DNase I footprinting analysis showed that *brn-2* protein binds selectively to five sites within the promoter and in cotransfection assays increased transcription from the CRH promoter 40-fold (Li *et al.,* 1993).

The N-terminus of N-Oct 3 harbors homopolymeric regions of 21 glycines and 21 glutamines, which are separated by a 36-amino-acid spacer. The glycine stretch is encoded mainly by GGC repeats and the glutamine-rich region by CAG residues (Schreiber *et al.,* 1993). Clusters of trinucleotide repeats may be unstable under certain conditions and have been recently found to cause human genetic disorders: fragile X syndrome (Fu *et al.,* 1991; Kremer *et al.,* 1991; Caskey *et al.,* 1992), chromosome X-linked spinal bulbar muscular dystrophy (SMBA) (La Spada *et al.,* 1991), myotonic dystrophy (Brook *et al.,* 1992; Buxton *et al.,* 1992; Fu *et al.,* 1992; Harley *et al.,* 1992) and Huntington disease (Huntington's Disease Collaborative Research Group, 1993) all involve the abnormal expression of G+C-rich triple repeats. The mechanisms leading to the occurrence of instability of trinucleotide repeats await identification.

To gain more insight into the putative role of

N-Oct 3 in diseases, we have mapped the gene to chromosomal region 6q16 using Southern hybridization and radioactive and fluorescence *in situ* hybridization (Figs. 5A–5C). Both chromosomal assignments and assignments predicted from mouse–human chromosomal homologies (Nadeau *et al.*, 1992) show that none of the other human POU genes were linked to each other (Xia *et al.*, 1993). They are widely distributed throughout the human genome, indicating that their coordinate regulation of expression is not the result of activation of one or a few chromosomal regions. Regulation is most probably mediated by *trans*-activating factors that interact with the promoter regions of each POU gene. Genes that have been assigned to the same region as the POUF3 gene include the cannabinoid receptor CNR (6q14–q15), the trophoblast glycoprotein TPBG (6q14–q15), the two γ-aminobutyric acid receptors rho $\frac{1}{2}$ (6q14–q21), and the ecto-5′-nucleotidase NT5 (6q14–q21).

Several disease loci have been located to 6q16 and neighboring bands by linkage analysis. Among these, only the phenotype of North Carolina macular dystrophy (NCMD) at 6q14–6q16.2 (Small *et al.*, 1992a) is *restricted to brain, the site of expression of N-Oct 3.* NCMD is a macular dystrophy causing impaired central vision (Small *et al.*, 1991). Inheritance is autosomal dominant; the disease course is generally stable, but the phenotypes are highly variable (Small *et al.*, 1992b). It is of considerable biological and medical interest to address experimentally the question whether the POUF3 gene is sensitive to a trinucleotide repeat mutation mechanism and whether it may play a role in NCMD disease.

With regard to the hypothesis of an involvement of N-Oct 3 in human diseases, it is interesting that the tumorigenicity of malignant melanoma cells can be suppressed by the introduction of human chromosome 6 or mouse chromosome 4 into these cells (Trent *et al.*, 1990). These chromosomes share a homologous region, contained in human chromosome 6q12–q21, as evidenced by the assignment of *brn-2* to mouse chromosome 4 (Xia *et al.*, 1993). Abnormalities of this human chromosomal region have been found in a range of different tumors, including cutaneous melanomas (Trent *et al.*, 1983; Pathak *et al.*, 1983; Becher *et al.*, 1983; Rauth *et al.*, 1994). In all of these tumors, mutations of tumor-suppressor genes on human chromosome 6q may be involved. Analysis of melanoma metastases and primary cultures of tumors revealed a range of Oct factors comigrating with activities previously described in mouse brain (Thomson *et al.*, 1993). Melanoma extracts express the melanocytic-associated Oct-M1 and Oct-M2 octamer-binding factors that comigrate with the N-Oct 3 and N-Oct 5 (Schreiber *et al.*, 1990) factors of brain nuclear extracts (Thomson *et al.*, 1993). Thus, Oct-M1 and Oct-M2 are most probably identical to N-Oct 3 and N-Oct 5, respectively. It may be of interest to investigate whether mutations of N-Oct 3 might be associated with tumorigenicity of malignant melanoma cells.

ACKNOWLEDGMENTS

We thank Olaf Zilian for helping in the isolation of phage DNA and Oliver Clay for technical assistance with computer imaging. We are also most grateful to Winship Herr for helpful discussions. This work was supported by the Swiss National Science Foundation (Grant 3100-028402.90/2).

REFERENCES

Ausubel, F. M., Bent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1989). "Current Protocols in Molecular Biology," Greene Publishing Associates and Wiley Interscience, New York.

Becher, R., Givas, Z., Karakousis, C., and Sandberg, A. A. (1983). Non-random chromosome changes in malignant melanoma. *Cancer Res.* **43:** 5010–5016.

Bird, A. P. (1986). CpG-rich islands and the function of DNA methylation. *Nature* **321:** 209–213.

Bodner, M., Castrillo, J. L., Theill, L. E., Deerinck, T., Ellisman, M., and Karin, M. (1988). The pituitary-specific transcription factor GHF-1 is a homeobox-containing protein. *Cell* **55:** 505–518.

Breathnach, R., and Chambon, P. (1981). Organization and expression of eukaryotic split genes coding for proteins. *Annu. Rev. Biochem.* **50:** 349–383.

Brook, J. D., McCurrach, M. E., Harley, H. G., Buckler, A. J., Church, D., Aburatani, H., Hunter, K., Stanton, V. P., Thirion, J. P., Hudson, T., Sohn, R., Zemelmann, B., Snell, R. G., Rundle, S. A., Crow, S., Davies, J., Shelbourne, P., Buxton, J., Jones, C., Juvonen, V., Johnson, K., Harper, P. S., Shaw, D. J., and Housman, D. E. (1992). Molecular basis of myotonic dystrophy: Expansion of a trinucleotide (CTG) repeat at the 3′ end of a transcript encoding a protein kinase family member. *Cell* **68:** 799–808.

Burglin, T. R., Finney, M., Coulson, A., and Ruvkun, G. (1989). *Caenorhabditis elegans* has scores of homeobox-containing genes. *Nature* **341:** 239–243.

Buxton, J., Shelbourne, P., Davies, J., Jones, C., Van Tongeren, T., Aslanidis, C., de Jong, P., Jansen, G., Anvret, M., and Riley, B. (1992). Detection of an unstable fragment of DNA specific to individuals with myotonic dystrophy. *Nature* **355:** 545–546.

Caskey, C. T., Pizzuti, A., Fu, Y., Fenwick, R. G., and Nelson, D. L. (1992). Triplet repeat mutations in human disease. *Science* **256:** 784–789.

Clerc, R. G., Corcoran, L. M., Lebowitz, J. H., Baltimore, D., and Sharp, P. A. (1988). The B-cell specific Oct-2 protein contains POU box- and homeo box-type domains. *Genes Dev.* **2:** 1570–1581.

Currie, R. A. (1994). Mechanisms of transcriptional regulation by the otamer family proteins. *Transcr. Mech. Reg.* **3:** 425–441.

Devereux, J., Haeberli, P., and Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.* **12:** 387–395.

Faisst, S., and Meyer, S. (1992). Compilation of vertebrate-encoded transcription factors. *Nucleic Acids Res.* **20:** 3–26.

Finney, M., Ruvkun, G., and Horvitz, H. R. (1988). The *C. elegans* cell lineage and differentiation gene unc-86 encodes a protein with a homeodomain and extended similarity to transcription factors. *Cell* **55:** 757–769.

Fries, R., Solinas, S., and Gunawardana, A. (1991). Chromosomal assignment of marker loci to monitor marker spacing. *In* "Strategies, Techniques and Applications" (L. B. Schook, H. A. Lewin, and D. G. McLaren, Eds.), pp. 159–176, Dekker, New York.

Fu, Y., Kuhl, D. P. A., Pizzuti, A., Pieretti, M., Sutcliffe, J. S., Richards, S., Verkerk, A. J. M. H., Holden, J. J. A., Fenwick, R. G., Warren, S. T., Oostra, B. A., Nelson, D. L., and Caskey, C. T. (1991). Variation of the CGG repeat at the fragile X site results in genetic instability: Resolution of the Sherman paradox. *Cell* **67:** 1047–1058.

Fu, Y., Pizzuti, A., Fenwick, R. G., King, J., Rajnarayan, S., Dunne, P. W., Dubel, J., Nasser, G. A., Ashizawa, A., De Jong, P., Wieringa, B., Korneluk, R., Perryman, M. B., Epstein, H. F., and Caskey, C. T. (1992). An unstable triplet repeat in a gene related to myotonic muscular dystrophy. *Science* **255:** *1256–1259.*

Fujii, H., and Hamada, H. (1993). A CNS-specific POU transcription factor, Brn-2, is required for establishing mammalian neural cell lineages. *Neuron* **11:** 1197–1206.

Hara, Y., Rovescalli, A. C., Kim, Y., and Nierenberg, M. (1992). Structure and evolution of four POU domain genes expressed in mouse brain. *Proc. Natl. Acad. Sci. USA* **89:** 3230–3284.

Harley, H. G., Brook, J. D., Rundle, S. A., Crow, S., Reardon, W., Buckler, A. J., Harper, P. S., Housman, D. E., and Shaw, D. J. (1992). Expansion of an unstable DNA region and phenotypic variation in myotonic dystrophy. *Nature* **355:** 545–546.

He, X., Treacy, M. N., Simmons, D. M., Ingraham, H. A., Swanson, L. W., and Rosenfeld, M. G. (1989). Expression of a large family of POU-domain regulatory genes in mammalian brain development. *Nature* **340:** 35–41.

Herr, W., Sturm, R. A., Clerc, R. G., Corcoran, L. M., Baltimore, D., Sharp, P. A., Ingraham, H. A., Rosenfeld, M. G., Finney, M., Ruvkun, G., and Horvitz, H. R. (1988). The POU domain: A large conserved region in the mammalian pit-1, oct-1, oct-2, and *Caenorhabditis elegans,* unc-86 gene products. *Genes Dev.* **2:** 1513–1516.

Huntington's Disease Collaborative Research Group (1993). A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. *Cell* **72:** 971–983.

Ingraham, H. A., Chen, R., Mangalam, H. J., Elsholtz, H. P., Flynn, S. E., Lin, C. R., Simmons, D. M., Swanson, L., and Rosenfeld, M. G. (1988). A tissue-specific transcription factor containing a homeodomain specifies a pituitary phenotype. *Cell* **55:** 519–529.

ISCN (1981). An international system for human cytogenetic nomenclature-high-resolution banding. *Cytogenet. Cell Genet.* **31:** 1–32.

Javahery, R., Khachi, A., Lo, K., Zenzie-Gregory, B., and Smale, S. T. (1994). DNA sequence requirements for transcriptional initiator activity in mammalian cells. *Mol. Cell. Biol.* **14:** 116–127.

Ko, H. S., Fast, P., McBride, W., and Staudt, L. M. (1988). A human protein specific for the immunoglobulin octamer DNA motif contains a functional homeobox domain. *Cell* **55:** 135–144.

Kremer, E. J., Pritchard, M., Lynch, M., Yu, S., Holman, K., Baker, E., Warren, S. T., Schlessinger, D., Sutherland, G. R., and Richards, R. I. (1991). Mapping of DNA instability at the fragile X to a trinucleotide repeat sequence $p(CCG)_n$. *Science* **253:** 1711–1714.

La Spada, A. R., Wilson, E. M., Lubahn, D. B., Harding, A. E., and Fischbeck, K. H. (1991). Androgen receptor gene mutations in X-linked spinal and bulbar muscular atrophy. *Nature* **352:** 77–79.

Le Moine, C. L., and Young, W. S. (1992). RHS2, a POU domain-containing gene, and its expression in developing and adult brain. *Proc. Natl. Acad. Sci. USA* **89:** 3285–3289.

Li, P., He, X., Gerrero, M. R., Mok, M., Aggarwal, A., and Rosenfeld, M. G. (1993). Spacing and orientation of bipartite DNA-binding motifs as potential functional determinants for POU domain factors. *Genes Dev.* **7:** 2483–2496.

Li, S., Crenshaw, E. B., Rawson, E. J., Simmons, D. M., Swanson, L. W., and Rosenfeld, M. G. (1990). Dwarf locus mutants lacking three pituitary cell types result from mutations in the POU-domain gene pit-1. *Nature* **347:** 528–533.

Lichter, P., Tang, C. J., Call, K., Hermanson, G., Evans, G. A., Housman, D., and Ward, D. C. (1990). High resolution mapping of human chromosome 11 by in situ hybridization with cosmid clones. *Science* **247:** 64–69.

Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982). "Molecular Cloning: A Laboratory Manual," Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Mathis, J. M., Simmons, D. M., He, X., Swanson, L. W., and Rosenfeld, M. G. (1992). Brain 4: A novel mammalian POU domain transcription factor exhibiting restricted brain-specific expression. *EMBO J.* **11:** 2551–2561.

Mitchell, P. J., Timmons, P. M., Herbert, J. M., Rigby, P. W., and Tijan, R. (1991). Transcription factor AP-2 is expressed in neural crest cell lineages during mouse embryogenesis. *Genes Dev.* **5:** 105–119.

Müller, M. M., Ruppert, S., Schaffner, W., and Matthias, P. (1988). A cloned octamer transcription factor stimulates transcription from lymphoid-specific promoters in non-B cells. *Nature* **336:** 544–551.

Nadeau, J. H., Davisson, M. T., Doolittle, D. P., Grant, P., Hillyard, A. L., Kosowsky, M. R., and Roderick, T. H. (1992). Comparative map for mice and humans. *Mamm. Genome* **3:** 480–536.

Pathak, S., Drwinga, H. L., and Hsu, T. C. (1983). Involvement of chromosome 6 rearrangements in human malignant melanoma cell lines. *Cytogenet. Cell Genet.* **36:** 573–579.

Philipp, J., Mitchell, P. J., Malipiero, U., and Fontana, A. (1994). Cell type specific regulation of expression of transcription factor AP-2 in neuroectodermal cells. *Dev. Biol.* **165:** 602–614.

