

The POU protein Oct-6 is a nucleocytoplasmic shuttling protein

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ABSTRACT

Like many POU domain proteins, Oct-6 plays important roles during vertebrate development. In accord with its function as a transcriptional regulator during neurogenesis and myelination, Oct-6 is predominantly found in the nucleus. Nuclear import is mediated by a nuclear localization signal at the N-terminal end of the POU homeodomain. Here we show, that Oct-6 in addition contains a nuclear export signal so that Oct-6 is able to shuttle constantly between nucleus and cytoplasm. This nuclear export signal is also localized in the POU homeodomain as part of helix 2 and the connecting loop to DNA recognition helix 3. It conforms to the consensus of hydrophobic leucine-rich export sequences and mediates export from the nucleus via CRM1/Exp1. Several amino acid substitutions or insertions that inactivate this nuclear export sequence, reduce DNA-binding of Oct-6 to its octamer recognition element slightly, but interfere strongly with Oct-6-dependent transcriptional activation, thus arguing that nuclear export and nucleocytoplasmic shuttling are essential aspects of Oct-6 function. Importantly, the nuclear export signal identified for Oct-6 is conserved in most, if not all other vertebrate POU proteins. Nuclear export might therefore be of general relevance for POU protein function throughout development.

INTRODUCTION

POU proteins form a subgroup of homeodomain proteins. They are characterized by possession of a POU domain in which the homeodomain is connected by a short linker region to an N-terminally located POU-specific domain (1). Both the POU-specific and the POU homeodomain are DNA-binding

domains of the helix–turn–helix type. In the context of POU proteins, both regions influence DNA-binding specificity and together define the octamer recognition elements for POU proteins, thus explaining why POU proteins have recognition sequences that differ significantly from those of other homeodomain proteins.

POU proteins can be grouped into classes I–VI according to sequence similarities both within and outside the POU domain (2). For example, class III consists of the related POU proteins Brn-1, Brn-2, Brn-4 and Oct-6 (3) which show strong expression in various parts of the nervous system (4) and exert important, partially redundant functions during nervous system development (5–12). Oct-6, which is also known as Tst-1 and SCIP, is for instance important for the proper development and positioning of neuronal subpopulations in the central nervous system and for the development of Schwann cells which represent one of the main types of glial cells in the peripheral nervous system and are found along vertebrate nerves. A fraction of Schwann cells insulate large diameter axons in peripheral nerves with myelin sheaths. In Oct-6-deficient mice, these myelinating Schwann cells become transiently arrested before terminal differentiation so that myelin sheaths are formed only with substantial temporal delay (5,6). The delay in terminal differentiation is even further enhanced upon additional deletion of Brn-2 in the mouse (7) arguing that these class III POU domain proteins function redundantly in developing Schwann cells and can partially compensate each other's loss. Functional equivalency of class III proteins during Schwann cell development has also been confirmed in a mouse model in which Oct-6 has been replaced by Brn-1 (13).

In contrast to the biology of class III POU proteins, their molecular mode of action is only poorly understood. Very few target genes or interacting partners have been identified. Additionally, the subcellular localization has not been analyzed in detail. Oct-6, for instance, is usually found within the nucleus and possesses a classical nuclear localization signal (NLS) within its POU homeodomain that is highly conserved in all POU proteins (14). However, in certain neuronal

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

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subpopulations of the adult mouse brain, Oct-6 has been found to be cytoplasmic as well as nuclear (15). This argues that Oct-6 may either be actively prevented from entering the nucleus or may have the capacity to leave the nucleus.

The best characterized nuclear export pathway involves the export factor CRM1/Exp1 as well as short hydrophobic and leucine-rich amino acid sequences within the protein to be translocated (16). These leucine-rich nuclear export sequences (NES) directly interact with CRM1/Exp1. Originally identified in the human immunodeficiency virus type-1 (HIV-1) Rev protein and the protein kinase A inhibitor (17,18), leucine-rich NES have in the meantime been identified in many more proteins, including members of several transcription factor families (19–26). Here, we show that Oct-6 also contains a leucine-rich NES within its homeodomain which confers the ability to constantly shuttle between nucleus and cytoplasm. Experimental evidence suggests that the ability to leave the nucleus is important for Oct-6 function. Given the fact that this NES is as highly conserved as the previously identified NLS, nucleocytoplasmic shuttling may be an essential mechanism to regulate POU protein function in general.

MATERIALS AND METHODS

Plasmid constructs

Using the Quick Change mutagenesis kit (Stratagene), the following mutations were introduced by site-directed mutagenesis into the mouse Oct-6 cDNA (accession no. X54628): L370A (Mut1); L374A, L376A (Mut2); L374T, L376I (Mut3); Q375STYGR (Mut4). Wild-type Oct-6 and mutated Oct-6 cDNAs were cloned into the eukaryotic expression vector pCMV5. Additionally, wild-type and mutant Oct-6 cDNAs were fused to the C-terminal end of green fluorescent protein (GFP) and similarly cloned into pCMV5. Sequences corresponding to the POU domain of Oct-6 (both wild-type and mutant versions, amino acids 240–396) were generated by PCR such that they carried a start codon and Kozak sequence and could be inserted between the EcoRI and BamHI sites of pEGFP-N1 (Clontech Laboratories, Palo Alto, CA) to yield fusion proteins consisting of an N-terminal POU domain and a C-terminal GFP moiety. Using an analogous strategy pEGFP-Brn-4-POU was generated in which the POU domain of Brn-4 is fused to GFP. A bacterial expression vector for a protein with the Oct-6 NES (amino acid 367–376) inserted between an N-terminal glutathione-S-transferase (GST) and a C-terminal GFP moiety was generated by insertion of a double-stranded synthetic oligonucleotide between the BamHI and NheI site of pGEX-GFP (27). In addition to fusions with the wild-type NES, additional ones were generated carrying the Mut1 and Mut2 of the Oct-6 NES. The control plasmids pEGFP-SOX10, pEGFP-SOX10-ΔNES, pGEX-SOX10-NES-GFP and pGEX-SOX10-ΔNES-GFP have been described before (25) as well as the HSVoct-luc luciferase vector (14).

Cell culture, transfections and luciferase assays

HeLa, 3T3, Neuro2a and COS-7 cells were kept in DMEM supplemented with 10% (v/v) fetal calf serum (FCS). U138 glioblastoma cells were grown in RPMI-1640 containing 10% (v/v) FCS. Transient transfection of COS-7 cells for

preparation of protein extracts was performed in 100 mm plates with 10 μg cytomegalovirus (CMV)-expression plasmid using DEAE dextran precipitates. For luciferase assays, U138 cells were transfected in 35 mm plates using calcium phosphate precipitates with 0.5 μg of luciferase reporter plasmid and 0.5 μg of pCMV5 expression plasmids, if not stated otherwise. Neuro2a cells were transfected in 24-well plates using Superfect reagent (Qiagen) with 0.25 μg of reporter and pCMV5 expression plasmid each. The total amount of plasmid DNA was kept constant in each transfection series using empty pCMV5. In some luciferase assays, leptomycin B (A.G. Scientific Inc., San Diego, CA) was added to the medium at a final concentration of 2.5 ng/ml. 48 h post-transfection, cells were harvested for extract preparation, immunocytochemistry or luciferase assays (28).

