POU domain proteins are important regulators of development and terminal differentiation based upon their transcriptional activity in the nucleus. Here, we analyzed the mechanism underlying the nuclear localization of Tst-1/Oct6, a member of this family that regulates events during neurogenesis and myelination. Nuclear localization of Tst-1/Oct6 was dependent on the POU domain, as its deletion prevented access to the nucleus, whereas its transfer to the amino terminus of β-galactosidase was sufficient to prompt nuclear accumulation of this normally cytosolic protein. Interestingly, nuclear localization and high affinity DNA binding were two independent functions of the POU domain and could be separated in several mutants. While specific high affinity binding to DNA required the presence of both the POU-specific and the POU homeodomain, the POU-specific domain was dispensable for nuclear localization of Tst-1/Oct6. Rather, the nuclear localization function was selectively contained within the POU homeodomain. Specifically, a basic cluster (GRKRRKKRT) preceding helix 1 of the homeodomain was shown by deletion mutagenesis to be involved in the nuclear localization of Tst-1/Oct6. This sequence, which is highly conserved among POU domain proteins, was by itself capable of translocating β-galactosidase to the nucleus defining it as the bona fide nuclear localization signal of Tst-1/Oct6 and presumably other POU domain factors.

POU domain proteins constitute a class of transcription factors that all share a highly conserved DNA-binding domain and are usually expressed during various critical, tightly controlled phases of embryonic development or cellular differentiation (for review see Refs. 1–5). A typical expression pattern for POU domain proteins is displayed by the class III member Tst-1, which is also known as Scip or Oct6 (6–12). In the mouse, it is transcribed from an intronless gene at the distal end of chromosome 4 that exhibits the features of an expressed retrotransposon (13, 14). During development, expression of this protein, which hereafter will be referred to as Tst-1/Oct6, is detected in embryonic stem cells, skin, neuronal subpopulations, and in precursors of myelinating glia (6, 11, 15–17). In the adult, expression of Tst-1/Oct6 persists in pyramidal neurons of layer 5 in the cerebral cortex and in skin (1, 6, 18).

Schwann cells which represent the myelinating glia of the peripheral nervous system have been used extensively to study the function of Tst-1/Oct6. In these cells, transient expression of Tst-1/Oct6 can be induced by axons and by agents that raise the intracellular cAMP level (10, 19). Expression of Tst-1/Oct6 correlates with a period of rapid cell division which immediately precedes the onset of myelination (17). In line with its supposed role during differentiation of myelinating glial cells, targeted expression of a dominant negative form of Tst-1/Oct6 in Schwann cells led to severe disturbances in the normal myelination program (20).

Like all other POU domain proteins characterized so far (2, 3, 5), Tst-1/Oct6 has to exert its function in the nucleus. Nuclear proteins obligatorily enter this cellular compartment via the nuclear pore complex after being synthesized in the cytoplasm (21). In general, they are actively transported through the nuclear pore. This active transport requires energy (22, 23), soluble factors, such as importin-α, importin-β, and Ran/TC4 (24–27), and a nuclear localization signal (NLS)1 within the protein targeted for nuclear import (for review see Ref. 28). NLSs were first discovered in the yeast Matα2 protein (29, 30) and in SV40 large T-antigen (31, 32) and are recognized by NLS-binding proteins such as the importin-α-importin-β complex. After the initial NLS recognition in the cytosol which is mainly mediated by importin-α, the complex docks to the nuclear pore via importin-β, before it gets translocated through the pore in a Ran/TC4-dependent manner (25). NLSs have been identified in a variety of nuclear proteins ranging in size from less than a hundred to more than a thousand amino acids (33), including polymerases (34), kinases and phosphatases (35, 36), transcription factors (37–39), histones (40), growth factors (41, 42), tumor suppressors (43, 44), and various viral proteins (31, 32, 45–48). Here, we have characterized the nuclear localization signal of Tst-1/Oct6, which is localized in the protein’s DNA-binding domain as a structure highly conserved among POU domain proteins in general. Its identification allows insights into the evolution of POU domain proteins and points to ways of regulating the access of POU domain proteins to the nucleus.