Rauth, S., Green, A., Bratescu, L., and Das Gupta, T. K. (1994). Chromosome abnormalities in metastatic melanoma. *In Vitro Cell. Dev. Biol.* **30A:** 79–84.

Sanger, F., Nicklen, S., and Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74:** 5463–5467.

Scheidereit, C., Cromlish, J. A., Gerster, T., Kawakami, K., Balmaceda, C. G., Currie, R. A., and Roeder, R. G. (1988). A human lymphoid-specific transcription factor that activates immunoglobulin genes is a homeobox protein. *Nature* **336:** 551–557.

Schreiber, E., Harshman, K., Kemler, I., Malipiero, U., Schaffner, W., and Fontana, A. (1990). Astrocytes and glioblastoma cells express novel octamer-DNA binding proteins distinct from the ubiquitous Oct-1 and B cell type Oct-2 proteins. *Nucleic Acids Res.* **18:** 5495–5503.

Schreiber, E., Merchant, R. E., Wiestler, O. D., and Fontana, A. (1994). Primary brain tumors differ in their expression of octamer deoxyribonucleic acid binding transcription factors from long-term cultured glioma cell lines. *Neurosurgery* **34:** 129–135.

Schreiber, E., Tobler, A., Malipiero, U., Schaffner, W., and Fontana, A. (1993). cDNA cloning of human N-Oct 3, a nervous-system specific POU domain transcription factor binding to the octamer DNA motif. *Nucleic Acids Res.* **21:** 253–258.

Smale, S. T., and Baltimore, D. (1989). The 'initiator' as a transcriptional control element. *Cell* **57:** 103–113.

Small, K. W., Hermsen, V., Gurney, N., Fetkenhour, C. L., and Folk, J. C. (1992b). North Carolina macular dystrophy and central areolar pigment epithelial dystrophy. *Arch. Ophthalmol.* **110:** 515–518.

Small, K. W., Killian, F., and McLean, W. C. (1991). North Carolina's dominant progressive foveal dystrophy: How progressive is it? *Br. J. Ophthalmol.* **75:** 401–406.

Small, K. W., Weber, J. L., Roses, A., Lennon, F., Vance, J. M., and Pericak-Vance, M. A. (1992a). North Carolina macular dystrophy is assigned to chromosome 6. *Genomics* **13:** 681–685.

Solinas Toldo, S., Fries, R., Steffen, P., Neibergs, H. L., Barendse, W., Womack, J. E., Hetzel, D. J. S., and Stranzinger, G. (1993). Physically mapped, cosmid-derived microsatellite markers as anchor loci on bovine chromosomes. *Mamm. Genome* **4:** 720–727.

Sturm, R. A., Das, G., and Herr, W. (1988). The ubiquitous octamer-

binding protein Oct-1 contains a POU domain with a homeo box subdomain. *Genes Dev.* **2:** 1582–1599.

Suzuki, N., Rohdewold, H., Neuman, T., Gruss, P., and Schöler, H. R. (1990). Oct-6: A POU transcription factor expressed in embryonal stem cells and in the developing brain. *EMBO J.* **9:** 3723–3732.

Thomson, J. A. F., Parsons, P. G., and Sturm, R. A. (1993). In vivo and in vitro expression of octamer binding proteins in human melanoma metastases, brain tissue, and fibroblasts. *Pigment Cell Res.* **6:** 13–22.

Treacy, M. N., and Rosenfeld, G. M. (1992). Expression of a family of POU-domain protein regulatory genes during development of the central nervous system. *Annu. Rev. Neurosci.* **15:** 139–165.

Trent, J. M., Rosenfeld, S. B., and Meyskens, F. L. (1983). Chromosome 6q involvement in human malignant melanoma. *Cancer Genet. Cytogenet.* **9:** 177–180.

Trent, J. M., Stanbridge, E. J., McBride, H. L., Meese, E. U., Casey, G., Araujo, D. E., Witkowski, C. M., and Nagle, R. B. (1990). Tumorigenicity in human melanoma cell lines controlled by introduction of human chromosome 6. *Science* **247:** 568–571.

Verrijzer, C. P., and Van der Vliet, P. C. (1993). POU domain transcription factors. *Biochim. Biophys. Acta* **173:** 1–21.

Wegner, M., Drolet, D. W., and Rosenfeld, M. G. (1993). POU-domain proteins: Structure and function of developmental regulators. *Curr. Opin. Cell Biol.* **5:** 488–498.

Williams, T., Admon, A., Lüscher, B., and Tjian, R. (1988). Cloning and expression of AP-2, a cell type-specific transcription factor that activates inducible enhancer elements. *Genes Dev.* **2:** 1557–1569.

Xia, Y.-R., Andersen, B., Mehrabian, M., Diep, A. T., Warden, C. H., Mohandas, T., McEvilly, R. J., Rosenfeld, M. G., and Lusis, A. J. (1993). Chromosomal organization of mammalian POU domain factors. *Genomics* **18:** 126–130.

4.2 N-Oct 5 is generated by *in vitro* proteolysis of the neural POU-domain protein N-Oct 3

Suzana Atanasoski, Edgar Schreiber, Adriano Fontana, and Winship Herr (1996). Submitted for publication

Classification: Biochemistry

N-Oct 5 is generated by *in vitro* proteolysis of the neural POU-domain protein N-Oct 3

Suzana Atanasoski*†, Edgar Schreiber†‡, Adriano Fontana†,
and Winship Herr*§

*Cold Spring Harbor Laboratory,
1 Bungtown Road, P.O. Box 100,
Cold Spring Harbor, NY 11724

†University Hospital of Zurich,
Department of Internal Medicine,
Division of Clinical Immunology,
Haeldeliweg 4, CH-8044 Zurich, Switzerland

‡present address: Calydon Inc.,
1014 Hamilton Ct.,
Menlo Park, CA 94025

§Corresponding author: Tel: (516) 367-8401
FAX: (516) 367-8454
e-mail: herr@cshl.org

ABSTRACT

The neural POU-domain proteins N-Oct 3 and N-Oct 5 were first identified in electrophoretic mobility retardation assays through their ability to bind to the octamer sequence ATGCAAAT. These two N-Oct factors are detected in extracts from tumor-derived and normal neural cells. They are present differentially, however, in extracts from melanocytes and melanoma cells: N-Oct 3 is present in extracts from both melanocytes and melanoma cells, whereas N-Oct 5 is more evident in extracts from metastatic melanoma cells. We show here that a cDNA encoding N-Oct 3 directs synthesis of both the N-Oct 3 and N-Oct 5 proteins and that the N-Oct 5 protein in neural and melanoma-cell extracts is also related to N-Oct 3. N-Oct 5, however, is apparently not expressed *in vivo*: It is not detected if cells are rapidly lysed in SDS or if extracts are prepared with appropriate protease inhibitors that includes the serine-protease inhibitor 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF). These data suggest that N-Oct 5 is a specific *in vitro* proteolytic cleavage product of N-Oct 3 and is not directly related to melanocyte malignancy.

INTRODUCTION

In eukaryotes, transcription is regulated by the combinatorial action of sequence-specific DNA-binding proteins. In non-vertebrate eukaryotes, these transcriptional regulators are commonly identified genetically. In vertebrates, they are more often identified biochemically in cell extracts through binding assays such as DNase I protection and electrophoretic mobility retardation. Many transcriptional regulators belong to families of sequence-specific DNA-binding proteins that recognize a particular *cis*-regulatory element. For this reason, the electrophoretic mobility retardation assay is particularly revealing because it allows individual family members to be discriminated from one another by the distinct mobilities of the

protein–DNA complexes they form on a single *cis*-regulatory site.

Indeed, electrophoretic mobility retardation was used to show that the DNA octamer sequence ATGCAAAT found in a variety of cellular and viral promoters is recognized by the related proteins Oct-1, a broadly expressed transcription factor, and Oct-2, a cell-specifically expressed transcription factor (1-3). Subsequently, this assay allowed identification of a family of human and mouse neural octamer-binding proteins (4, 5); these neural (N) proteins are known as N-Oct 2, N-Oct 3, N-Oct 4, and N-Oct 5 in humans. Studies indicate that these Oct and N-Oct proteins belong to the large POU-domain family of eukaryotic transcriptional regulators – a family of proteins that perform functions important in cell differentiation and development (reviewed in ref. 6, 7), and share a conserved POU DNA-binding domain (reviewed in ref. 8).

Cloning of human POU-domain protein-encoding cDNAs (9) indicates that the N-Oct 3 protein is the human homolog of the rodent Brn-2 protein (10-12). In mouse cells, expression of Brn-2 is required for establishment of neural cell lineages (13), and Brn-2-deficient mice fail to establish specific subpopulations of neurons in the hypothalamus, demonstrating a role in neural cell differentiation (14, 15). In humans, N-Oct 3 is expressed in both normal and cancer cells of many different cell-types originating from the neuroectoderm (5, 16-20). In melanocytes, which are neuroectodermal, N-Oct 3 plays a role in cell differentiation and in the development of malignant melanomas (20).

The other N-Oct protein N-Oct 5 is also present, together with N-Oct 3, in extracts from neural cells, but these two N-Oct factors are present differentially in extracts from melanocytes and melanoma cells: only N-Oct 3 is detected in cultured human melanocytes (16), whereas both N-Oct 3 and N-Oct 5 are detected in many secondary melanoma cell lines (18). These results suggest a causal relationship between N-Oct 5 expression and melanoma development.

Ectopic expression of N-Oct 3 in nonneuronal cells results in both N-Oct 3 and an N-Oct 5-like protein in cell extracts, suggesting that N-Oct 5 arises by alternate expression of the gene encoding N-Oct 3, perhaps by alternate translational initiation (9). Because the N-Oct 5

protein is expressed in many, but not all, N-Oct 3-expressing cells, such alternate expression of the gene encoding N-Oct 3 might be regulated, and this regulation may be related to the oncogenic transformation of melanocytes.

Here, to understand the regulation of N-Oct 5 expression, we have investigated the origin of the N-Oct 5 protein. Our studies show that N-Oct 5 is indeed related to N-Oct 3, but does not result from alternate translational initiation. Indeed, N-Oct 5 apparently does not exist *in vivo*. Rather, N-Oct 5 results from proteolytic cleavage of N-Oct 3 during *in vitro* extract preparation. Generation of N-Oct 5 is not eliminated by the commonly used protease inhibitors PMSF and leupeptin, but can be inhibited by a cocktail of protease inhibitors including the PMSF-like serine-protease inhibitor 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF). These results demonstrate one instance in which a member of a family of DNA-binding proteins, originally detected by electrophoretic mobility retardation, has no evident *in vivo* counterpart.

MATERIALS and METHODS

Expression constructs.

The human N-Oct 3 expression vectors are derived from pBS-N-Oct 3 (9). An *N-Oct 3*-containing Eco47III/EcoRV fragment from pBS-N-Oct 3 was inserted between end-filled XbaI and BamHI sites of the CMV-promoter expression vector pCGATG$^-$ (21) yielding pCGATG$^-$N-Oct 3; pCGATG$^-$N-Oct 3 contains 22 bp of 5' and 465 bp of 3' untranslated *N-Oct 3* sequences and expression of N-Oct 3 is driven by the *N-Oct 3* AUG translational initiation codon. To produce an amino-terminally epitope-tagged N-Oct 3 expression plasmid (pCGN–N-Oct 3), we replaced the *N-Oct 3* 5' untranslated sequences and initiation codon in pCGATG$^-$N-Oct 3 with the initiation codon and influenza virus hemagglutinin (HA) epitope-encoding sequences of pCGN (22); in this construct, the HA epitope tag-encoding sequences are fused via an XbaI site to position 2 of the N-Oct 3-coding sequences. To construct a carboxy-

terminally epitope-tagged N-Oct 3 expression plasmid (pCGATG$^-$N-Oct 3–C-tag), the HA epitope-containing sequence GGSYPYDVPDYAS was inserted immediately upstream of the *N-Oct 3* termination codon.

The NotI^a *N-Oct 3*-deletion construct (pCGATG$^-$N-Oct 3–C-tag ΔNotI^a) was prepared by removal of a unique 112-bp NotI fragment in the 5' region of the N-Oct 3–C-tag coding sequences, resulting in a translational frame shift. The NotI^b *N-Oct 3*-deletion construct (pCGATG$^-$N-Oct 3–C-tag ΔNotI^b) was prepared by end-fill of the NotI sites prior to reclosure of the plasmid by ligation; the end-fill step maintained the N-Oct 3 translational reading frame. The veracity of the constructs was veryfied by DNA sequence analysis.

Tissue culture, transfection, and extract preparation.
A2058 human melanoma cells (American Type Culture Collection, Rockville, Maryland) and COS cells were grown in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum, 200 U/ml penicillin and 100 μg/ml streptomycin.
COS cells were transfected by calcium phosphate coprecipitation as described by Tanaka et al. (23). Each expression construct (1 μg) was transfected along with 1 μg of the internal reference plasmid expressing the GAL4 DNA-binding domain (residues 1-94; ref. (24) and pUC119 carrier DNA (up to 20 μg of total DNA). Forty hours after transfection, nuclear extracts were prepared in the presence of the protease inhibitors PMSF and leupeptin as described previously (25). Where indicated, the extraction buffer contained a cocktail of protease inhibitors (Boehringer Mannheim) containing AEBSF (1 mg/ml), EDTA (0.5 mg/ml), leupeptin (10 μg/ml), pepstatin (10 μg/ml) and aprotinin (1 μg/ml). Nuclear extracts from A2058 melanoma cells were prepared from confluent cell cultures as described above. Nuclear extracts from mouse primary astrocyte cultures were kindly provided by Dr. J. Philipp (University Hospital of Zurich).

Mobility retardation analysis and DNA-binding sites.
Protein-DNA binding reactions were as described by Schreiber et al. (25).

The DNA probes contained a single copy of the wild-type (ATGCAAAT) or mutant (CGGCAAAT) histone H2B octamer site inserted into the *Hin*dIII site of the pUC119 polylinker (26). Probes ~145 bp in length were radioactively labeled by PCR amplification with pUC119 polylinker-specific ^{32}P-end-labeled primers.

Generation of rabbit anti-N-Oct 3 antiserum.