Protein preparations, electrophoretic mobility shift assays (EMSA) and western blotting

Whole cell extracts were prepared from COS-7 cells 48 h post-transfection. Cells from one 100 mm plate were lysed in the presence of 10 μg leupeptin and aprotinin each in ice-cold 20 mM HEPES (pH 7.9), 10 mM KCl, 5 mM MgCl₂, 0.5 mM EDTA, 10% glycerol, 2 mM DTT, 0.1% Triton-X 100 and 300 mM NaCl. After extraction for 15 min under constant rotation on a rotary mixer (Labinco, Breda, Netherlands) cell debris was removed from the extract by centrifugation.

For EMSA, 0.5 ng of ³²P-labeled HSVoct oligonucleotide were incubated with COS-7 cell extract for 20 min on ice in a 20 μl reaction mixture as described using poly(dIdC) as unspecific competitor (14). Samples were loaded on to native 5% polyacrylamide gels and electrophoresed in 0.5 × TBE buffer [45 mM Tris, 45 mM boric acid and 1 mM EDTA (pH 8.3)] at 120 V for 1.5 h. Gels were dried and exposed for autoradiography.

Western blotting was performed on these extracts as described (14) using a rabbit polyclonal antibody against Oct-6 [1:3000 dilution, ref. (29)] or a mouse monoclonal antibody against GFP (1:5000 dilution, Roche Diagnostics) as primary antibodies and horseradish peroxidase coupled protein A or horseradish peroxidase coupled goat anti-mouse IgGs as secondary detection reagents with the enhanced chemiluminescence detection system (Amersham).

Purification and microinjection of GST-GFP fusion proteins

GST-GFP hybrid proteins were expressed in *Escherichia coli* BL21 and purified on glutathione Sepharose 4B (Pharmacia Biotech, Germany) as described previously (25). Eluted proteins were analyzed by SDS-PAGE and Coomassie blue staining. Fractions were pooled, concentrated by ultrafiltration with a Nanosep filter (Pall-Filtron, USA) and microinjected into the nuclei of HeLa cells at 1.5 mg/ml in combination with Cy3-coupled rabbit IgG as injection control (1.0 mg/ml, Biotrends, Köln, Germany) by using a CompiC INJECT computer-assisted injection system (Cell-biology Trading, Hamburg, Germany). Cells were fixed 2 h post-injection with 4% paraformaldehyde, and the injected proteins were detected by immunofluorescence analysis. In some experiments, 2.5 ng/ml leptomycin B was added 2 h before microinjection.

Heterokaryon assays and immunofluorescence microscopy

Nucleocytoplasmic shuttling of Oct-6 and its mutants was analyzed in a heterokaryon assay, as initially described by Pinol Roma *et al.* (30) and modified in (25). Briefly, transfected NIH 3T3 cells were seeded on glass coverslips together with an equal number of HeLa cells and were incubated overnight. Cells were incubated with 50 µg/ml cycloheximide 30 min before the fusion and throughout the experiment. In some experiments, 2.5 ng/ml leptomycin B was added 3 h before cell fusion. Subsequently, cells were washed in phosphate-buffered saline (PBS), and heterokaryons were formed by incubating the cells for 2 min in 50% polyethylene glycol 8000 in DMEM. Cells were returned to fresh medium containing 50 µg/ml cycloheximide (and 2.5 ng/ml leptomycin B when needed). After 2 h of incubation at 37°C, cells were fixed with 4% paraformaldehyde, washed with PBS, treated with 5 µg/ml of DAPI (Sigma, Taufkirchen, Germany) for 5 min and mounted in Mowiol (Calbiochem, La Jolla, CA) for indirect immunofluorescence analysis using a Leica inverted microscope (DMIRB). At least 25 heterokaryons were analyzed for each experiment and nuclear shuttling was only scored positive when a minimum of 80% of the heterokaryons showed a positive staining of the investigated protein. Images were recorded with a cooled MicroMax CCD camera (Princeton Instruments, Trenton, NJ) and processed using the IPLab spectrum and Adobe Photoshop software packages.

RESULTS

The POU homeodomain of Oct-6 contains a putative leucine-rich NES

The presence of Oct-6 in the cytoplasm of subpopulations of neurons in the adult brain (15) prompted us to inspect the primary amino acid sequence of Oct-6 for potential NES. Interestingly, amino acids 367–376 (Figure 1a) closely conformed to the consensus for leucine-rich NES (Figure 1b) with an isoleucine and three leucines in the proper spacing. They furthermore were the only obvious candidate for a nuclear export sequence. The putative NES is located in the POU homeodomain at the C-terminal end of helix 2 and in the following loop to helix 3 and should therefore be accessible for the nuclear export machinery.

Oct-6 is a nucleocytoplasmic shuttling protein

Previous studies had identified an NLS within the POU homeodomain of Oct-6 that is responsible for nuclear localization (Figure 1a) (14). If Oct-6, in addition, carries an NES, it should not only be able to enter, but also to leave the nucleus. To study the ability of Oct-6 to shuttle between nucleus and cytoplasm, we generated fusion proteins between GFP and Oct-6 and between GFP and the isolated POU domain of Oct-6 (Figure 2a and b). When transfected into NIH 3T3 cells, these fusion proteins are predominantly nuclear (Figure 2c and data not shown) similar to Oct-6 itself as described previously (31). While this could mean that Oct-6 is permanently located in the nucleus, this finding is also compatible with a situation in which nuclear import rates significantly exceed those for nuclear export.