MATERIALS AND METHODS  

Plasmid Constructs—Tst-1/Oct6 expression plasmid pCMV/Tst-1 as well as the mutants pCMV/Tst-1 ΔN (missing amino acids 4–240), pCMV/Tst-1 ΔPOU (missing amino acids 241–395), pCMV/Tst-1 ΔPOUΔuc (missing amino acids 336–395), pCMV/Tst-1 ΔNC (missing amino acids 396–448), pCMV/Tst-1 ΔNC (missing amino acids 4–240 and 396–448), and pCMV/Tst-1 ΔC (carrying a double point mutation at amino acids 383 and 384 in the recognition helix of the POU homeodo---

1 The abbreviations used are: NLS, nuclear localization signal; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactosidase; PBS, phosphate-buffered saline.

* This work was supported by Grant We 13265/1 from the Deutsche Forschungsgemeinschaft (to M. W.) and a grant from the National Institutes of Health (to M. G. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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β-Galactosidase histochemistry was carried out as described (54). Shortly, cells were fixed for 5 min at 4 °C in PBS containing 2% formaldehyde and 0.2% glutaraldehyde. After washing, cells were stained in PBS containing 1 mg/ml X-Gal, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 2 mM MgCl2.

RESULTS

Tst-1/Oct6 Is a Predominantly Nuclear Protein—Using electrophoretic mobility shift assays as well as Western blot analysis, Tst-1/Oct6 has been shown in previous studies to be present in nuclear extracts from embryonic stem cells, embryonic carcinoma cells, proliferating Schwann cells, oligodendrocyte precursors, and stably transformed fibroblasts (9, 11, 13, 15). To be able to study the cellular distribution of Tst-1/Oct6 in more detail, we used rabbit polyclonal antibodies raised against full-length Tst-1/Oct6 protein that contained a 6×His tag fused to its amino terminus and was purified from baculovirus-infected Sf9 cells. When tested on nuclear and cytosolic extracts prepared from embryonic stem cells or U138 glial cells transiently transfected with Tst-1/Oct6, this antibody recognized specifically and at high dilution a protein of 47,000 that was enriched in the nucleus (Fig. 1, and data not shown). This protein was absent from U138 cells not transfected with an expression vector for Tst-1/Oct6 showing that this protein was indeed Tst-1/Oct6. A small fraction of Tst-1/Oct6 was also found in the cytosolic extracts. It is unclear at present whether this is an artifact of the extract preparation or represents truly cytosolic Tst-1/Oct6.

The POU Domain Targets Tst-1/Oct6 to the Nucleus—To be able to map the region of Tst-1/Oct6 responsible for its predominantly nuclear localization, we used a series of mutants in which single changes of the domain of the Tst-1/Oct6 protein had been removed (Table I). Their DNA binding activity and ability for transcriptional activation were assessed in standard electrophoretic mobility shift assays and transient transfection assays. As summarized in Table I, the results correlated well with previously published data mapping DNA binding to the POU domain and transactivation function to the amino-terminal part of the protein (50, 55, 56).

To determine the intracellular localization of Tst-1/Oct6 deletion mutants, we cotransfected Tst-1/Oct6 and the luciferase expression plasmid pRSVluc into various cell types and performed cell fractionation studies. The results obtained in U138 glial cells transfected with the wild-type Tst-1/Oct6 in approx. Fig. 1, but identical results were also obtained in CV1 and HeLa cells (data not shown). The quality of cytoplasmic and nuclear extracts was assessed in each case by Western blot analysis with a rabbit polyclonal antiserum directed against luciferase. Because luciferase is known to be a cytoplasmic protein present in the peroxisomes, it should preferably be detected in the cytoplasmic fraction, while it should be absent from the nuclear fraction. As shown in the bottom panel of Fig. 1, most of the luciferase is indeed localized in the cytoplasm, showing that contamination of the nuclear fraction with cytosol was minimal. Contamination of cytosol with nuclear proteins was equally low as evidenced by the distribution pattern of a high molecular weight band that exhibited weak cross-reactivity with the anti-Tst-1/Oct6 antiserum (data not shown). U138 cells transfected with wild-type Tst-1/Oct6 contained a 47,000 protein not present in mock-transfected cells, which was highly enriched in the nuclear fraction (Fig. 1). A similar pattern of distribution was also found for mutant Tst-1/Oct6 proteins that either had all sequences carboxyl-terminal (ΔC) or amino-terminal (ΔN) of the POU domain deleted.