A 10-amino-acid peptide with the sequence CGGTLPGAED, containing N-Oct 3 residues 416–424 (underlined) was synthesized, coupled to key hole limpet haemocyanin, and inoculated into a New Zealand white rabbit with 300 µg of antigen in Freund's incomplete adjuvant at 3 week intervals (27). An immune response was detected by mobility retardation analysis after the sixth inoculation. For mobility retardation assays, the anti-N-Oct 3 antiserum, purified over protein A Sepharose, was added to the nuclear extract in a 15-min preincubation step prior to probe addition.

Immunoblot analysis.

For immunoblotting, 5 µl of either nuclear or whole-cell extracts of transfected COS cells were used. For whole-cell extracts, cells were rapidly lysed in SDS-sample buffer by boiling for 5 min and cellular debris was subsequently removed by centrifugation at 12,000 x g for 10 min as described (28). Soluble proteins were resolved by 7% SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell NC^{TM}). The membranes were probed with the 12CA5 anti-HA monoclonal antibody and immunoreactive proteins detected by chemiluminescence (Amersham).

RESULTS

The N-Oct 3 cDNA encodes both the N-Oct 3 and N-Oct 5 proteins.

Schreiber et al. (9) showed that transient expression of the human N-Oct 3-encoding cDNA in monkey COS cells yields N-Oct 3 and N-Oct 5

octamer site-specific DNA-binding activities in an electrophoretic mobility retardation assay. To study the relationship of these two DNA-binding activities, we individually tagged the amino and carboxyl termini of N-Oct 3 with an epitope derived from the influenza hemagglutinin (HA) protein (29), expressed these constructs in COS cells, and tested whether epitope-tagged forms of N-Oct 3 and N-Oct 5 were present.

Figure 1 shows the results of such an experiment. Cell extracts containing transiently expressed untagged and epitope-tagged N-Oct 3 proteins were assayed by electrophoretic mobility retardation analysis in the presence and absence of an epitope tag-specific antibody. For the electrophoretic mobility retardation analysis, we used wild-type (lanes 1-9) and mutant (lanes 10-15) octamer-site probes originating from the histone H2B promoter. As an internal control for COS-cell transient expression efficiency, we co-expressed the DNA-binding domain of the yeast GAL4 transcriptional activator (residues 1-94; ref. 24).

As expected, a mock-transfected cell extract generated a single octamer site-dependent complex (compare lane 2 with lane 1 and with lane 11). As shown with an Oct-1-specific monoclonal antibody (YL15; ref. 30), this complex is formed by endogenous Oct-1 (data not shown). Unexpectedly, transient expression of the internal-reference GAL4 DNA-binding domain resulted in a fast migrating complex on both the wild-type and mutant octamer-site probes (compare lanes 3 and 12 with lanes 2 and 11). As shown with GAL4-specific antibodies and comparison to binding to a wild-type GAL4-binding site probe (data not shown), this complex represents weak binding of the GAL4 DNA-binding domain to our octamer-site probes and thus serves as a direct internal control in the octamer-site mobility retardation assays.

Transient expression of the wild-type (WT) or amino- (N-tag) or carboxy- (C-tag) terminally tagged N-Oct 3-encoding cDNAs resulted in the generation of multiple N-Oct factor–DNA complexes each of which was octamer-site specific (compare lanes 4, 6, and 8 with lanes 13-15). Expression of the wild-type N-Oct 3-encoding cDNA (lane 4) resulted in three protein–DNA complexes that correspond to N-Oct 3 and two N-Oct 5-species with slightly different mobilities (called N-Oct 5a and 5b; ref.

9). Consistent with the expected larger size of the epitope-tagged proteins, expression of the N-tag and C-tag N-Oct 3-encoding cDNAs resulted in a retardation in the mobility of the N-Oct 3–DNA complexes compared to the untagged, wild-type N-Oct 3 protein–DNA complex (compare lanes 6 and 8 with lane 4). Furthermore, unlike the wild-type protein, these epitope-tagged N-Oct 3 proteins were recognized by the anti-epitope-tag antibody (compare lanes 5, 7 and 9). The mobility of the N-Oct 5–DNA complex, however, was only affected when the epitope was attached to the carboxyl terminus of N-Oct 3 (compare lanes 4, 6, and 8) and only this C-tag-N-Oct 5 protein was recognized by the anti-epitope-tag antibody (compare lanes 5, 7, and 9). These results indicate that the N-Oct 5 proteins are indeed related to N-Oct 3 but lack amino-terminal N-Oct 3 sequences.

Melanoma and astrocyte N-Oct 5 proteins are also N-Oct 3 related.

Having demonstrated that N-Oct 5 proteins are related to N-Oct 3 in a transient expression assay, we asked whether endogenous N-Oct 5–DNA complexes found in cell extracts are also related to N-Oct 3 or simply co-migrate with transiently-expressed N-Oct 5–DNA complexes. To address this question, we developed polyclonal antibodies specific for N-Oct 3. Because POU-III-class POU-domain proteins, of which N-Oct 3 is a member, are very closely related (6), we raised a polyclonal antiserum against a short nine-amino-acid peptide sequence in the carboxy-terminal region of N-Oct 3 that is not conserved among the class III POU-domain proteins. We tested the effects of the anti-N-Oct 3-peptide antiserum on the mobility retardation patterns generated by N-Oct 3-expressing and nonexpressing COS-cell extracts, and extracts from untransfected human A2058 melanoma cells and primary mouse astrocytes as shown in Figure 2.

We assayed the extracts using wild-type (1-13) and mutant (14-18) octamer-site probes. The resulting N-Oct patterns were octamer specific (compare lanes 2, 5, 8 and 11 with lanes 15-18), except for a nonspecific complex in the COS- and melanoma-cell extracts that formed with both the wild-type (lanes 2, 5 and 8) and mutant (lanes 15-17) H2B octamer-site probes. This nonspecific complex co-migrated

with the octamer-specific complex in the astrocyte extract that results from N-Oct 4 binding (5) (compare lanes 11 and 18).

Consistent with the carboxy-terminal location of the N-Oct 3 epitope, when antiserum was added to binding reactions with extracts of N-Oct 3-transfected COS cells, the anti-N-Oct 3 antiserum recognized both the N-Oct 3 and N-Oct 5 complexes (compare lane 7 with lane 6), but did not affect the endogenous Oct-1– or the GAL4–DNA complexes (compare lanes 3 and 4, and 6 and 7). Whereas the N-Oct 3 antiserum did not affect the complexes formed by the neural-specific factors N-Oct 2 and N-Oct 4 (compare lanes 12 and 13), it did inhibit formation of the endogenous N-Oct 3– and N-Oct 5–DNA complexes generated by the melanoma-cell and astrocyte extracts (compare lanes 9 and 10, and 12 and 13). These results indicate that, in addition to transiently expressed N-Oct 5 from the N-Oct 3 cDNA, endogenous N-Oct 5 is also N-Oct 3 related.

N-Oct 5 expression is dependent on N-Oct 3 expression.
The results described above show that exogenously and endogenously expressed N-Oct 5 proteins are related to N-Oct 3, but apparently lack amino-terminal N-Oct 3 sequences. Synthesis of N-Oct 5 could result from post-translational modification of N-Oct 3. Alternatively, N-Oct 5 synthesis might not be dependent on N-Oct 3 synthesis. For example, as suggested previously (9), N-Oct 5 synthesis could result from translational initiation at two internal N-Oct 3 AUG codons (see AUG^3 and AUG^4 in Fig. 3B, below). Inconsistent with this hypothesis, however, point mutations in these two codons did not alter N-Oct 5a or 5b expression (data not shown).

To determine whether or not N-Oct 5 synthesis is dependent on N-Oct 3 synthesis, we designed the experiment shown in Figure 3. We tested the effects on N-Oct 5 expression of two deletions of amino-terminal N-Oct 3 coding sequences. Both of these deletions, called $\Delta NotI^a$ and $\Delta NotI^b$, result from removal of the same NotI restriction fragment. In $\Delta NotI^a$, the religation of the NotI sites causes a translational frame shift such that, if translation initiates in this open reading frame at the N-Oct 3 initiation codon, the truncated 160-

amino-acid $\Delta NotI^a$ protein illustrated in Figure 3B is expressed. This deletion, however, should not affect expression of an N-Oct 3-related protein that either (i) initiates translation downstream of the NotI sites or (ii) results from translation of a spliced N-Oct 3 transcript that removes the sequences affected by this deletion.

In the $\Delta NotI^b$ deletion, the four-base overhangs resulting from NotI cleavage were end-filled before religation, thus restoring the N-Oct 3 translational reading frame, but shortening the protein as illustrated in Figure 3B. If N-Oct 5 expression is dependent on N-Oct 3 synthesis, then we expect this deletion to restore N-Oct 5 expression — unless the deletion itself directly affects the synthesis of N-Oct 5.

Figure 3A shows the result of this experiment. As expected, the $\Delta NotI^a$ deletion causing a translational frame-shift affects synthesis of N-Oct 3 (compare lanes 6 and 4); it also affects, however, the synthesis of N-Oct 5, suggesting that N-Oct 5 synthesis is dependent on N-Oct 3 synthesis. Consistent with this hypothesis, when the N-Oct 3 translational reading frame is restored in the $\Delta NotI^b$ construct, N-Oct 5 expression is restored (compare lanes 4, 6, and 8), but, unlike the mobility of the N-Oct 3–DNA complex, the mobility of the N-Oct 5–DNA complex is unaffected by the $\Delta NotI^b$ deletion (compare lanes 4 and 8). These results suggest that the N-Oct 5 protein(s) results from post-translational processing of N-Oct 3. For example, proteolytic cleavage of N-Oct 3 at a site(s) downstream of the $\Delta NotI^b$ deleted sequences but upstream of the POU-domain sequences would explain these results.

N-Oct 5 proteins are present in nuclear extracts but not after rapid cell lysis.

In the experiments described above, we examined N-Oct 3 and N-Oct 5 expression indirectly in a DNA-binding assay. To study N-Oct 3 and N-Oct 5 expression directly, we performed immunoblot analyses. Because the antipeptide N-Oct 3 antiserum described above was not useful for immunoblot analysis (data not shown), we assayed the synthesis of the carboxyl epitope-tagged (C-tag) N-Oct 3 and N-Oct 5 proteins after transient expression in COS cells, using the epitope tag-specific antibody. We compared the pattern of N-Oct 3 and N-Oct 5 expression

in nuclear extracts used in the electrophoretic mobility retardation assays, and extracts prepared by rapid cell lysis in the presence of SDS. Figure 4 shows the result of such an experiment.

Consistent with the presence of both N-Oct 3 and N-Oct 5 polypeptides in the nuclear extract, the extract from transfected cells (lane 2) but not mock-transfected cells (lane 1) contained three immunoreactive bands that probably correspond to the N-Oct 3 and N-Oct 5a and 5b polypeptides as indicated. Although the sample prepared by rapid cell lysis contained N-Oct 3 (as well as cross-reactive species not related to N-Oct 3; compare lanes 3 and 4 to lane 1), the N-Oct 5a and 5b-related species were missing (compare lanes 2 and 4). This unexpected result suggests that the N-Oct 5a and 5b species are generated upon extract preparation. Together with the aforedescribed results, this finding suggests that N-Oct 5 is generated by proteolysis during extract preparation, even though protease inhibitors PMSF and leupeptin were present in the extraction buffer.

N-Oct 5 arises from proteolytic cleavage of N-Oct 3 *in vitro*. To test this hypothesis, we prepared extracts from mock- and N-Oct 3-transfected COS cells, and from untransfected A2058 human melanoma cells in the absence of protease inhibitors and the presence of a more extensive cocktail of protease inhibitors. This more extensive cocktail included aprotinin, leupeptin, pepstatin, and the PMSF-like serine protease-inhibitor AEBSF (Pefabloc^{R} SC; Boehringer Mannheim), rather than our usual set of protease inhibitors leupeptin and PMSF. Figure 5 shows the results of such an experiment. Extracts containing either transiently expressed N-Oct 3 (lanes 4 and 5) or endogenous melanoma-cell N-Oct 3 (lanes 6 and 7) produced the N-Oct 5 complex only when prepared in the absence of the more extensive cocktail of protease inhibitors (compare lanes 4 and 6 to lanes 5 and 7). These results indicate that the N-Oct 5 POU-domain protein (5, 9, 16–18, 20) is a product of *in vitro* N-Oct 3 proteolysis and has no *in vivo* counterpart. These results also indicate that this protease cocktail can aid in the preparation of cell extracts with minimal protein degradation.

DISCUSSION

Alternate forms of transcription factors have been shown to arise from a single mRNA species through alternate translational initiation and to possess distinct activities. One well documented example are the liver-enriched leucine-zipper proteins LAP and LIP: LAP, a translational activator, and LIP, a transcriptional repressor, are translated from a single mRNA species by use of two AUGs in the same open reading frame (31). We anticipated that the N-Oct 3 and N-Oct 5 proteins might be similarly related. We found instead, however, that N-Oct 5 is probably not present *in vivo*, but instead is most likely generated *in vitro* by proteolysis of N-Oct 3 protein during nuclear extract preparation.

The nature of the protease or proteases that generate the characteristic breakdown products of the N-Oct 3 protein is unknown. Their presence, however, is not restricted to the tissues derived from the neuroectoderm because the N-Oct 5 proteins are also detected in extracts from transfected nonneuronal cells (e.g., COS cells). The efficacy of the serine-protease inhibitor AEBSF in preventing synthesis of N-Oct 5 suggests that the protease responsible is a serine protease. Thomson et al. (18) found that, in cells of the melanocyte lineage, N-Oct 5 is only prominent in extracts of metastatic melanoma cells and not in melanocytes and non-metastatic melanocyte cell lines. Because our results show that N-Oct 5 is generated by *in vitro* proteolysis, it is possible that the protease activity responsible for N-Oct 3 cleavage *in vitro* is either upregulated or otherwise altered in metastatic tumor cells compared to normal and non-metastatic cells. It is unlikely, however, that the changes in levels of N-Oct 5 in extracts from normal and cancerous melanocytes is directly relevant to tumor progression *in vivo*.