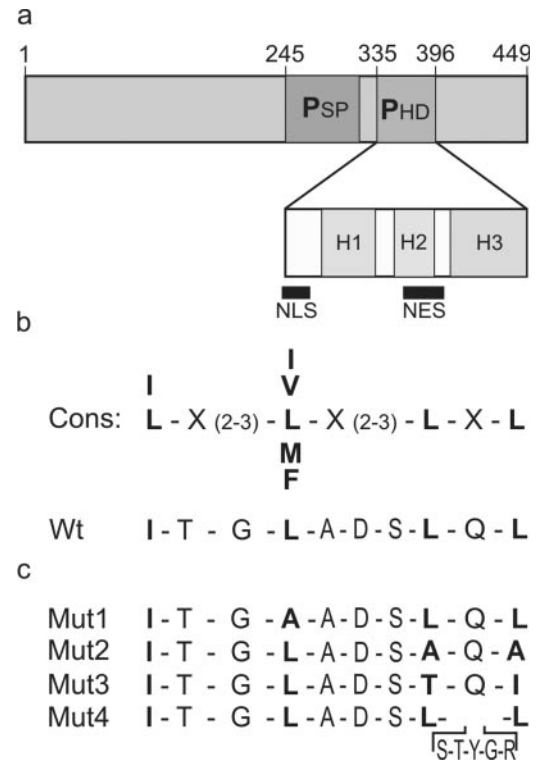


Figure 1. Identification and mutation of the putative NES of Oct-6. (a) schematic representation of Oct-6 with its POU domain consisting of POU-specific domain (P_{SP}) and POU homeodomain (P_{HD}) with its 3 helices H1, H2 and H3. Locations of the previously identified NLS (14) and the putative NES within the POU homeodomain are highlighted. (b) comparison of the putative NES in Oct-6 (Wt, amino acids 367–376) with the consensus sequence (cons) of leucine-rich NES (16). (c) Mutations introduced into the NES of Oct-6 and used throughout this study.

To differentiate between these two possibilities, we performed heterokaryon assays in which mouse NIH 3T3 cells were first transfected and then fused to untransfected human HeLa cells under conditions where protein synthesis is suppressed by cycloheximide treatment. Nuclei from NIH 3T3 and HeLa cells can be distinguished in such heterokaryons by DAPI staining which is punctate for mouse nuclei and homogeneous for human nuclei. Proteins with nucleocytoplasmic shuttling activity such as a fusion protein of GFP with the transcription factor Sox10 (25) were able to leave the nuclei of NIH 3T3 cells and enter those of HeLa cells (Figure 3a). The ability to leave the nuclei of NIH 3T3 cells was furthermore dependent on the presence of an intact NES, as the same protein with mutated NES was no longer able to undergo nucleocytoplasmic shuttling (Figure 3b). When the GFP–Oct-6 fusion was analyzed in this heterokaryon assay, significant amounts of this protein were found in HeLa nuclei 2 h after heterokaryon formation (Figure 3c). Thus we conclude, that Oct-6 is also a nucleocytoplasmic shuttling protein. As both NLS and putative NES are located within the POU domain of Oct-6, we also studied whether the isolated POU domain could replace full length Oct-6 in its ability to mediate nucleocytoplasmic shuttling in a heterokaryon assay when fused to GFP. This was indeed the case (Figure 3d).

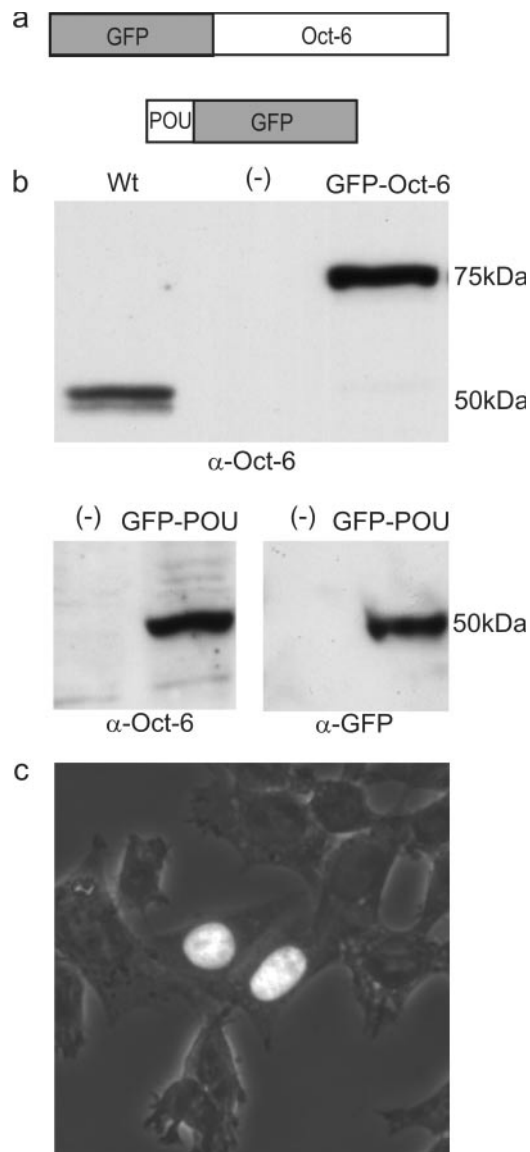


Figure 2. Characterization of GFP-Oct-6 and GFP-Oct-6-POU. (a) schematic representation of the GFP fusion constructs. (b) western blot analyses of extracts from COS-7 cells expressing either Oct-6 (Wt), GFP-Oct-6 or GFP-Oct-6-POU (GFP-POU) using anti-Oct-6 (α -Oct-6) or anti-GFP (α -GFP) antibodies. Protein sizes are indicated on the right side of the panels. (-), control COS-7 extract. (c) Subcellular localization of GFP-Oct-6 in transiently transfected NIH 3T3 cells by direct fluorescence microscopy.

The leucine-rich sequence in the POU homeodomain of Oct-6 is necessary for nucleocytoplasmic shuttling

To study the role of the putative NES, we introduced several mutations into this leucine-rich sequence (Figure 1c). In Mut1, the leucine residue at position 370 was changed to an alanine residue. In contrast, leucine residues at position 374 and 376 were targeted in Mut2 and Mut3. In Mut2, both leucines were replaced by alanines, whereas Mut3 carried a threonine instead of leucine-374 and an isoleucine instead of leucine-376. L376I is a conservative substitution that should not interfere with NES function, so that altered nuclear export in Mut3 should primarily be a consequence of the change at position 374. Mut4 differs from the other mutants in that none of the