When a mutant was assayed that just contained the POU domain of Tst-1/Oct6 (ΔN), no immunoreactivity could be observed in Western blot analysis (Fig. 1A). The fact that this mutant was not detected was explained by the specificity of the

main) have been described before (49, 50). Additional point mutants of Tst-1/Oct6 were created as follows. In pCMV/Tst-1 PM1 amino acids 262 and 263 were changed from two arginines to aspartic acid and serine; in pCMV/Tst-1 PM2 amino acids 269 and 270 were changed from threonine and glutamine to methionine and glutamic acid; in pCMV/Tst-1 PM3 amino acids 286 and 287 were changed from serine and glutamine to valine and aspartic acid; in pCMV/Tst-1 PM4 amino acids 292 and 294 were changed from arginine and glutamic acid to glutamic acid and glutamine; in pCMV/Tst-1 PM5 amino acids 338–340 were changed from two lysines and one arginine to three alanines; in pCMV/Tst-1 PM6 amino acids 387 and 388 were changed from two arginines to valine and aspartic acid; and in pCMV/Tst-1 dm amino acids 389 and 393 were changed from glutamine to arginine to a glycine and a tryptophan. The control plasmid pCMV/asTst-1 contained the region coding for Tst-1/Oct6 inserted in antisense orientation relative to the cytomegalovirus promoter. pRSVluc and pH5Oct-luc which contain the firefly luciferase gene under the control of the Rous sarcoma virus-cytomegalovirus promoter. pRSVluc and pH5Oct-luc which contain the firefly luciferase gene under the control of the Rous sarcoma virus-long terminal repeat and a combination of herpes simplex virus octamer motif (5'-GCATGCTAATGATATTCTTT-3') and rat prolactin minimal promoter, respectively, have been described before (50, 51).

all β-galactosidase expression vectors were based on pCMVlacz (a gift of Dr. G. E. DiMattia, London Regional Cancer Centre, London, Canada) which contained the lacZ gene under the control of the CMV promoter. This lacZ gene lacked the first eight nonessential amino acids and instead contained a eukaryotic translation consensus sequence at its 5' end. A small t intron A and polyadenylation signal at its 3' end. A short sequence encompassing the nuclear localization signal from the T-antigen of SV40 (32) was added to the 5' end of the lacZ gene, yielding pCMVlacZISV40tNLS. Similarly, fragments from the tst-1/ oct6 gene were added as NcoI fragments to the 5' end of lacZ. pCMVlacZ(POU) contained sequences coding for amino acids 239–404 of Tst-1/Oct6 (pCMVlaczPOULuc) and contained sequences coding for amino acids 239–324; and pCMVlacZ(POU)m contained sequences coding for amino acids 328–404. Plasmid pCMVlacZ(Tst-1 NLS) was obtained by inserting a short sequence corresponding to amino acids 334–341 of Tst-1/Oct6 (GRKRRKKRT) between NcoI and NruI sites directly behind the start methionine.

Expression, Transfection, and Preparation of Protein Extracts—CV1 cells were maintained in Dulbecco's modified Eagle's medium and U138 glioblastoma cells in RPMI 1640, both supplemented with 10% fetal calf serum. One day prior to transfection, CV1 or U138 cells were plated at a density of 2 × 10⁵ per 60-mm plate in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Cells were transfected with 2 μg of Tst-1/Oct6 expression vector and 2 μg of luciferase plasmid using the calcium-phosphate technique as described (49, 52). Cells were harvested 48 h posttransfection for luciferase assays (49) or for the preparation of extracts. Extracts were prepared as described (53) with minor modifications (52). Cells from two 60-mm plates yielded 300 μl of cytosolic extract and 150 μl of nuclear extract.

Western Blot Analysis—Western blot analysis was performed as described (52). Detection of luciferase activity was by autoradiography of 5 μg of cytoplasmic extract and 25 μg of nuclear extract was achieved using rabbit polyclonal antisera against either luciferase (gift of Dr. S. Subramani, La Jolla) or against full-length Tst-1/Oct6 purified from baculovirus-infected Sf9 cells. The amount of cytoplasmic and nuclear extract corresponded to the equivalent of 1.3 × 10⁵ cells.