Our experiments do not identify the precise site of N-Oct 3 cleavage. They do show, however, that two unusual sequence features in the amino-terminal region of N-Oct 3, a 21-amino-acid stretch of glycines (labeled G_{21} in Fig. 3B) and a 21-amino-acid stretch of glutamines (labeled Q_{21} in Fig. 3B), are not the sites responsible for N-

Oct 5 synthesis: These sequence motifs either lie upstream of G_{21} or are deleted by, as in the case of Q_{21}, the $NotI^b$ deletion, which does not affect N-Oct 5 synthesis (Figure 3). The NotI deletional analysis described in Figure 3 places the proteolytic cleavage of N-Oct 3 between the Q_{21} segment and the POU domain. Examination of the amino acid sequence of this region, however, does not reveal any obvious candidate sites for the generation of N-Oct 5 proteins. The N-Oct 3 cleavage sites may represent novel proteolytic target sequences; additionally, the structure of the N-Oct 3 protein may expose this portion of the protein making it more accessible for proteases.

Effects of proteases on the stability of transcription factors in cell extracts may not be limited to the human N-Oct 3 protein. For example, the mouse neural octamer-site binding proteins Oct-8, Oct-9, and Oct-10 have only been reported in electrophoretic mobility retardation assays (4); because they display similar migration patterns to the N-Oct 5 proteins, they may also represent products of *in vitro* proteolysis not relevant to *in vivo* Oct-factor function. Identification of transcription factors other than N-Oct 3 affected by proteolysis during extract preparation will require further investigation. The more extensive use of protease inhibitors during extract preparation described here may prove useful in such studies.

ACKNOWLEDGMENTS

We thank J. Philipp for the primary mouse astrocyte-cell extract; M. Tanaka, and A. Wilson for helpful discussions; D. Aufiero and R. Whitaker for technical assistance; M. Cleary, R. Freiman, C. Hinkley, and W. Tansey for comments on the manuscript; and J. Duffy, M. Ockler, and P. Renna for help in preparing the figures. These studies were supported by U.S. Public Health Service grant CA-13106 to W.H. and Swiss National Science Foundation grant 3100-042900.95-1 to A.F.

REFERENCES

1. Singh, H., Sen, R., Baltimore, D. & Sharp, P. A. (1986) *Nature* **319,** 154-158.

2. Landolfi, N. F., Capra, J. D. & Tucker, P. W. (1986) *Nature* **323,** 548-551.

3. Staudt, L. M., Singh, H., Sen, R., Wirth, T., Sharp, P. A. & Baltimore, D. (1986) *Nature* **323,** 640-643.

4. Schöler, H. R., Hatzopoulos, A. K., Balling, R., Suzuki, N. & Gruss, P. (1989) *EMBO J.* **8,** 2543-2550.

5. Schreiber, E., Harshman, K., Kemler, I., Malipiero, U., Schaffner, W. & Fontana, A. (1990) *Nucleic Acids Res.* **18,** 5495-5503.

6. Rosenfeld, M. G. (1991) *Genes Dev.* **5,** 897-907.

7. Ruvkun, G. & Finney, M. (1991) *Cell* **64,** 475-478.

8. Herr, W. & Cleary, M. A. (1995) *Genes Dev.* **9,** 1679-1693.

9. Schreiber, E., Tobler, A., Malipiero, U., Schaffner, W. & Fontana, A. (1993) *Nucleic Acids Res.* **21,** 253-258.

10. He, X., Treacy, M. N., Simmons, D. M., Ingraham, H. A., Swanson, L. W. & Rosenfeld, M. G. (1989) *Nature* **340,** 35-41.

11. Hara, Y., Rovescalli, A. C., Kim, Y. & Nirenberg, M. (1992) *Proc Natl Acad Sci U S A* **89,** 3280-4.

12. Li, P., He, X., Gerrero, M. R., Mok, M., Agarwal, A. & Rosenfeld, M. G. (1993) *Genes Dev.* **7,** 2483-2496.

13. Fujii, H. & Hamada, H. (1993) *Neuron* **11,** 1197-1206.

14. Nakai, S., Kawano, H., Yudate, T., Nishi, M., Kuno, J., Nagata, A., Jishage, K., Hamada, H., Fujii, H., Kawamura, K., Shiba, K. & Noda, T. (1995) *Genes Dev.* **9,** 3109-3121.

15. Schonemann, M. D., Ryan, A. K., McEvilly, R. J., O'Connell, S. M., Arias, C. A., Kalla, K. A., Li, P., Sawchenko, P. E. & Rosenfeld, M. G. (1995) *Genes Dev.* **9,** 3122-3135.

16. Sturm, R. A., Bisshop, F., Takahashi, H. & Parsons, P. G. (1991) *Cell Growth and Differ.* **2,** 519-524.

17. Schreiber, E., Himmelmann, A., Malipiero, U., Tobler, A., Stahel, R. & Fontana, A. (1992) *Cancer Res.* **52,** 6121-6124.

18. Thomson, J. A. F., Parsons, P. G. & Sturm, R. A. (1993) *Pigment Cell Res.* **6,** 13-22.

19. Schreiber, E., Merchant, R. E., Wiestler, O. D., Fontana, A. (1994) *Neurosurgery* **34,** 129-35.

20. Thomson, J. A. F., Murphy, K., Baker, E., Sutherland, G. R., Parsons, P. G. & Sturm, R. A. (1995) *Oncogene* **11,** 691-700.

21. Das, G. & Herr, W. (1993) *J. Biol. Chem.* **268,** 25026-25032.

22. Tanaka, M. & Herr, W. (1990) *Cell* **60,** 375-386.

23. Tanaka, M., Grossniklaus, U., Herr, W., Hernandez, N. (1988) *Genes Dev.* **2,** 1764-1778.

24. Carey, M., Kakidani, H., Leatherwood, F., Mostashari, F. & Ptashne, M. (1989) *J. Mol. Biol.* **209,** 423-432.

25. Schreiber, E., Matthias, P., Mueller, M. M. & Schaffner, W. (1989) *Nucleic Acids Res.* **17,** 6419.

26. Cleary, M. A., Stern, S., Tanaka, M. & Herr, W. (1993) *Genes Dev.* **7,** 72-83.

27. Harlow, E. & Lane, D. (1988) *Antibodies: A laboratory manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

28. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A laboratory manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.).

29. Field, J., Nikawa, J., Broek, D., MacDonald, B., Rodgers, L., Wilson, I. A., Lerner, R. A. & Wigler, M. (1988) *Mol. Cell. Biol.* **8,** 2159-2165.

30. Lai, J.S., Cleary, M.A., Herr, W. (1992) *Genes Dev.* **6,** 2058-2065.

31. Descombes, P. & Schibler, U. (1991) *Cell* **67,** 569-579.

FIGURES

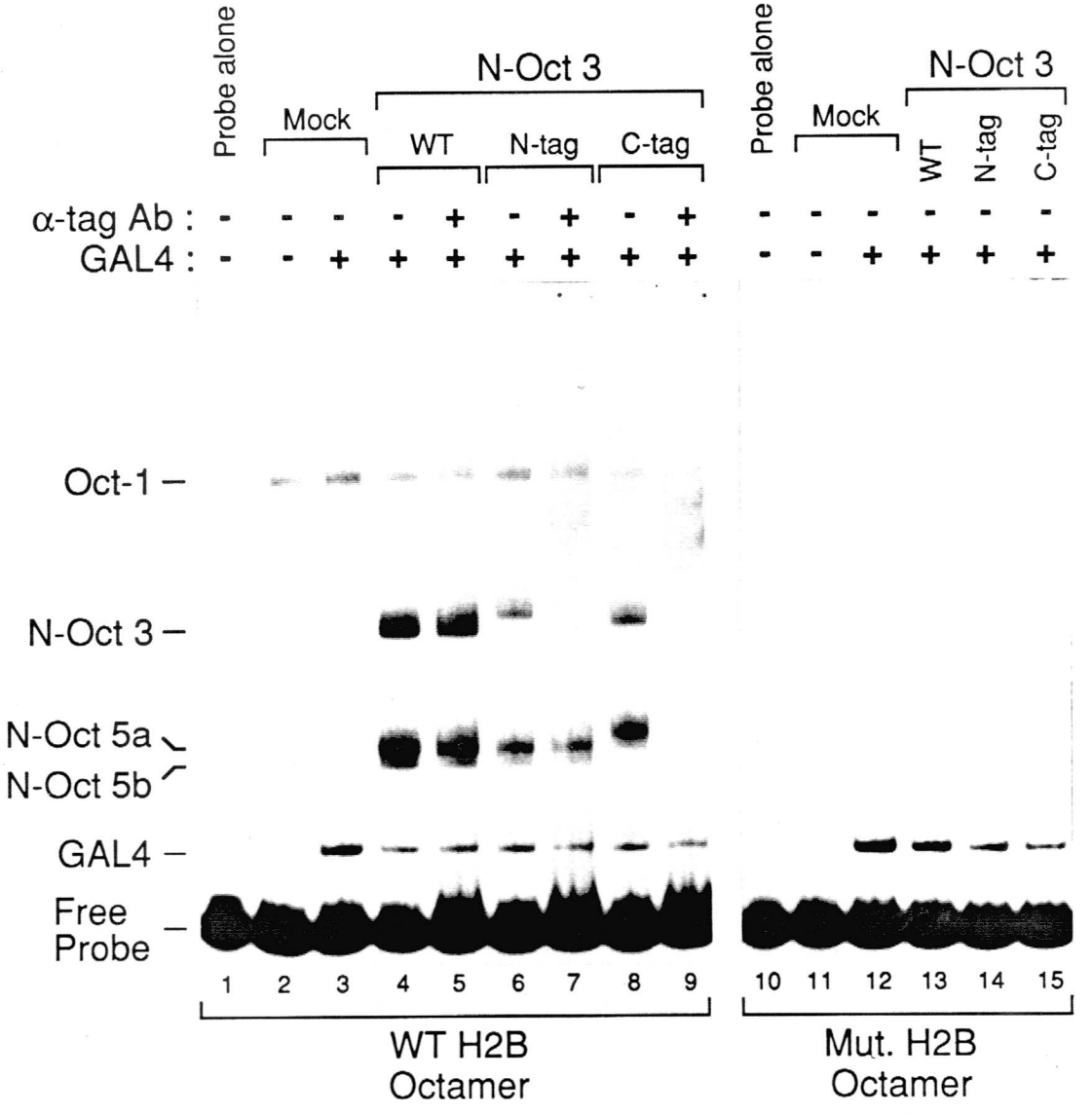

Probe alone
Mock
N-Oct 3
WT
N-tag
C-tag
Probe alone
Mock
N-Oct 3
WT
N-tag
C-tag
α-tag Ab : - - - - + - + - +
GAL4 : - - + + + + + + +
- - - - - - - - -
- - + + + + + + +
Oct-1
N-Oct 3
N-Oct 5a
N-Oct 5b
GAL4
Free Probe
1 2 3 4 5 6 7 8 9
10 11 12 13 14 15
WT H2B Octamer
Mut. H2B Octamer

Figure 1

N-Oct 5 proteins are derived from N-Oct 3. Electrophoretic mobility retardation analysis of transiently expressed wild-type and epitope-tagged forms of N-Oct 3/N-Oct 5 on a wild-type (lanes 1-9) and mutant (lanes 10-15) histone H2B octamer site. Extracts were derived from COS cells transfected with pUC119 DNA (Mock, lanes 2, 3, 11, 12), a construct expressing wild-type N-Oct 3 (lanes 4, 5, 13), or a construct expressing N-Oct 3 tagged with an influenza virus HA epitope at either the amino terminus (N-tag, lanes 6, 7, 14) or the carboxyl terminus (C-tag, lanes 8, 9, 15). Extracts in lanes 5, 7, and 9 were incubated with the anti-epitope-tag monoclonal antibody 12CA5, which recognizes the HA epitope (29). Where indicated, a GAL4 DNA-binding domain (residues 1-94) expression construct was transfected along with the N-Oct 3 constructs as an internal transfection control. The positions of the endogenously expressed Oct-1, the ectopically expressed N-Oct and GAL4 factors, and the free probe are indicated to the left of the figure.