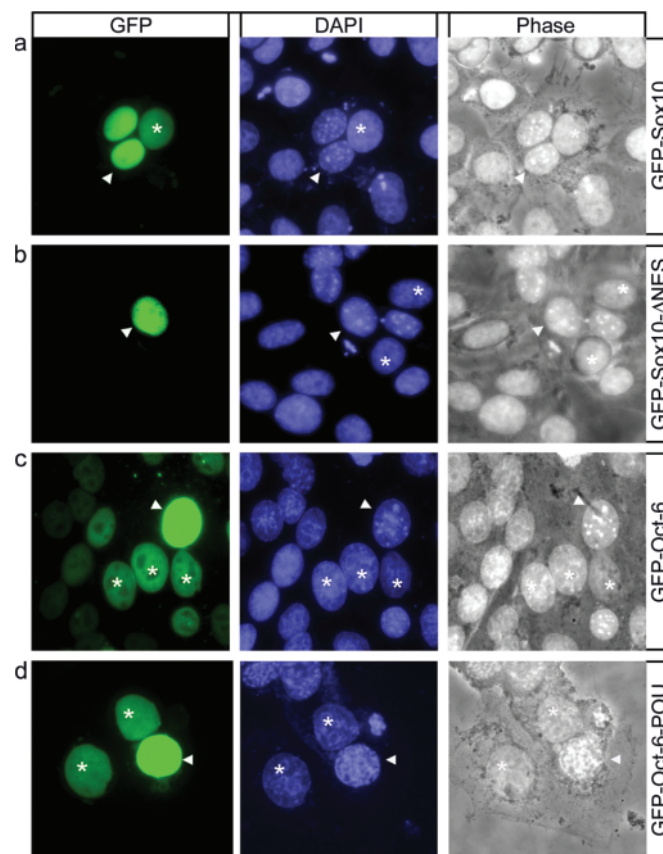


Figure 3. Oct-6 shuttles between the nucleus and the cytoplasm as revealed by heterokaryon assays. NIH 3T3 cells were transfected with expression plasmids for GFP-SOX10 (a) GFP-Sox10- Δ NES (b) GFP-Oct-6 (c) and GFP-Oct-6-POU (d) before they were subjected to heterokaryon formation with HeLa cells. After 2 h, cells were fixed, counterstained with DAPI (middle panel) and analyzed for GFP autofluorescence (left panel). Right panels show the heterokaryons in phase contrast. Donor mouse nuclei are marked by an arrowhead and recipient human nuclei by asterisks.

essential leucines of the NES was replaced. Instead, Q375 was replaced by five amino acids so that the loop between helix 2 and helix 3 of the POU homeodomain was extended and the spacing altered between leucine-374 and leucine-376. Amino acid changes in Mut3 and Mut4 were chosen because the introduced amino acids are found at these positions in naturally occurring homeodomain proteins (Mut3 as in chicken Mix.1, accession no. U34615; Mut4 as in a homeodomain transcription factor from rice, accession no. AP001111).

The consequences of these mutations on nucleocytoplasmic shuttling were first analyzed in the context of the GFP fusion with the POU domain of Oct-6. As evident from Figure 4b–e, all mutations effectively retained the GFP fusion protein in the nuclei of transfected NIH 3T3 cells and thus interfered with nucleocytoplasmic shuttling. Shuttling was only observed for the GFP fusion protein with the wild-type version of the POU domain (Figure 4a). These results strongly support the notion that the putative NES is indeed required for nuclear export.

The leucine-rich sequence in the POU homeodomain of Oct-6 is sufficient for nuclear export

To analyze whether the identified leucine-rich sequence in the POU homeodomain of Oct-6 is sufficient to mediate nuclear

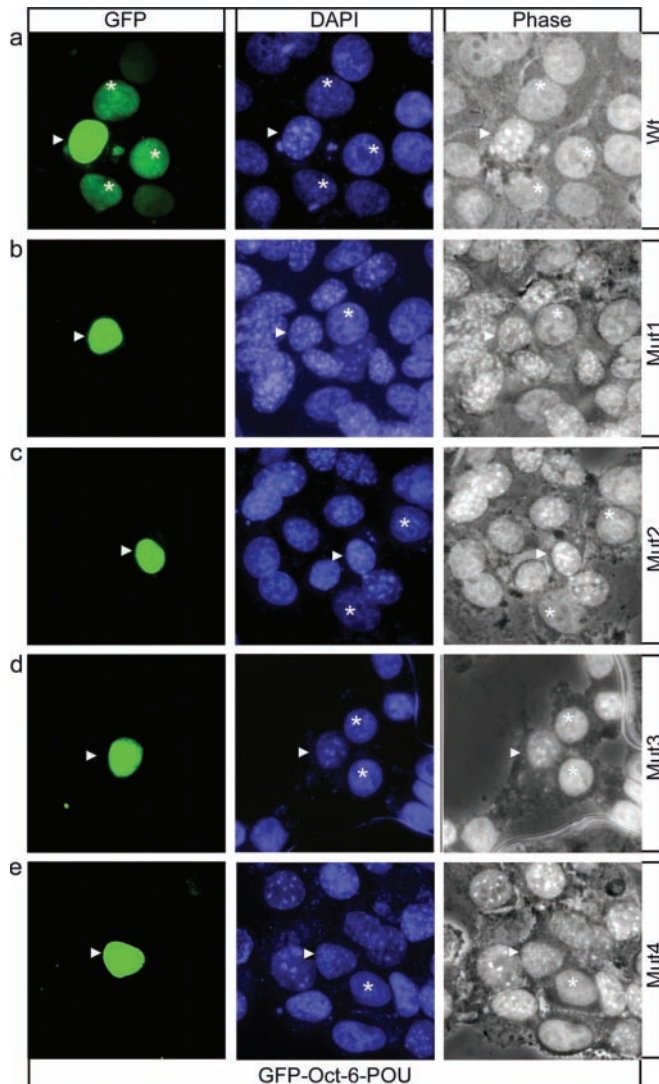


Figure 4. The leucine-rich sequence in the POU homeodomain of Oct-6 is required for nucleocytoplasmic shuttling. NIH 3T3 cells were transfected with expression plasmids for wild-type (Wt) GFP-Oct-6-POU (a), and versions which carried the Mut1 (b), the Mut2 (c), the Mut3 (d) or the Mut4 (e) mutation in their POU domain (see also Figure 1c). After cycloheximide treatment, transfected NIH 3T3 cells were subjected to heterokaryon formation with HeLa cells. After 2 h, cells were fixed, counterstained with DAPI (middle panel) and analyzed for GFP autofluorescence (left panel). Right panels show the heterokaryons in phase contrast. Donor mouse nuclei are marked by an arrowhead and recipient human nuclei by asterisks.

export, we inserted the NES into an artificial GST–GFP protein between the GST and the GFP moieties. The recombinant protein was produced in bacteria and purified from bacterial extracts via its GST part. The purified proteins were then microinjected into the nuclei of HeLa cells in a mixture with mouse IgGs. If the NES is indeed functional in the context of this artificial protein, the microinjected protein should be able to leave the nucleus while the IgG remains in the nucleus and serves as a control for nuclear injection.

Proteins served as controls in which the previously identified NES of Sox10 was similarly inserted into the GST–GFP chimera, either in its wild-type sequence or in a mutant version known to interfere with NES function (25). As shown in

Figure 5a and b, only the wild-type NES of Sox10 mediated the nuclear export of the GST–GFP chimera, whereas the one with the mutant NES remained in the nucleus. When the putative NES of Oct-6 was used instead of the Sox10 NES, a significant fraction of the microinjected protein was able to leave the HeLa cell nucleus and enter the cytoplasm, whereas the control IgG remained confined to the nucleus (Figure 5c). Nevertheless Oct-6 NES driven export was clearly less efficient than observed for the Sox10 NES. While this could hint at a generally lower activity of the Oct-6 NES, it is equally possible that we simply failed to transfer enough NES flanking sequences to the GST–GFP chimera which in some cases have been shown to be critical for full activity as NES activity is context-dependent (32). Importantly, the mutant versions of the Oct-6 NES were completely unable to relocate the microinjected GST–GFP proteins to the cytoplasm (Figure 5d and e) thus confirming that the identified leucine-rich sequence in the POU homeodomain of Oct-6 is indeed able to function as a transferable NES and is therefore sufficient to mediate nuclear export.