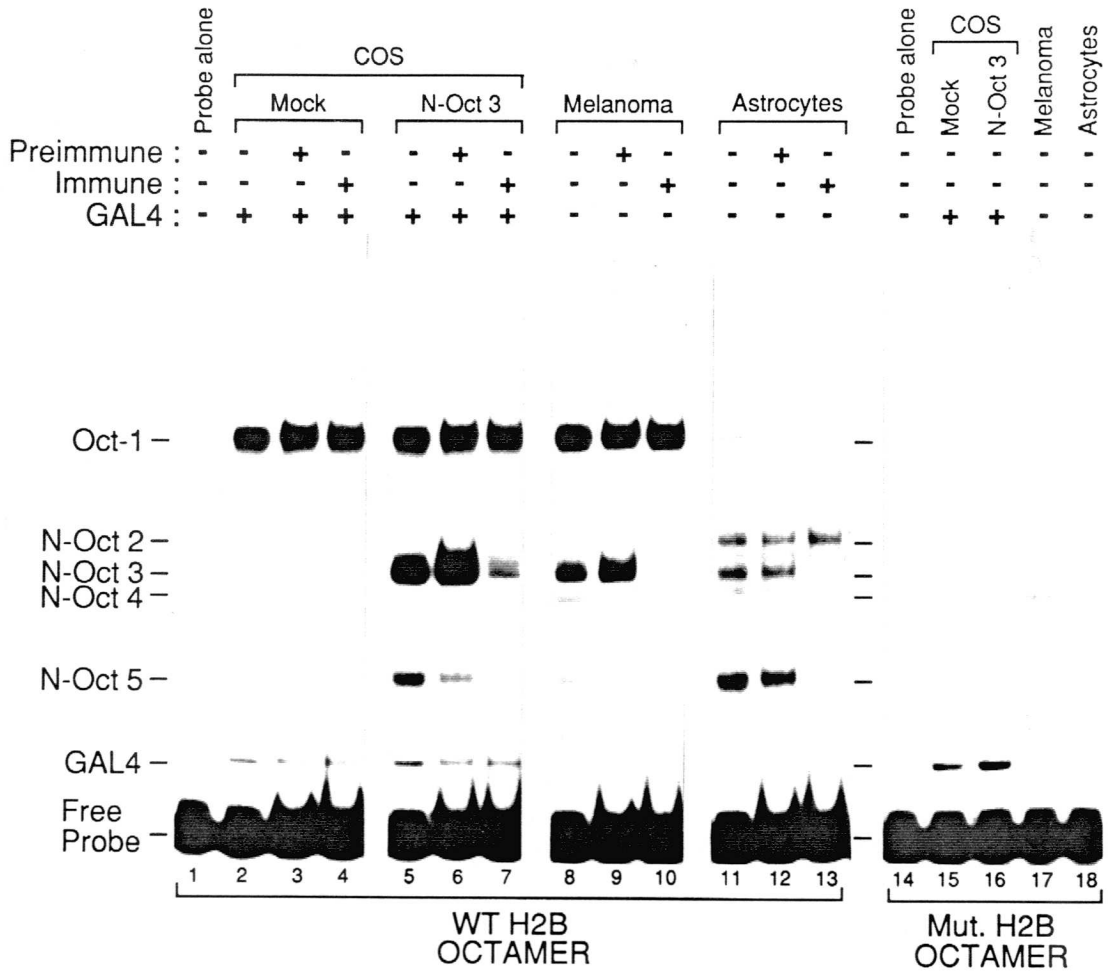
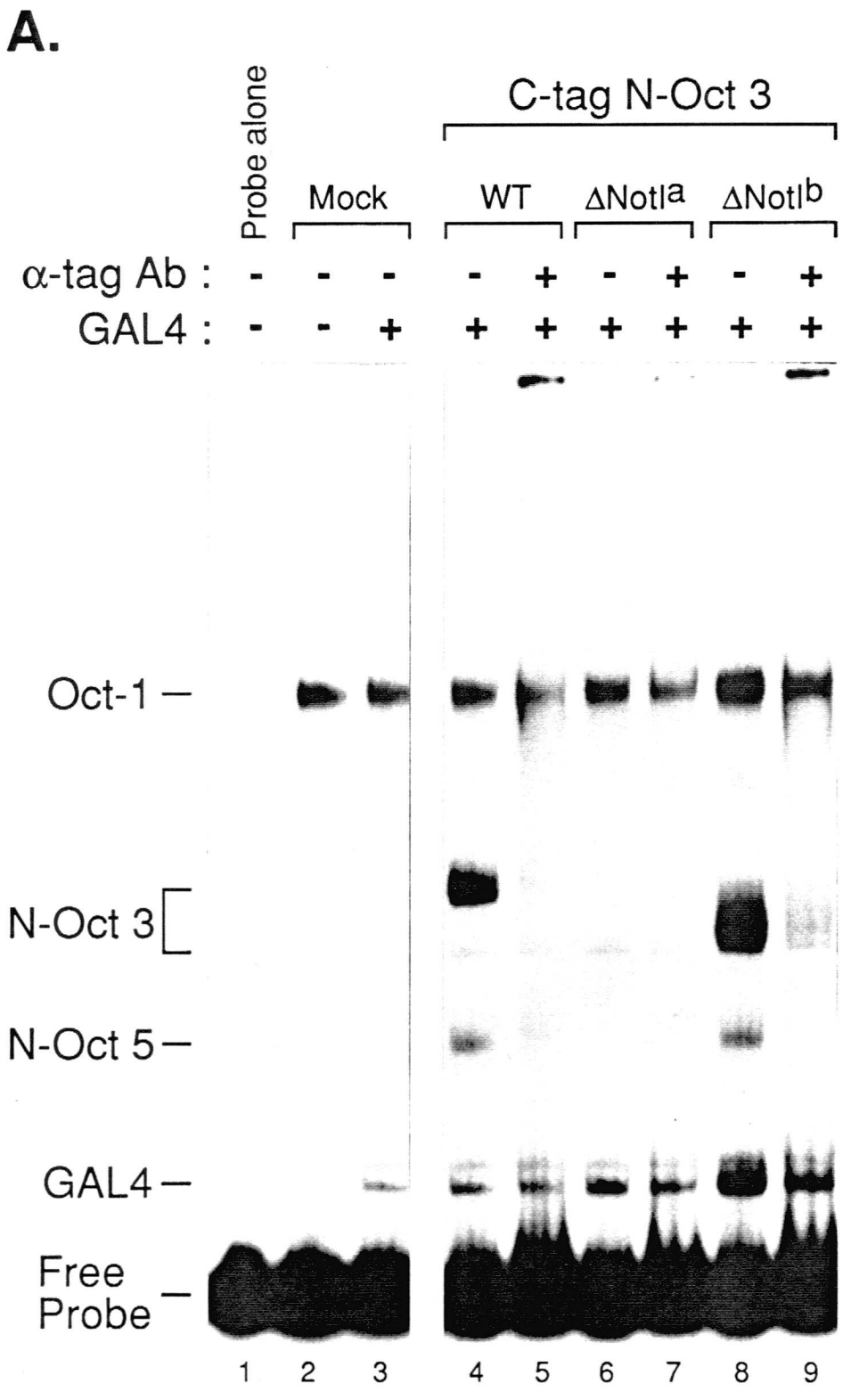

Probe alone
COS
Mock
N-Oct 3
Melanoma
Astrocytes
Probe alone
COS
Mock
N-Oct 3
Melanoma
Astrocytes
Preimmune :
Immune :
GAL4 :
Oct-1
N-Oct 2
N-Oct 3
N-Oct 4
N-Oct 5
GAL4
Free Probe
1 2 3 4 5 6 7 8 9 10 11 12 13
14 15 16 17 18
WT H2B OCTAMER
Mut. H2B OCTAMER

Figure 2

N-Oct 5 proteins in melanoma and astrocytes are also related to N-Oct 3. Nuclear extracts from A2058 melanoma cells (lanes 8, 9, 10 and 17), mouse primary astrocytes (lanes 11, 12, 13 and 18), and COS cells transfected with pUC119 DNA (lanes 2, 3, 4 and 15) or an N-Oct 3 expression vector (lanes 5, 6, 7 and 16) were analyzed by electrophoretic mobility retardation analysis with either a wild-type (lanes 1-13) or mutant H2B octamer site-containing probe (lanes 14-18). Reactions were incubated in the presence (+) or the absence (-) of either pre-immune serum or polyclonal antiserum to N-Oct 3 as indicated at the top of each lane. Abbreviations are as described in Fig. 1.


A.
Probe alone
C-tag N-Oct 3
Mock
WT
ΔNotIa
ΔNotIb
α-tag Ab : - - - - + - + - +
GAL4 : - - + + + + + + +
Oct-1
N-Oct 3
N-Oct 5
GAL4
Free Probe
1 2 3 4 5 6 7 8 9

B.

C-tag N-Oct 3

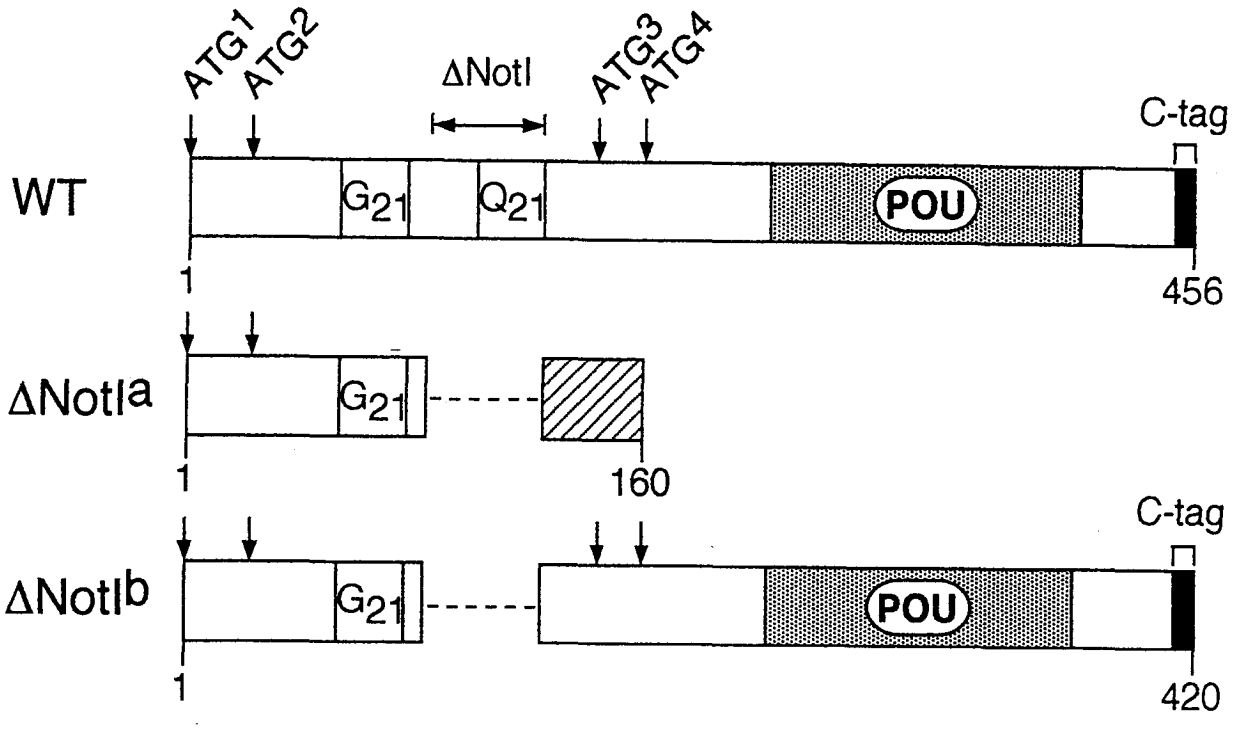

ATG1
ATG2
ΔNotI
ATG3
ATG4
C-tag
WT
G21
Q21
POU
1
456
ΔNotIa
G21
1
160
C-tag
ΔNotIb
G21
POU
1
420

Figure 3

Translation of N-Oct 5 does not initiate at internal translation start sites in the *N-Oct 3* mRNA. (*A*) Electrophoretic mobility retardation analysis of extracts from COS cells transfected with C-tag N-Oct 3 (lanes 4 and 5) or C-tag N-Oct 3 carrying a NotI deletion that either disrupts the expression of C-tag N-Oct 3 (C-tag N-Oct 3 ΔNotI^a, lanes 6 and 7) or shortens the C-tag N-Oct 3 product (C-tag N-Oct 3 ΔNotI^b, lanes 8 and 9). The fragment spanning the NotI deletion is shown in Fig. 3B. Extracts in lanes 5, 7 and 9 were incubated with anti-epitope-tag monoclonal antibody (12CA5). Mock lanes and abbreviations are as described in Fig. 1. (*B*) Schematic drawing of N-Oct 3, showing the POU-domain in the C-terminal region and the stretch of 21 glycines (G_{21}) and 21 glutamines (Q_{21}) in the N-terminal part of the protein. The locations of the N-Oct 3 initiating AUG codon (AUG^1) and the internal AUG codons (AUG^2, AUG^3 and AUG^4) as well as the region of the NotI deletion (ΔNotI) are indicated. (WT) Wild type. (ΔNotI^a) NotI-deletion construct that causes a translational frame shift. (ΔNotI^b) NotI-deletion construct that shortens the N-Oct 3 product but does not cause a translational frame shift.

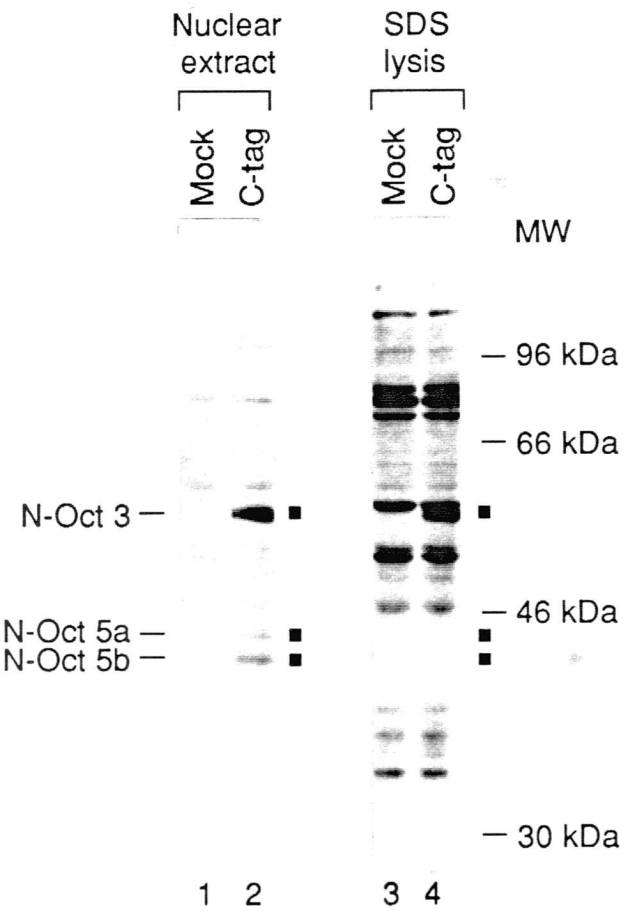

Nuclear extract
SDS lysis
Mock
C-tag
Mock
C-tag
MW
— 96 kDa
— 66 kDa
N-Oct 3 —
— 46 kDa
N-Oct 5a —
N-Oct 5b —
— 30 kDa
1 2 3 4

Figure 4

N-Oct 5a and N-Oct 5b proteins are not found in vivo. Proteins from either nuclear extracts (lanes 1 and 2) or SDS-lysed COS cells (lanes 3 and 4) transfected with pUC119 DNA (lanes 1 and 3) or a C-tag N-Oct 3 expression construct (lanes 2 and 4) were separated on a 10% SDS polyacrylamide gel and immunoblotted with the 12CA5 monoclonal antibody which recognizes the HA epitope of the C-tag N-Oct 3. Molecular weight standards (Amersham, Rainbow^{TM} colored protein high molecular weight marker) are shown to the right of the figure. The closed squares identify the N-Oct 3 and N-Oct 5a/5b pattern similar to that detected in electrophoretic mobility retardation analysis shown in Fig 1.