The leucine-rich sequence in the POU homeodomain is the only NES within Oct-6

To analyze whether the identified NES represents the major export signal within Oct-6, we also tested the effect of the Mut1 and Mut2 mutation on nucleocytoplasmic shuttling in the context of GFP fusions with full length Oct-6. As already observed for the GFP fusions with the isolated POU domain, both mutations effectively prevented the GFP–Oct-6 fusions from leaving the NIH 3T3 nuclei (Figure 6b and c), whereas the corresponding fusion with wild-type Oct-6 showed nucleocytoplasmic shuttling activity in parallel assays and efficiently relocated to HeLa cell nuclei in heterokaryons (Figure 6a). These results indicate that the identified NES is the major export signal in Oct-6.

Nuclear export of Oct-6 is CRM1/Exp1-dependent

Proteins with leucine-rich NES are usually exported in a CRM1/Exp1-dependent manner (33). This CRM1/Exp1-dependent nuclear export can be specifically inhibited by leptomycin B (17,18). When heterokaryon assays were performed with cells pretreated with leptomycin B, the wild-type GFP–Oct-6 fusion behaved similar to the Mut1 and Mut2 versions and failed to leave the NIH 3T3 nuclei (Figure 7a). Similarly, GST–GFP chimeras with a wild-type Oct-6 NES completely remained in the microinjected nuclei of leptomycin B-treated HeLa cells (Figure 7b). This argues that nuclear export of Oct-6 via its leucine-rich NES is CRM1/Exp1-dependent.

Nuclear export influences the transcriptional activity of Oct-6

The NES of Oct-6 is localized in the POU homeodomain. Its amino acids are part of helix 2 and the connecting turn to helix 3, and thus do not directly contact DNA. Because of its vicinity to the DNA recognition helix, mutations in the NES might however influence DNA-binding. Therefore, we compared the DNA-binding ability of wild-type Oct-6 and the NES mutants (Figure 8). For this purpose, COS-7 cell extracts containing wild-type Oct-6 or one of the mutants were titrated by western blot analysis. The amounts were adjusted so that the levels of

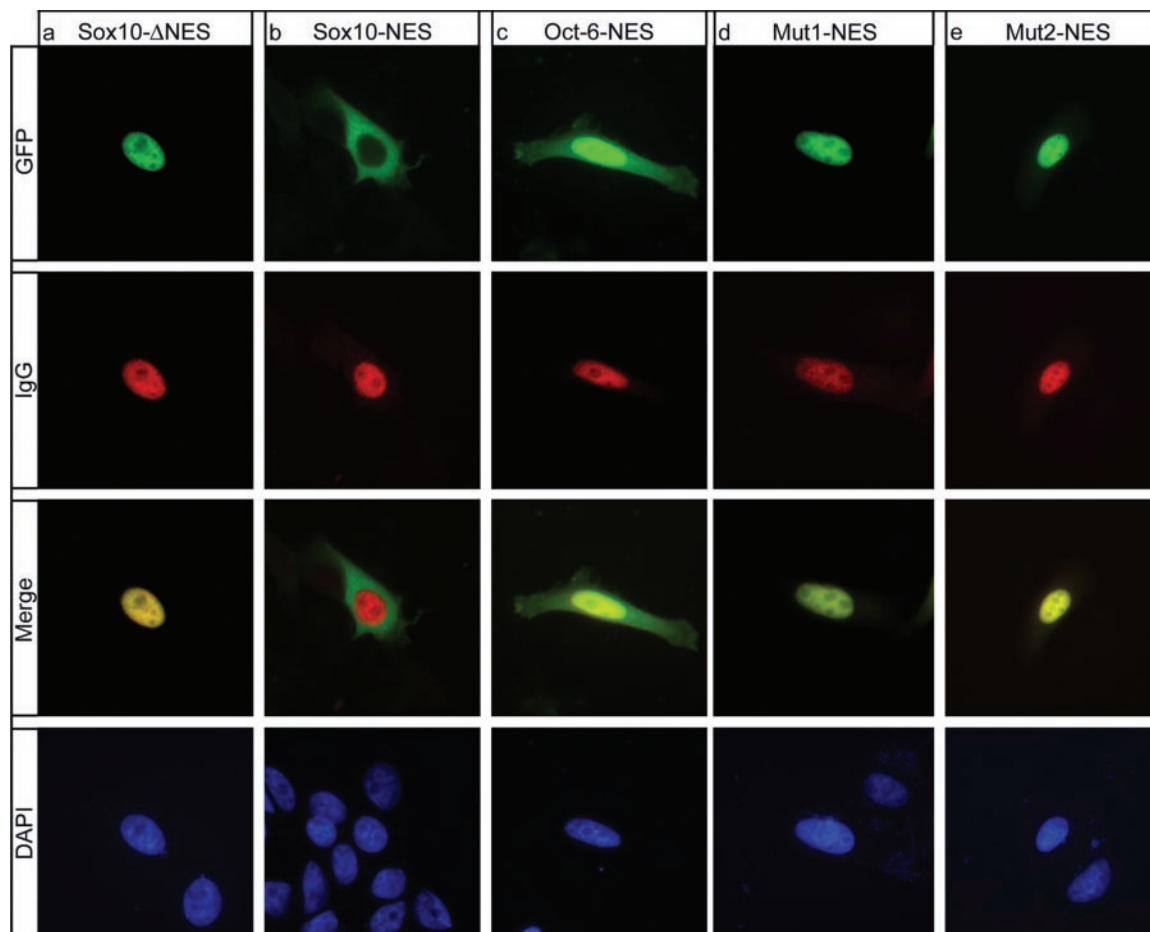


Figure 5. The hydrophobic leucine-rich sequence in Oct-6 functions as a NES when transferred to a heterologous protein. Purified GST–GFP chimeric proteins were microinjected into the nuclei of HeLa cells together with rabbit Cy3-coupled IgG as a marker for the nuclear injection site. 45 min after injection, the cells were fixed and counterstained with DAPI (last row). The injected rabbit IgG was detected exclusively within the nucleus (second row), whereas the GST–GFP chimeras containing either the NES of Sox10 (b, Sox10-NES) or of Oct-6 (c, Oct-6 NES) were significantly translocated from the nuclear injection site into the cytoplasm as revealed by the green autofluorescence of the GFP moiety (first row). In contrast those GST–GFP chimeras containing mutant versions of the Sox10 NES (a, Sox10- Δ NES) or the Oct-6 NES (d, Mut1-NES; e, Mut2-NES) failed to leave the microinjected nucleus. Merged pictures of the upper two rows are shown in the third row.