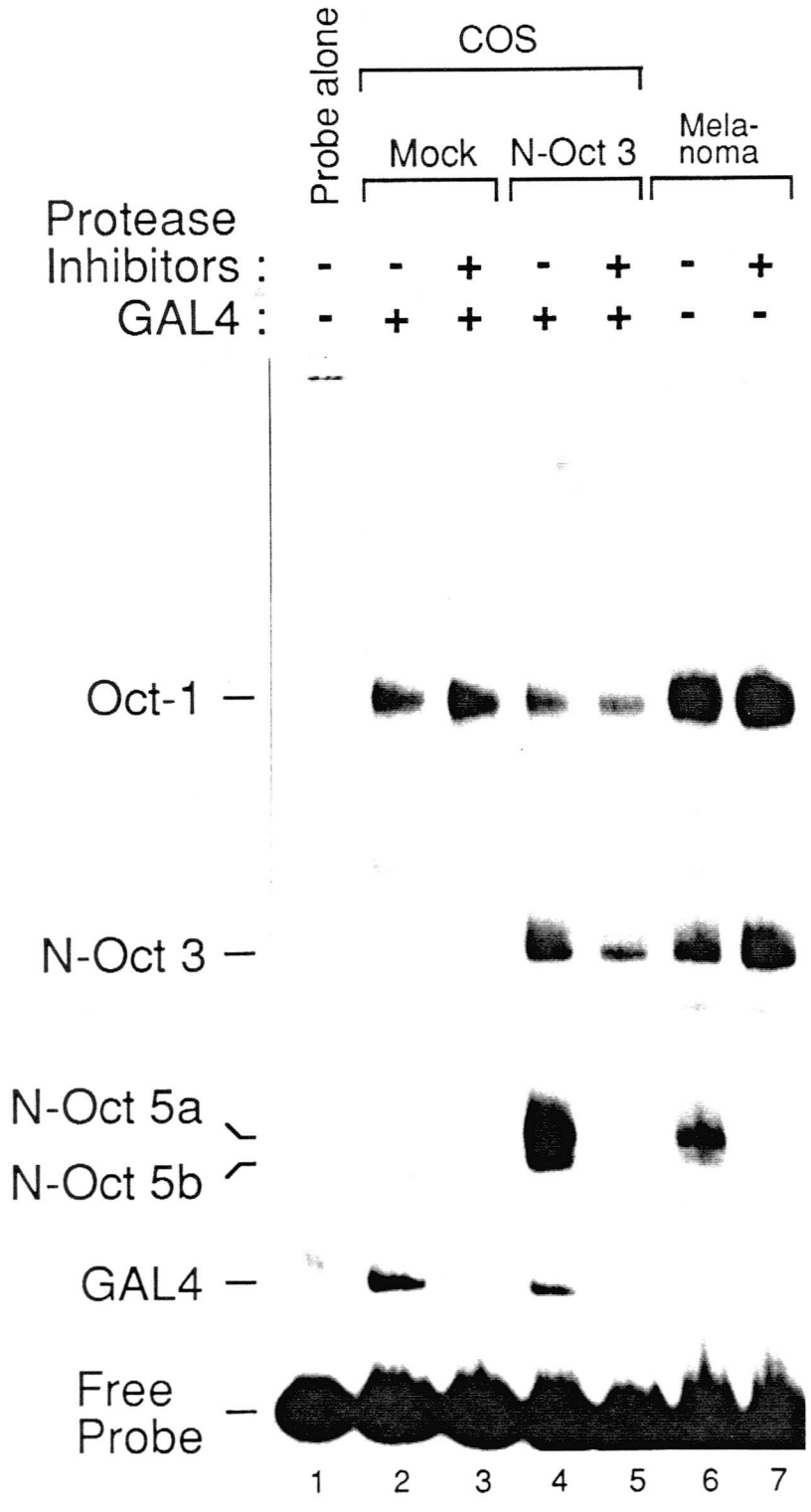

Probe alone
COS
Mock
N-Oct 3
Mela-noma
Protease Inhibitors :
GAL4 :
Oct-1
N-Oct 3
N-Oct 5a
N-Oct 5b
GAL4
Free Probe
1 2 3 4 5 6 7

Figure 5

A cocktail of protease inhibitors containing aprotinin, leupeptin, pepstatin, and the PMSF-like serine protease-inhibitor AEBSF prevent proteolytic cleavage of N-Oct 3 to form N-Oct 5. Nuclear extracts from A2058 melanoma cells (lanes 6 and 7) and COS cells transfected with pUC119 DNA (lanes 2 and 3) or the N-Oct 3 expression vector (lanes 4 and 5) were incubated with an H2B octamer probe. Extracts were prepared in presence (+) or absence (-) of the protease inhibitors aprotinin, leupeptin, pepstatin, and AEBSF. Abbreviations are as described in Fig. 1.

4.3 The brain-specific POU-box gene Brn4 is a sex-linked transcriptions factor on the human and mouse X Chromosomes

P. J. Douville, S. Atanasoski, A. Tobler, A. Fontana, M. E. Schwab (1994). Mammalian Genome **5**, 180-182

Mammalian Genome 5, 180–182 (1994).

Mammalian Genome

The brain-specific POU-box gene *Brn4* is a sex-linked transcription factor located on the human and mouse X Chromosomes

P.J. Douville,[1,*] S. Atanasoski,[2] A. Tobler,[2] A. Fontana,[2] M.E. Schwab[1]

[1]Brain Research Institute, University of Zurich, August-Forel-Strasse 1, CH-8029 Zurich, Switzerland
[2]Division of Clinical Immunology, Department of Internal Medicine, University Hospital Zurich, Häldiweg 4, CH-8044 Zurich, Switzerland

Received: 18 August 1993 / Accepted: 17 November 1993

POU-box genes encode homeodomain-containing proteins belonging to a multigene family of transcription factors. They have been implicated in determining cell lineage during development and are characterized by the POU domain, which comprises two DNA-binding subdomains, the POU-specific and POU-homeodomain and a less conserved linker region (Herr et al. 1988; He et al. 1989). Both subdomains contain helix-turn-helix motifs originally identified in prokaryotic transcriptional regulators such as the lambda repressor (Hoschschild et al. 1983). The family has been divided into six classes according to sequence homology within the POU domain (Rosenfeld 1992; Okamoto et al. 1993).

Brn4, a class III member, is exclusively expressed in the developing and adult nervous system and shares particularly high homology in the POU domain with other class III members (Hara et al. 1992; Mathis et al. 1992; Le Moine and Young 1992). Bandshift experiments have demonstrated that Brn4 is capable of binding the so-called octamer motif (ATGCAAAT) found in many cellular and viral promoters and enhancers (Schaffner 1989) and to transactivate octamer-containing promoters (Mathis et al. 1992). Although the target genes of Brn4 have not been identified, it might be expected to transcriptionally regulate some target genes containing the octamer or related motifs. Intriguingly, *Brn4* is intronless as are all other class III POU factors found to date (Hara et al. 1992). It has been postulated that this class of POU factors arose by an ancestral retrotransposition and later gene duplication, which occurred before humans and rodents diverged, to generate the other class III family members (Kuhn et al. 1991; Hara et al. 1992). Despite 80 million years of separate evolution, amino acid sequence identity has been strictly conserved between mouse and human class III homologs. This suggests that little mutational variation was allowed in this gene, underscoring its functional importance and cell type-specific expression in the developing and adult central nervous systems. Thus, one might expect genetic abnormalities within this gene to have major repercussions in the nervous system, perhaps because of alterations of normal development or disruption of cell identity, as has been found with some other POU genes, for example, *Pit-1* and *unc86* (Li et al. 1990; Pfäffle et al. 1992; Radovick et al. 1992; Finney et al 1988). We therefore undertook to genetically map the *Brn4* locus in the mouse and to determine its chromosomal localization in the human to see if it were linked with previously described mouse mutants or human hereditary diseases.

To map the mouse *Brn4* gene, we used a mouse interspecific backcross DNA panel derived from (C57BL/6J × *Mus spretus*)F_1 × C57BL/6J (E. Birkenmeier and L. Rowe, The Jackson Laboratory, Bar Harbor, Me.). We began by looking for polymorphisms in the *Brn4* gene in both parental species of mice (C57BL/6J and *Mus spretus*). Polymerase chain reaction (PCR) amplification of *Brn4* from both species yielded an expected 970-bp product encompassing almost the entire coding sequence. No other PCR products of alternate size were detected, confirming that *Brn4* is an intronless gene in both species of mice. These results forced us to screen for restriction fragment length polymorphisms (RFLPs) rather than length polymorphisms. We detected an informative RFLP after digestion with *Ddel* (Fig. 1A). This polymorphism was due to an additional *Ddel* site in the *Mus spretus Brn4* allele which is also present in a rat Brn4 cDNA (Le Moine and Young 1992). In the rat sequence this *Ddel* site is found at the 3′ end of the linker and does not result in an amino acid substitution. Given the phylogenetic conservation of Brn4, it is unlikely that the *Mus spretus Ddel* polymorphism results in an amino acid substitution.

**Present address:* Institute for Molecular Biology II, University of Zurich, Winterthurerstr. 190, CH-8057 Zurich, Switzerland

Correspondence to: P.J. Douville

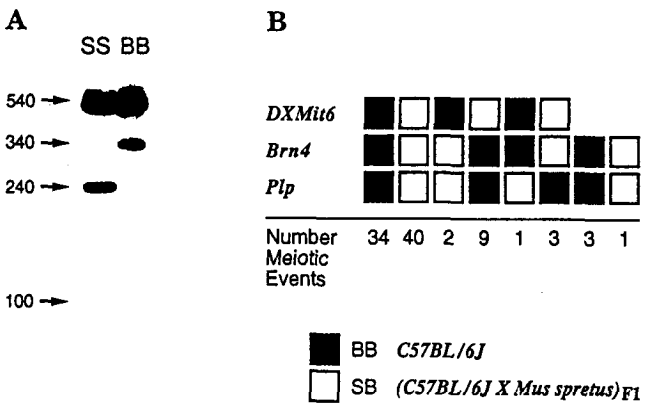


Fig. 1. (A) Southern blot identification of a *Ddel* RFLP in *Brn4* alleles PCR amplified from *C57BL/6J* (BB) and *Mus spretus* (SS). For PCR amplification of *Brn4* alleles from mouse DNA, we used an oligonucleotide primer (5′-CCTTTCCGCAATCCTCAG-3′) derived from the 5′ end of the mouse Brn4 coding region and an antisense primer (5′-TGTTTTCACCGTGTGCGA-3′) from the 3′ end of the coding region. PCR amplification was carried out from 50 ng of genomic DNA in 10 mM Tris pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTPs, and 0.65 U AmpliTaq (Perkin-Elmer Corp., NJ., USA) under the following cycling conditions: 1 min denaturation at 94°C, 1 min annealing at 54°C and 2 min extension at 72°C for 35 cycles. The amplification products were digested with the restriction enzyme *Ddel* and analyzed by electrophoresis in 10% poly(NAT) gels (Elchrom AG, Horgen, Switzerland) for species-specific typing. The sizes in base pairs for each band are indicated on the side. (B) Haplotype analysis of 93 backcross progeny mice. The genotypes of individual backcross mice are represented by the filled (BB) and open (SB) boxes. The numbers of mice with each haplotype are indicated underneath the boxes. RFLPs for *DXMit6* and *Plp* were defined previously.

The mouse interspecific backcross panel was screened for the *Ddel* RFLP and the allele distribution was compared to that from previously typed markers. Each of the *Ddel*-digested DNAs from the (C57BL/6J × *Mus spretus*)F_1 × C57BL/6J interspecific backcross mice displayed either the homozygous (BB) or the heterozygous (SB) F_1 pattern. The allele distribution of *Brn4* in the backcross mouse panel most closely resembled that of the proteolipoprotein gene *Plp* (Eicher et al. 1992; Willard and Riordan 1985). In 89 out of 93 meiotic events examined, *Brn4* cosegregated with the *Plp* locus (Fig. 1B), indicating that the mouse *Brn4* locus is linked to *Plp* on the X Chromosome (Chr). *Brn4* also showed linkage to the DNA marker *DXMit6* (Dietrich et al. 1992; E. Birkenmeier and L. Rowe, unpublished observations) with the two loci cosegregating in 78 out of 89 meiotic events examined (Fig. 1B). The best gene order (Bishop, 1985) ± the standard error (Green, 1981) indicated that *Brn4* was 4.3 ± 2.1 cM (95% confidence limits are 1.2 and 10.6 cM) proximal to *Plp* and 12.4 ± 3.5 cM (95% confidence limits are 6.3 and 21.0 cM) distal to *DXMit6*. We consider it unlikely that this *Brn4* locus is a pseudogene because Southern blots of mouse genomic DNA strongly suggest that *Brn4* is a single-copy gene (Hara et al. 1992).

Brn4 is the first transcription factor from the POU family to display sex linkage, and mutations at this locus would be expected to be phenotypically expressed in males. Our mouse linkage data indicate that *Brn4* does not map to regions where previously described mouse mutants have been mapped. Given its widespread CNS distribution, it is possible that mutations at this locus are lethal. More trivially, it may be that mouse mutants of this locus have not yet been described.

To localize the human BRN4 gene, we used a ^{32}P-labeled 215-bp restriction fragment from a human Brn4 cDNA clone which comprised the 5′ end of the clone ending at nucleotide 750 according to the mouse DNA sequence numbering (Hara et al. 1992). This probe was hybridized to a DNA panel from human × hamster somatic cell lines (BIOS Laboratories, New Haven, Conn.). We detected a unique human-specific Brn4 band in three *Eco*R1-digested hybrid cell line DNA samples that corresponded to those harboring the human X Chr (Table 1). We did not detect a human-specific band in cell lines that did not contain the X Chr. Our level of sensitivity was probably not sufficient to detect the human-specific band in one cell line (968) in which only 65% of the cells contain the human X Chr. From these data we concluded that the human BRN4 locus was located on the X Chr. It is unlikely that we detected another class III POU gene because our human 5′ Brn4 probe did not include the highly conserved POU-domain. The fact that we detected a uniquely hybridizing human band and localization to a single chromosome strongly implies that BRN4 is a single-copy gene in human.