wild-type Oct-6 and the mutants were comparable (Figure 8a). Increasing amounts of these adjusted extracts were then incubated with the octamer element of the Herpes simplex virus immediate early promoter (HSVoct, ref. 14) in EMSA and complex formation with the HSVoct probe was evaluated for each mutant. Of the 4 mutants analyzed, only Mut2 had a severely reduced DNA-binding activity. Mut1, Mut3 and Mut4 on the other hand, all still bound to the HSVoct probe with considerable strength. Compared to the wild-type, \sim 5-fold more protein had to be used for the Mut1, Mut3 and Mut4 mutants to obtain a complex of similar strength with the HSVoct probe (Figure 8b). Binding kinetics were very similar to the wild-type for all mutants, as no differences could be detected in the on-rate or the off-rate of complex formation (data not shown).

All mutants with deficient nuclear export signal were analyzed after transient transfection in U138 and Neuro2a cells for their ability to activate transcription of the Oct-6-dependent luciferase reporter HSVoct-luc (14). Whereas standard amounts of wild-type Oct-6 reproducibly activated expression of this reporter 5- to 20-fold depending on the cell line, none of the nuclear export mutants did, independent of their ability to

bind to DNA (Figure 8c). Increases in the amount of wild-type Oct-6 led to concomitant increases in reporter gene activation until saturating Oct-6 levels were reached (Figure 8d). In contrast, even saturating amounts of the nuclear export mutants were unable to elicit Oct-6-dependent reporter gene expression. Furthermore, treatment of transfected U138 cells with leptomycin B decreased the transactivation capacity of wild-type Oct-6 by 70% (Figure 8e).

The NES is conserved in all POU proteins and in many homeodomain proteins

Sequence inspection revealed that the sequence of the Oct-6 NES is conserved in most other POU proteins (Figure 9a). In fact, the only POU protein with significant deviation from the NES consensus is Brn-5. In all other cases, isoleucine and leucine residues are conservatively replaced by similarly hydrophobic residues. Some other homeodomain proteins also contain the NES as shown for Bicoid, or slight spacing variations of the NES as in the case of Hox proteins (Figure 9a). To get an idea whether this sequence is also functional in other POU proteins, we chose to fuse the POU domain of Brn-4 to

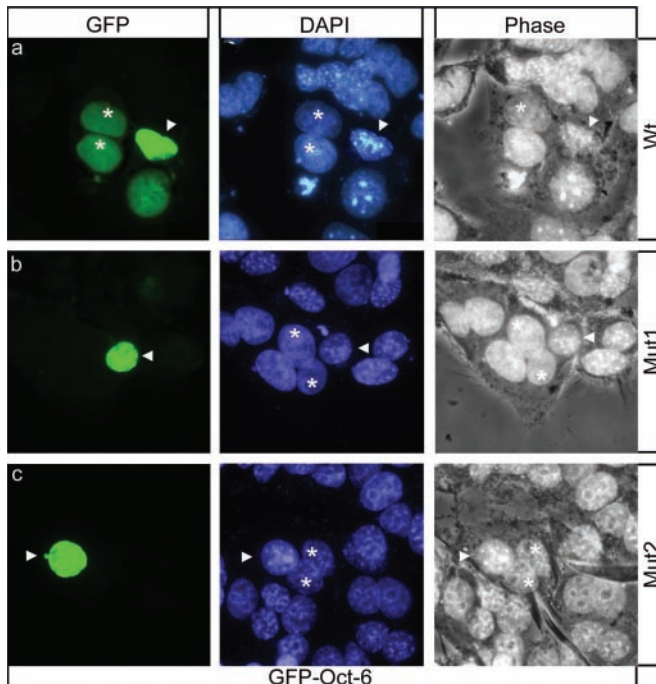


Figure 6. The NES in the POU domain is the dominant NES of Oct-6. NIH 3T3 cells were transfected with expression plasmids for wild-type (Wt) GFP-Oct-6 (a) and versions which carried the Mut1 (b) or the Mut2 (c) mutation. After cycloheximide treatment, transfected NIH 3T3 cells were subjected to heterokaryon formation with HeLa cells. After 2 h, cells were fixed, counterstained with DAPI (middle panel) and analyzed for GFP autofluorescence (left panel). Right panels show the heterokaryons in phase contrast. Donor mouse nuclei are marked by an arrowhead and recipient human nuclei by asterisks.

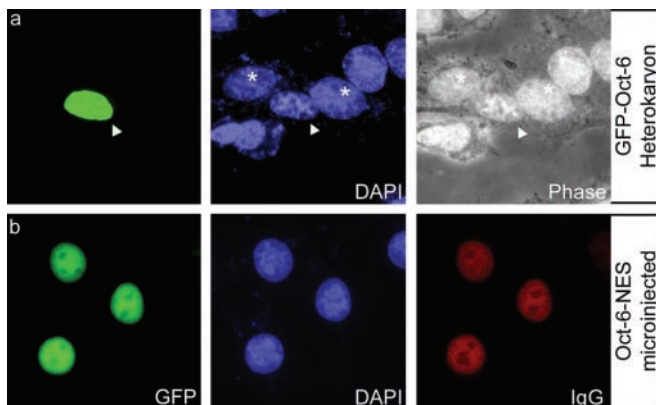


Figure 7. Nucleocytoplasmic shuttling of Oct-6 is CRM1/Exp1-dependent. (a) NIH 3T3 cells were transfected with an expression plasmid for GFP-Oct-6 and pretreated with leptomycin B, before they were subjected to heterokaryon formation with HeLa cells. After 2 h in the continued presence of leptomycin B, cells were fixed, counterstained with DAPI (middle panel) and analyzed for GFP autofluorescence (left panel). Right panels show the heterokaryons in phase contrast. (b) A GST-GFP chimera carrying the NES of Oct-6 was microinjected into the nuclei of leptomycin B-pretreated HeLa cells together with rabbit Cy3-coupled IgG as a marker for the nuclear injection site. Cells were kept under leptomycin B. 45 min after injection, the cells were fixed and counterstained with DAPI (middle row). The injected rabbit IgG (right panel) as well as the GST-GFP chimera carrying the NES of Oct-6 (left panel) were detected exclusively within the nucleus.

GFP and analyze the resulting fusion protein in heterokaryon assays. In accord with the assumption that the conserved leucine-rich sequence is also functional as an NES in Brn-4, nucleocytoplasmic shuttling was detected for the GFP-Brn-4 fusion (Figure 9b).

DISCUSSION

Whereas transcription factors had originally been thought to spend their complete life in the cell nucleus, members of several transcription factor families have been shown in the meantime to be able to both enter and leave the nucleus. These include the STAT and Smad transcription factors, several bZIP proteins, nuclear receptors and other zinc finger proteins as well as some Sox proteins (19–26). To our knowledge, Oct-6 is the first POU protein shown to possess nucleocytoplasmic shuttling activity.

Similar to nuclear import, which is an active process that requires the presence of at least one NLS in the protein to be transported into the nucleus, nuclear export depends on the presence of nuclear export signals. In many cases, these NES are hydrophobic, leucine-rich stretches. This is also the case for the NES identified in Oct-6 which contains one isoleucine and three leucines in characteristic spacing.

We have previously identified the major NLS of Oct-6 at the N-terminal end of the POU homeodomain (14). It is intriguing that the newly identified NES is also part of the POU homeodomain. One single domain is thus involved in nuclear import, DNA-binding and nuclear export. The NES localization in helix 2 and the connecting loop to helix 3 of the POU homeodomain ensures that the NES is not masked by DNA-binding so that even DNA-bound Oct-6 is not necessarily precluded from interaction with the nuclear export machinery. Given the fact that most hydrophobic leucine-rich NES are recognized by CRM1/Exp1, it is not surprising that Oct-6 nuclear export is also CRM1/Exp1-dependent.

Although Oct-6 has the capacity to constantly move between nucleus and cytoplasm, it is usually detected in the nucleus. However, this static picture only reflects the fact that nuclear import rates exceed those for nuclear export. That this is not always the case, is indicated by the cytoplasmic occurrence of significant amounts of Oct-6 in select neuronal subpopulations of the adult mouse brain (15). Cytoplasmic localization has also been detected for other POU proteins such as Brn-2 and Oct-1 (34,35).

The balance between nuclear export and import is often subject to regulatory mechanisms. Phosphorylation events, in particular, have been shown to alter NLS or NES activity (23,36,37). In case of Oct-6, the NLS rather than the NES is a likely target for regulatory post-translational modifications as it is flanked on both sides by several putative phosphorylation sites.

Nuclear export is an essential regulatory mechanism to control the activity of transcription factors in response to different cues including oxidative stress, cell-density or extracellular environment (22,23,37,38). In principle, many ways can be envisaged by which nuclear export influences activity of a transcription factor. Nuclear export may sequester transcription factors away from their target genes. This could be important for POU