The location of the human BRN4 gene on the X Chr is not surprising given the very conserved nature of the mouse and human X Chrs (Amar et al. 1988; Davisson et al. 1991; O'Brien and Marshall Graves 1991). In the mouse it is found in a linkage group that includes the phosphoglycerate kinase 1 locus (*Pgk1*) and the *Plp* locus that is syntenic with the human X Chr (Amar et al. 1988). On the human long arm of the X Chr these loci define the region spanned from Xq13.3 to Xq21.3-q22 (Amar et al. 1988; Willard and Riordan 1985), and the gene order in the mouse strongly suggests that the human BRN4 locus lies within this region.

Numerous disease loci have been assigned to the middle of the long arm of the human X Chr by linkage analysis. Among these, however, only the phenotype of X-linked spastic paraplegia at Xq21-q22 (Keppen et al. 1987) is restricted to the CNS, the site of expression of Brn4. This disorder is characterized mostly by spastic gait and hyperflexia. Another interesting disorder that also maps to Xq21.3-q22 is a form of hypopituitarism, X-linked pituitary dwarfism IV (Phelan et al. 1971; Schimke et al. 1971), which is associated with growth hormone deficiency (Conley et al. 1991). Interestingly, an autosomal form of hypopituitarism with a dwarf phenotype in mice and humans has been described that is caused by mutations in the POU-box gene *Pit-1* (Li et al. 1990; Pfäffle et al. 1992; Radovick et al. 1992). Phenotypically, the autosomal form of hypopituitarism is characterized by a growth hormone deficiency whose gene contains Pit-1 recognition motifs to which Brn-4 can also bind (Mathis et al. 1992). This interesting correlation may implicate Brn-4 as a potential candidate gene involved in the pathogenesis of pituitary dwarfism IV. However, at least some affected patients show a complicated form of hypopituitarism with a concomitant X-linked agammaglobulinemia (XLA). The gene responsible for XLA has recently been identified as a member of the *src* family of tyrosine kinases (Vetrie et al. 1993), and the mouse homolog (*btk*) maps close to *Brn4* on the mouse X Chr (Thomas et al. 1993). Since on the one

Table 1. Segregation of human BRN4 in human/rodent hybrids.

Hybridization/ Chromosome	Human Chromosome 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y
+/+	0	0	0	1	3	2	0	3	0	0	1	0	0	2	0	0	0	0	0	0	0	1	3	0
−/−	29	32	27	31	4	29	30	28	29	29	32	27	24	23	28	29	31	27	21	29	23	27	32	28
+/−	3	3	3	2	0	1	3	0	3	3	2	3	3	1	3	3	3	3	3	3	3	2	0	3
−/+	4	1	6	2	29	4	3	5	4	4	1	6	9	10	5	4	2	6	12	4	10	6	1	5
% Discordance	19	11	25	11	80	14	17	14	19	19	8	25	33	31	22	19	14	25	42	19	36	22	3	22

Note: (+) or (−) indicates the presence or absence of the human BRN4 fragment in relation to the presence (+) or absence (−) of a particular human chromosome.

hand Brn-4 is not expressed in tissues of the immune system, and on the other hand panhypopituitarism does not necessarily lead to XLA, it is possible that this complicated form of hypopituitarism may represent a syndrome involving several genes in close proximity. Nevertheless, it will be interesting to determine whether mutations of the human BRN4 gene are associated with one of these disorders.

Acknowledgments. We thank Edward Birkenmeier and Lucy Rowe for helpful discussions and analysis of our mouse linkage data (NIH Biomedical Research Support Grant 2S07RR056545, and General Research Support Funds of The Jackson Laboratory). We also thank Oliver Clay for expert computer assistance and Fritz Ochsenbein for graphical assistance. We are indebted to Walter Schaffner and Koichi Matsuo for critical reading of the manuscript. This work was supported by a grant from the Swiss National Science Foundation (31-29981,90) to M.E. Schwab. P.J. Douville was supported by a fellowship from the Medical Research Council of Canada.

References

Amar, L.C., Dandolo, L., Hanauer, A., Ryder Cook, A., Arnaud, D., Mandel, J.-L., Avner, P. (1988). Conservation and reorganization of loci on the mammalian X chromosome: a molecular framework for the identification of homologous subchromosomal regions in man and mouse. Genomics 2, 220–230.

Bishop, D.T. (1985). The information content of phase-known matings for ordering genetic loci. Genet. Epidemiol. 2, 349–361.

Conley, M.E., Burks, A.W., Herrod, H.G., Puck, J.M. (1991). Molecular analysis of X-linked agammaglobulinemia with growth hormone deficiency. J. Pediatr. 119, 392–397.

Davisson, M.T., Lalley, P.A., Peters, J., Doolittle, D.P., Hillyard, A.L., Searle, A.G. (1991). Report of the comparative committee for human, mouse and other rodents. Cytogenet. Cell Genet. 58, 1152–1189.

Dietrich, W., Katz, H., Lincoln, S.E., Shin, H.S., Friedman, J., Dracopoli, N.C., Lander, E.S. (1992). A genetic map of the mouse suitable for typing intraspecific crosses. Genetics 131, 423–447.

Eicher, E.M., Lee, B.K., Washburn, L.L., Hale, D.W., King, T.R. (1992). Telomere-related markers for the pseudoautosomal region of the mouse genome. Proc. Natl. Acad. Sci. USA 89, 2160–2164.

Finney, M., Ruvkun, G., Horvitz, R.H. (1988). The *C. elegans* cell lineage and differentiation gene *unc-86* encodes a protein with a homeodomain and extended similarity to transcription factors. Cell 55, 757–769.

Green, E.L. (1981). Linkage recombination and mapping. In *Genetics and Probability in Animal Breeding Experiments,* E. Green, ed. (New York: MacMillan), pp. 77–113.

Hara, Y., Rovescalli, A.C., Kim, Y., Nirenberg, M. (1992). Structure and evolution of four POU domain genes expressed in mouse brain. Proc. Natl. Acad. Sci. USA 89, 3280–3284.

He, X., Treacy, M.N., Simmons, D.M., Ingraham, H.A., Swanson, L.W., Rosenfeld, M.G. (1989). Expression of a large family of POU-domain regulatory genes in mammalian brain development. Nature 340, 35–42.

Herr, W., Sturm, R.A., Clerc, R.G., Corcoran, L.M., Baltimore, D., Sharp, P.A., Ingraham, H.A., Rosenfeld, M.G., Finney, M., Ruvkun, G., Horvitz, H.R. (1988). The POU domain: a large conserved region in the mammalian *pit-1, oct-1, oct-2* and *Caenorhabditis elegans unc-86* gene products. Genes Dev. 2, 1513–1516.

Hochschild, A., Irwin, N., Ptashne, M. (1983). Repressor structure and the mechanism of positive control. Cell 32, 319–325.

Keppen, L.D., Leppert, M.F., O'Connell, P., Nakamura, Y., Stauffer, D., Lathrop, M., Lalovel, J.-M., White, R. (1987). Etiological heterogeneity in X-linked spastic paraplegia. Am. J. Hum. Genet. 41, 933–943.

Kuhn, R., Monuki, E.S., Lemke, G. (1991). The gene encoding the transcription factor SCIP has features of an expressed retroposon. Mol. Cell. Biol. 11, 4642–4650.

Le Moine, C., Young, W.S. III (1992). RHS2, a POU domain-containing gene, and its expression in developing and adult rat. Proc. Natl. Acad. Sci. USA 89, 3285–3289.

Li, S., Crenshaw, B.E. III, Rawson, E.J., Simmons, D.M., Swanson, L.W., Rosenfeld, M.G. (1990). Dwarf locus mutants lacking three pituitary cell types result from mutations in the POU-domain gene Pit-1. Nature 347, 528–533.

Mathis, J.M., Simmons, D.M., He, X., Swanson, L.W., Rosenfeld, M.G. (1992). Brain 4: a novel mammalian POU domain transcription factor exhibiting restricted brain-specific expression. EMBO J. 11, 2551–2561.

O'Brien, S.J., Marshall Graves, J.A. (1991). Report of the committee on comparative gene mapping. Cytogenet. Cell Genet. 58, 1124–1151.

Okamoto, K., Wakamiya, M., Noji, S., Koyoma, E., Taniguchi, S., Takemura, R., Copeland, N.G., Gilbert, D.J., Jenkins, N.A., Muramatsu, M., Hamada, H. (1993). A novel class of murine POU gene predominantly expressed in central nervous system. J. Biol. Chem. 268, 7449–7457.

Pfäffle, R.W., DiMattia, G.E., Parks, J.S., Brown, M.R., Wit, J.M., Jansen, M., Van der Nat, H., Van den Brande, J.L., Rosenfeld, M.G., Ingraham, H.A. (1992). Mutation of the POU-specific domain of Pit-1 and hypopituitarism without pituitary hypoplasia. Science 257, 1118–1121.

Phelan, P.D., Connelly, J., Martin, F.I.R., Wetenhall, H.N. (1971). X-linked recessive hypopituitarism. Birth Defects VII(6), 24–27.

Radovick, S., Nations, M., Du, Y., Berg, L.A., Weintraub, B.D., Wondisford, F.E. (1992). A mutation in the POU-homeodomain of Pit-1 responsible for combined pituitary hormone deficiency. Science 257, 1115–1118.

Rosenfeld, M.G. (1992). POU-domain transcription factors: pou-er-ful developmental regulators. Genes Dev. 5, 897–907.

Schaffner, W. (1989). How do different transcription factors binding to the same DNA sequence sort out their jobs? Trends Genet. 5, 37–39.

Schimke, R.N., Spaulding, J.J., Hollowell, J.G. (1971). X-linked congenital panhypopituitarism. Birth Defects VII(6), 21–23.

Thomas, J.D., Sideras, P., Edvard Smith, C.I., Vorechovsky, I., Chapman, V., Paul, W.E. (1993). Colocalization of X-linked agammaglobulinemia and X-linked immunodeficiency genes. Science 261, 355–361.

Vetrie, D., Vorechovsky, I., Sideras, P., Holland, J., Davies, A., Flinter, F., Hammarström, L., Kinnon, C., Levinsky, R., Bobrow, M., Edvard Smith, C.I., Bentley, D.R. (1993). The gene involved in X-linked agammaglobulinaemia is a member of the *src* family of protein-tyrosine kinases. Nature 361, 226–233.

Willard, H.F., Riordan J.R. (1985). Assignment of the gene for myelin proteolipid protein to the X chromosome: implications for X-linked myelin disorders. Science 230, 940–942.

5. REFERENCES

1. Shepherd, G. M. (1994) *Neurobiology* (Oxford University Press, Oxford).

2. LeDouarin, N. M. (1982) *The neural crest* (Cambridge University Press, Cambridge, England).

3. Stemple, D. L. & Anderson, D. J. (1992) *Cell* **71,** 973-85.

4. McKay, R. D. (1989) *Cell* **58,** 815-21.

5. Sternberg, P. W. & Horvitz, H. R. (1984) *Annu Rev Genet* **18,** 489-524.

6. Bantle, J. A. & Hahn, W. E. (1976) *Cell* **8,** 139-50.

7. Sentenac, A. (1985) *CRC Crit Rev Biochem* **18,** 31-90.

8. Maniatis, T., Goodbourn, S. & Fischer, J. A. (1987) *Science* **236,** 1237-45.

9. Struhl, K. (1989) *Trends Biochem Sci* **14,** 137-40.

10. Miller, J., McLachlan, A. D. & Klug, A. (1985) *EMBO J.* **4,** 1609-14.

11. Kornberg, T. B. (1993) *J Biol Chem* **268,** 26813-6.

12. Abel, T. & Maniatis, T. (1989) *Nature* **341,** 24-5.

13. Gehring, W. J. (1987) *Science* **236,** 1245-52.

14. Ingham, P. W. (1988) *Nature* **335,** 25-34.

15. Akam, M. (1989) *Cell* **57,** 347-9.

16. Rosenfeld, M. G. (1991) *Genes Dev.* **5,** 897-907.

17. Finney, M., Ruvkun, G. & Horvitz, H. R. (1988) *Cell* **55,** 757-69.

18. Burglin, T. R., Finney, M., Coulson, A. & Ruvkun, G. (1989)

Nature **341**, 239-43.

19. Bodner, M., Castrillo, J. L., Theill, L. E., Deerinck, T., Ellisman, M. & Karin, M. (1988) *Cell* **55**, 505-18.

20. Ingraham, H. A., Chen, R. P., Mangalam, H. J., Elsholtz, H. P., Flynn, S. E., Lin, C. R., Simmons, D. M., Swanson, L. & Rosenfeld, M. G. (1988) *Cell* **55**, 519-29.

21. Simmons, D. M., Voss, J. W., Ingraham, H. A., Holloway, J. M., Broide, R. S., Rosenfeld, M. G. & Swanson, L. W. (1990) *Genes Dev.* **4**, 695-711.

22. Snell, G. D. (1929) *Proc. Natl. Acad. Sci. USA* **15**, 733-734.

23. Eicher, E. M. & Beamer, W. G. (1980) *J. Hered* **71**, 187-90.

24. Li, S., Crenshaw, E. B. d., Rawson, E. J., Simmons, D. M., Swanson, L. W. & Rosenfeld, M. G. (1990) *Nature* **347**, 528-33.

25. Roux, M., Bartke, A., Dumont, F. & Dubois, M. P. (1982) *Cell Tissue Res.* **223**, 415-20.

26. Pfaffle, R. W., DiMattia, G. E., Parks, J. S., Brown, M. R., Wit, J. M., Jansen, M., Van der Nat, H., Van den, T. I. B. J. L., Rosenfeld, M. G. & Ingraham, H. A. (1992) *Science* **257**, 1118-21.

27. Scheidereit, C., Cromlish, J. A., Gerster, T., Kawakami, K., Balmaceda, C. G., Currie, R. A. & Roeder, R. G. (1988) *Nature* **336**, 551-7.