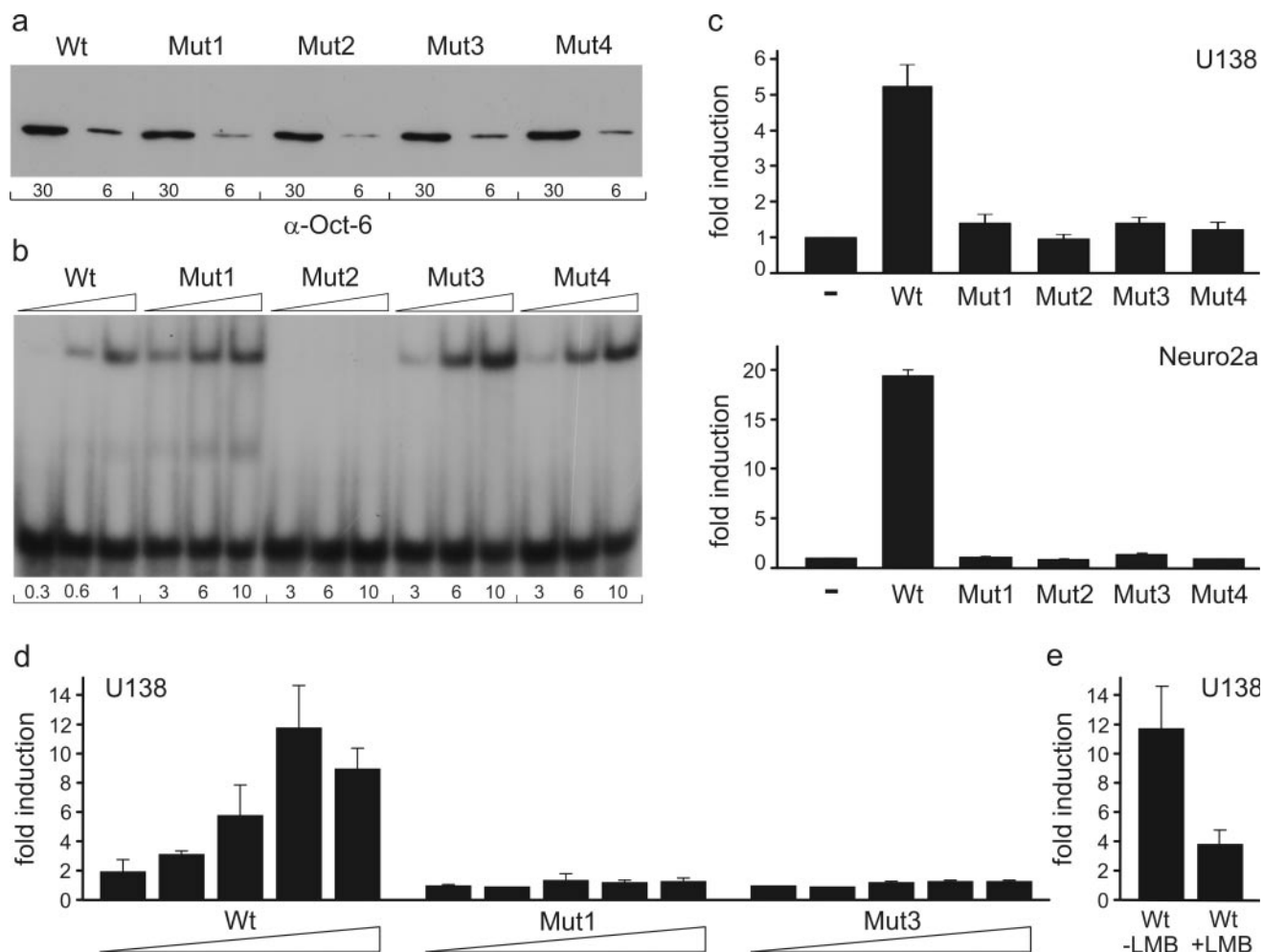


Figure 8. The NES modulates the transcriptional activity of Oct-6. (a) western blot analysis of COS-7 extracts containing wild-type (Wt) GFP-Oct-6-POU or its mutant versions Mut1, Mut2, Mut3 and Mut4 using an anti-Oct-6 antibody. Approximately equal amounts of each Oct-6 protein were loaded. (b) EMSA of wild-type (Wt) GFP-Oct-6-POU and its mutant versions Mut1, Mut2, Mut3 and Mut4 using HSVoct as probe. In both (a) and (b), the relative amount of each protein is indicated below the lanes. The highest amount of wild-type protein used in the electrophoretic mobility shift assay was arbitrarily set to 1. (c–e) Transient transfections in U138 and Neuro2a cells with a luciferase reporter under the control of the HSVoct-binding site and the β -globin minimal promoter (HSVoct-luc). The luciferase reporter was transfected either alone or in combination with Oct-6 in its wild-type form (Wt) or in mutant versions (Mut1, Mut2, Mut3 and Mut4). Whereas 0.5 μ g of Oct-6 expression plasmids were used in (c), increasing amounts (20 ng, 0.2 μ g, 0.5 μ g, 1 μ g and 2.5 μ g) were used in (d). Cells in (e) were kept in the absence (– LMB) or presence (+ LMB) of leptomycin B after transfection. Data from at least two independent experiments each performed in duplicates are presented as fold inductions \pm SEM with the activity for each luciferase reporter in the absence of co-transfected Oct-6 arbitrarily set to 1.

proteins as developmental regulators with often transient function either as a mechanism to accumulate potentially active protein in the cytoplasm before a rapid induction event or as a mechanism to inactivate still existing protein after the phase ended during which the protein was active. Accumulation of potentially active protein in the cytoplasm is observed for Oct-1 in early *Xenopus* embryos, where maternal Oct-1 protein is retained in the cytoplasm until mid-blastula transition (35). Rapid inactivation may be the reason for the differential localization of Brn-2 in the sub-ventricular and intermediate zones of the developing spinal cord. As precursor cells in the developing spinal cord mature and move from the sub-ventricular zone to the intermediate zone, Brn-2 becomes relocated from nucleus to cytoplasm probably as a means of rapid inactivation (34). Future experiments will have to address whether these mechanisms are also employed for the regulation of Oct-6 function.

Our studies also revealed that Oct-6 lost its transcriptional activity upon NES inactivation. Although the reduced DNA-binding of NES mutants almost certainly contributes to the observed loss of transcriptional activity, we do not consider this the sole cause as even saturating amounts of these NES mutants failed to activate Oct-6-dependent transcription despite their residual DNA-binding activity. Additionally, pharmacological inhibition of nuclear export by treatment with leptomycin B reduced the transcriptional activity of wild-type Oct-6. These results point to the possibility that repeated passage through the cytoplasm is required for Oct-6 to acquire and retain full activity as a transcription factor. A similar model has also been proposed for R-Smads and Sox proteins of group E after studying their nuclear export in cell and explant cultures (25,26,39). What acts on these transcription factors in the cytoplasm, for instance by introducing post-translational modifications, is unclear at the moment.

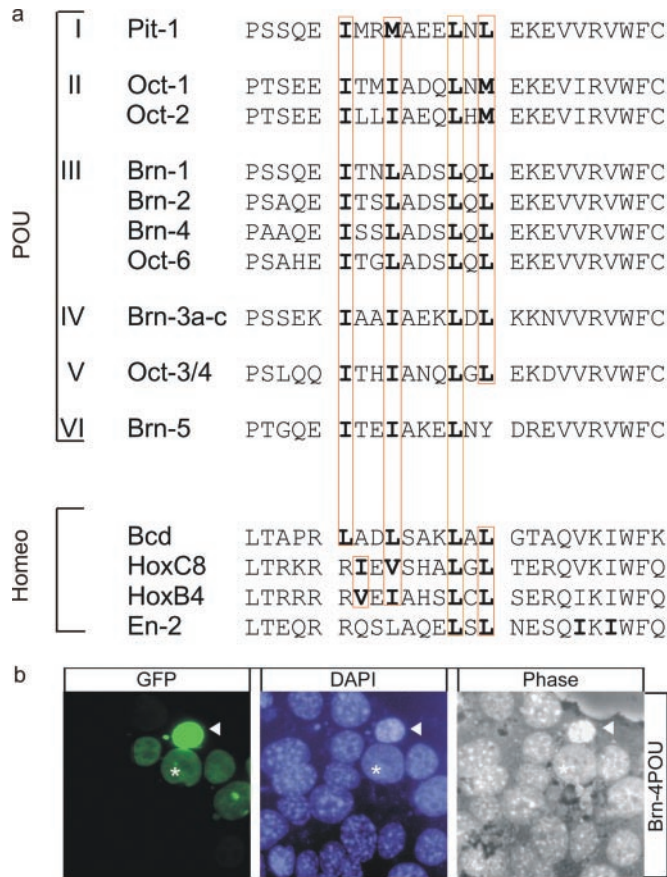


Figure 9. Conservation of the Oct-6 export sequence in other homeodomain proteins. (a) Comparison of the NES defined in this work with the corresponding regions of POU proteins representative of classes I–VI and select other homeodomain proteins. Bcd, Bicoid; En-2, Engrailed-2. (b) Nucleocytoplasmic shuttling activity of the Brn-4 POU domain. NIH 3T3 cells were transfected with an expression plasmid for wild-type GFP-Brn-4-POU. After cycloheximide treatment, transfected NIH 3T3 cells were subjected to heterokaryon formation with HeLa cells. After 2 h, cells were fixed, counterstained with DAPI (middle panel) and analyzed for GFP autofluorescence (left panel). Right panels show the heterokaryons in phase contrast. Donor mouse nuclei are marked by an arrowhead and recipient human nuclei by an asterisk.

The NES that we have identified in this study in Oct-6 is conserved in the homeodomain of most, if not all POU proteins. Brn-5 could be the exception, as the residue corresponding to L376 in the Oct-6 NES is replaced by a tyrosine residue (Figure 9a). Even more striking, other homeodomain proteins also contain the NES, such as the maternal effect protein Bicoid from *Drosophila melanogaster* which functions both within the nucleus and the cytoplasm. Other homeodomain proteins including many Hox proteins have hydrophobic leucine-rich sequences that differ only slightly from those of canonical NES and might as well be functional during nuclear export (Figure 9a). Other homeodomain proteins such as Engrailed proteins contain hydrophobic, leucine-rich sequences in helix 3 and the preceding loop in a position that strongly overlaps with the location of the Oct-6 NES (Figure 9a). Despite the fact that the hydrophobic residues do not exhibit the typical spacing of an NES, they have been shown to mediate nuclear export (40). Thus, many homeodomains appear to be capable of nuclear export. In evolutionary terms, it certainly makes sense to include into

a DNA-binding domain the necessary information for both nuclear import and export. Any new protein that acquires a homeodomain as its DNA-binding module during evolution would then automatically be able to reach its targets in the nucleus and leave it again. It might, however, also point to the fact that many homeodomain proteins have the capacity for nucleocytoplasmic shuttling and in fact might require this capacity to obtain their full activity or be correctly regulated during development.

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