28. Clerc, R. G., Corcoran, L. M., LeBowitz, J. H., Baltimore, D. & Sharp, P. A. (1988) *Genes Dev.* **2**, 1570-81.

29. Ko, H. S., Fast, P., McBride, W. & Staudt, L. M. (1988) *Cell* **55**, 135-44.

30. Monuki, E. S., Weinmaster, G., Kuhn, R. & Lemke, G. (1989) *Neuron* **3**, 783-93.

31. He, X., Treacy, M. N., Simmons, D. M., Ingraham, H. A., Swanson, L. W. & Rosenfeld, M. G. (1989) *Nature* **340**, 35-41.

32. Suzuki, N., Rohdewohld, H., Neuman, T., Gruss, P. & Scholer, H. R. (1990) *EMBO J.* **9,** 3723-32.

33. Tobler, A., Schreiber, E. & Fontana, A. (1993) *Nucleic Acids Res* **21,** 1043.

34. Faus, I., Hsu, H. J. & Fuchs, E. (1994) *Mol Cell Biol* **14,** 3263-75.

35. Hara, Y., Rovescalli, A. C., Kim, Y. & Nirenberg, M. (1992) *Proc Natl Acad Sci U S A* **89,** 3280-4.

36. Schreiber, E., Harshman, K., Kemler, I., Malipiero, U., Schaffner, W. & Fontana, A. (1990) *Nucleic Acids Res.* **18,** 5495-5503.

37. Schreiber, E., Tobler, A., Malipiero, U., Schaffner, W. & Fontana, A. (1993) *Nucleic Acids Res.* **21,** 253-258.

38. Thomas, K. R. & Capecchi, M. R. (1987) *Cell* **51,** 503-12.

39. Stoykova, A. S., Sterrer, S., Erselius, J. R., Hatzopoulos, A. K. & Gruss, P. (1992) *Neuron* **8,** 541-58.

40. Corcoran, L. M., Karvelas, M., Nossal, G. J., Ye, Z. S., Jacks, T. & Baltimore, D. (1993) *Genes Dev.* **7,** 570-82.

41. Fujii, H. & Hamada, H. (1993) *Neuron* **11,** 1197-206.

42. Thomson, J. A. F., Murphy, K., Baker, E., Sutherland, G. R., Parsons, P. G. & Sturm, R. A. (1995) *Oncogene* **11,** 691-700.

43. Sturm, R. A., Bisshop, F., Takahashi, H. & Parsons, P. G. (1991) *Cell Growth and Differ.* **2,** 519-524.

44. Schonemann, M. D., Ryan, A. K., McEvilly, R. J., O'Connell, S. M., Arias, C. A., Kalla, K. A., Li, P., Sawchenko, P. E. & Rosenfeld, M. G. (1995) *Genes Dev.* **9,** 3122-3135.

45. Nakai, S., Kawano, H., Yudate, T., Nishi, M., Kuno, J., Nagata, A., Jishage, K., Hamada, H., Fujii, H., Kawamura, K., Shiba, K. & Noda, T. (1995) *Genes Dev.* **9,** 3109-3121.

46. Mathis, J. M., Simmons, D. M., He, X., W., S. L. & Rosenfeld, M. G.

(1992) *EMBO J.***11,** 2551-2561.

47. Douville, P. J., Atanasoski, S., Tobler, A., Fontana, A. & Schwab, M. E. (1994) *Mamm Genome* **5,** 180-182.

48. de Kok, Y. J., van der Maarel, S. M., Bitner-Glindzicz, M., Huber, I., Monaco, A. P., Malcolm, S., Pembrey, M. E., Ropers, H. H. & Cremers, F. P. (1995) *Science* **267,** 685-8.

49. Sturm, R. A., Das, G. & Herr, W. (1988) *Genes Dev* **2,** 1582-99.

50. Andersen, B., Schonemann, M. D., Flynn, S. E., Pearse, R. V., Singh, H. & Rosenfeld, M. G. (1993) *Science* **260,** 78-82.

51. Billin, A. N., Cockerill, K. A. & Poole, S. J. (1991) *Mech Dev* **34,** 75-84.

52. Lloyd, A. & Sakonju, S. (1991) *Mech Dev* **36,** 87-102.

53. Atanasoski, S., Toldo, S. S., Malipiero, U., Schreiber, E., Fries, R. & Fontana, A. (1995) *Genomics* **26,** 272-80.

54. Le Moine, C. & Young, W. S. d. (1992) *Proc Natl Acad Sci U S A* **89,** 3285-9.

55. Johnson, W. A. & Hirsh, J. (1990) *Nature* **343,** 467-70.

56. Anderson, M. G., Perkins, G. L., Chittick, P., Shrigley, R. J. & Johnson, W. A. (1995) *Genes Dev* **9,** 123-37.

57. Agarwal, V. R. & Sato, S. M. (1991) *Dev Biol* **147,** 363-73.

58. Matsuzaki, T., Amanuma, H. & Takeda, H. (1992) *Biochem Biophys Res Commun* **187,** 1446-53.

59. Collum, R. G., Fisher, P. E., Datta, M., Mellis, S., Thiele, C., Huebner, K., Croce, C. M., Israel, M. A., Theil, T., Moroy, T. & et al. (1992) *Nucleic Acids Res* **20,** 4919-25.

60. Gerrero, M. R., Mcevilly, R. J., Turner, E., Lin, C. R., Oconnell, S., Jenne, K. J., Hobbs, M. V. & Rosenfeld, M. G. (1993) *Proc Natl Acad Sci USA* **90,** 10841-10845.

61. Theil, T., Mcleanhunter, S., Zornig, M. & Moroy, T. (1993) *Nucleic Acids Res* **21,** 5921-5929.

62. Lillycrop, K. A., Budrahan, V. S., Lakin, N. D., Terrenghi, G., Wood, J. N., Polak, J. M. & Latchman, D. S. (1992) *Nucleic Acids Res* **20,** 5093-6.

63. Ninkina, N. N., Stevens, G. E. M., Wood, J. N. & Richardson, W. D. (1993) *Nucleic Acids Res* **21,** 3175-3182.

64. Turner, E. E., Jenne, K. J. & Rosenfeld, M. G. (1994) *Neuron* **12,** 205-218.

65. Xiang, M. Q., Zhou, L. J., Peng, Y. W., Eddy, R. L., Shows, T. B. & Nathans, J. (1993) *Neuron* **11,** 689-701.

66. Theil, T., Rodel, B., Spiegelhalter, F. & Moroy, T. (1995) *J Biol Chem* **270,** 30958-64.

67. Treacy, M. N., Neilson, L. I., Turner, E. E., He, X. & Rosenfeld, M. G. (1992) *Cell* **68,** 491-505.

68. Okamoto, K., Okazawa, H., Okuda, A., Sakai, M., Muramatsu, M. & Hamada, H. (1990) *Cell* **60,** 461-72.

69. Rosner, M. H., Vigano, M. A., Ozato, K., Timmons, P. M., Poirier, F., Rigby, P. W. & Staudt, L. M. (1990) *Nature* **345,** 686-92.

70. Scholer, H. R., Ruppert, S., Suzuki, N., Chowdhury, K. & Gruss, P. (1990) *Nature* **344,** 435-9.

71. Hinkley, C. S., Martin, J. F., Leibham, D. & Perry, M. (1992) *Mol Cell Biol* **12,** 638-49.

72. Andersen, B., Schonemann, M. D., Pearse, R. V., Jenne, K., Sugarman, J. & Rosenfeld, M. G. (1993) *J Biol Chem* **268,** 23390-23398.

73. Greenstein, D., Hird, S., Plasterk, R. H., Andachi, Y., Kohara, Y., Wang, B., Finney, M. & Ruvkun, G. (1994) *Genes Dev* **8,** 1935-48.

74. Grosschedl, R. & Birnstiel, M. L. (1980) *Proc Natl Acad Sci U S A* **77**, 7102-6.

75. Muller, M. M., Gerster, T. & Schaffner, W. (1988) *Eur J Biochem* **176**, 485-95.

76. Zawel, L. & Reinberg, D. (1992) *Curr Opin Cell Biol* **4**, 488-95.

77. Buratowski, S. (1994) *Cell* **77**, 1-3.

78. Harrison, S. C. (1991) *Nature* **353**, 715-9.

79. Johnson, P. F. & McKnight, S. L. (1989) *Ann Rev Biochem* **58**, 799-839.

80. Mitchell, P. J. & Tjian, R. (1989) *Science* **245**, 371-8.

81. Ptashne, M. (1988) *Nature* **335**, 683-9.

82. Herr, W. & Cleary, M. A. (1995) *Genes Dev.* **9**, 1679-1693.

83. Herr, W., Sturm, R. A., Clerc, R. G., Corcoran, L. M., Baltimore, D., Sharp, P. A., Ingraham, H. A., Rosenfeld, M. G., Finney, M., Ruvkun, G. & et al. (1988) *Genes Dev* **2**, 1513-6.

84. Assa-Munt, N., Mortishiresmith, R. J., Aurora, R., Herr, W. & Wright, P. E. (1993) *Cell* **73**, 193-205.

85. Aurora, R. & Herr, W. (1992) *Mol Cell Biol* **12**, 455-467.

86. Ingraham, H. A., Flynn, S. E., Voss, J. W., Albert, V. R., Kapiloff, M. S., Wilson, L. & Rosenfeld, M. G. (1990) *Cell* **61**, 1021-33.

87. Verrijzer, C. P., Alkema, M. J., Vanweperen, W. W., Vanleeuwen, H. C., Strating, M. J. J. & Vandervliet, P. C. (1992) *EMBO J* **11**, 4993-5003.

88. Landolfi, N. F., Capra, J. D. & Tucker, P. W. (1986) *Nature* **323**, 548-551.

89. Falkner, F. G. & Zachau, H. G. (1984) *Nature* **310**, 71-4.

90. Parslow, T. G., Blair, D. L., Murphy, W. J. & Granner, D. K.

(1984) *Proc Natl Acad Sci U S A* **81,** 2650-4.

91. Staudt, L. M., Singh, H., Sen, R., Wirth, T., Sharp, P. A. & Baltimore, D. (1986) *Nature* **323,** 640-643.

92. Singh, H., Sen, R., Baltimore, D. & Sharp, P. A. (1986) *Nature* **319,** 154-158.

93. Klemm, J. D., Rould, M. A., Aurora, R., Herr, W. & Pabo, C. O. (1994) *Cell* **77,** 21-32.

94. Li, P., He, X., Gerrero, M. R., Mok, M., Aggarwal, A. & Rosenfeld, M. G. (1993) *Genes Dev* **7,** 2483-2496.

95. Voss, J. W., Wilson, L. & Rosenfeld, M. G. (1991) *Genes Dev* **5,** 1309-20.

96. Treacy, M. N., He, X. & Rosenfeld , M. G. (1991) *Nature* **350,** 577-584.

97. Kristie, T. M. & Sharp, P. A. (1990) *Genes Dev* **4,** 2383-96.

98. Stern, S., Tanaka, M. & Herr, W. (1989) *Nature* **341,** 624-30.

99. Stern, S. & Herr, W. (1991) *Genes Dev* **5,** 2555-66.

100. Pierani, A., Heguy, A., Fujii, H. & Roeder, R. G. (1990) *Mol Cell Biol* **10,** 6204-15.

101. Luo, Y., Fujii, H., Gerster, T. & Roeder, R. G. (1992) *Cell* **71,** 231-41.

102. Luo, Y. & Roeder, R. G. (1995) *Mol Cell Biol* **15,** 4115-24.

103. Gstaiger, M., Knoepfel, L., Georgiev, O., Schaffner, W. & Hovens, C. M. (1995) *Nature* **373,** 360-2.

104. Strubin, M., Newell, J. W. & Matthias, P. (1995) *Cell* **80,** 497-506.

105. Small, K. W., Weber, J. L., Roses, A., Lennon, F., Vance, J. M. & Pericak-Vance, M. A. (1992) *Genomics* **13,** 681-5.

106. Kelsell, R. E., Godley, B. F., Evans, K., Tiffin, P. A., Gregory, C. Y.,

Plant, C., Moore, A. T., Bird, A. C. & Hunt, D. M. (1995) *Hum. Mol. Genet.* **4,** 1653-1656.

107. Rauth, S., Green, A., Bratescu, L. & Das Gupta, T. K. (1994) *In Vitro Cell Dev Biol Anim* **30A,** 79-84.

108. Pathak, S., Drwinga, H. L. & Hsu, T. C. (1983) *Cytogenet Cell Genet* **36,** 573-9.

109. Trent, J. M., Rosenfeld, S. B. & Meyskens, F. L. (1983) *Cancer Genet Cytogenet* **9,** 177-80.

110. Becher, R., Gibas, Z., Karakousis, C. & Sandberg, A. A. (1983) *Cancer Res* **43,** 5010-6.

111. Mitchell, P. J., Timmons, P. M., Hebert, J. M., Rigby, P. W. & Tjian, R. (1991) *Genes Dev* **5,** 105-19.

112. Philipp, J., Mitchell, P. J., Malipiero, U. & Fontana, A. (1994) *Dev Biol* **165,** 602-14.

113. Schöler, H. R., Hatzopoulos, A. K., Balling, R., Suzuki, N. & Gruss, P. (1989) *EMBO J.* **8,** 2543-2550.

114. Thomson, J. A. F., Parsons, P. G. & Sturm, R. A. (1993) *Pigment Cell Res.* **6,** 13-22.

6. CURRICULUM VITAE

Personal	Born February 29, 1968, Frauenfeld, Switzerland
Nationality	Swiss
Education	
1975-1981	Primary school in Frauenfeld
1981-1987	Gymnasium in Frauenfeld, matriculation type B
1987-1992	Diploma study in Natural Sciences at the Swiss Federal Institute of Technology (ETH) in Zurich Degree in Biochemistry, Molecular Biology, Genetics, Immunology and Endocrinology
1990-1992	Postgraduate Teaching Degree in Chemistry (Höheres Lehramt für Chemie) at the ETH
1992-1996	Preparation of the PhD thesis under the supervision of Prof. A. Fontana at the University Hospital of Zurich, Department of Internal Medicine, Division of Clinical Immunology and Prof. M. Schachner at the Swiss Federal Institute of Technology (ETH) in Zurich, Department of Biology, Institute for Neurobiology