Electrophoretic Mobility Shift Assay—Double-stranded oligonucleotides containing the herpes simplex virus octamer motif (5'-GCATGCTAATGATATTCTTT-3’) were generated and radiolabeled with [α-³²P]dCTP. For electrophoretic mobility shift assays, 0.5 ng of labeled probe was incubated with nuclear extracts of cells transfected with Tst-1/Oct6 expression plasmids for 20 min at room temperature in 10 μl of reaction containing 1% Triton X-100, 100 μM NaCl, 5 mM MgCl₂, 2 mM dithiothreitol, 0.1 mM EDTA, 2 μg of poly(dI-dC), and 0.5 μg of bovine serum albumin. One-third of each reaction was loaded onto a 5% nondenaturing acrylamide gel. Electrophoresis was in 0.5 × TBE at 200 V for 3 h.

Immunocytochemistry and β-Galactosidase Histochemistry—CV1 cells were grown on chamber slides (Linfenco, Nutley, N.J., or Nunc, Roskilde, Denmark) and transfected as described above. 48 h posttransfection, the medium was removed, and the cells were washed twice with PBS. Cells were then fixed with 3% formaldehyde in PBS for 20 min and treated with 1% Triton X-100 in PBS for 5 min. After washing the cells twice with PBS, cells were incubated for 20 min with the polyclonal anti-Tst1 antiseraum (diluted 1:1000) in PBS containing 0.1% Tween 20 (PBST). After washing three times with PBST, cells were incubated 20 min with Cy3-conjugated goat anti-rabbit antibodies (Dianova) diluted 1:500 in PBST. Cells were washed extensively with PBST, mounted, and analyzed on an Axiovert microscope (Zeiss).
antiserum that reacted with epitopes in the regions both ami-
nino- and carboxyl-terminal of the POU domain, but not with
the POU domain itself. A bacterially expressed POU domain of
Tst-1/Oct6, for instance, was not recognized by the antiserum
(data not shown). Electrophoretic mobility shift analysis, on
the other hand, clearly revealed a specific complex of high
mobility, indicative of this mutant, that was predominantly
found in the nuclear extract and comigrated with the complex
formed by the bacterially expressed POU domain of Tst-1/Oct6
(Fig. 1B and data not shown).

To corroborate the importance of the POU domain for nu-
clear localization and address the possibility of additional nu-
clear localization signals outside the POU domain, we deleted
the POU domain from the protein and analyzed the cellular
distribution of this mutant (ΔPOU; Fig. 1A). Contrary to all
other mutants analyzed, this mutant was predominantly found
in the cytosolic fraction. These results imply that the POU
domain is both necessary and sufficient to target Tst-1/Oct6 to

Nuclear Localization of Tst-1/Oct6

The POU Homeodomain Is Essential for Nuclear Localiza-
tion—Analysis of nuclear localization by cell fractionation
has been shown in the past to be susceptible to artifacts (57).
During extract preparation, nuclear proteins that were not
bound to nuclear constituents leaked from the nucleus and
were recovered in the cytosol despite carrying a nuclear local-
ization signal. Thus it is conceivable that the ΔPOU mutant
was retrieved in the cytosolic fraction merely because of its
inability to bind to DNA not, however, because of the true
absence of a nuclear localization signal.

Therefore, we tried to corroborate and extend our cell frac-
tionation studies by immunocytochemistry on transiently
transfected CV1 cells. The antiserum raised against Tst-1/Oct6
yielded only a weak background staining when employed to
mock-transfected cells (Fig. 2A). Transfection with an expres-
sion plasmid for wild-type Tst-1/Oct6, on the other hand, led to
an intense staining of cell nuclei (Fig. 2B). Comparable nuclear
staining was also detected with mutants ΔN and ΔC, indicating
that these regions of Tst-1/Oct6 are dispensable for nuclear
localization (Fig. 2, C and F). In contrast, the ΔPOU mutant
was present predominantly in the cytosol, leading to a uniform,
dispersed labeling of the entire cell (Fig. 2D). This obvious
agreement between cell fractionation studies and immunocyto-
chemistry strongly confirms the role of the POU domain in
targeting Tst-1/Oct6 to the cell nucleus.

Intriguingly, a comparable loss of nuclear localization was
observed with a mutant, which had only the POU homeo-
domain removed but still retained the POU-specific domain
(ΔPOU HD, Fig. 2E). This clearly points to the importance of
the POU homeodomain for nuclear localization.

The POU Homeodomain Is Sufficient to Direct β-Galactosid-
ase to the Cell Nucleus—For a closer inspection we transferred
parts of Tst-1/Oct6 to a heterologous protein and evaluated the
localization of the corresponding fusions. The fusion partner
was cytosolic β-galactosidase (Fig. 3B). By the amino-terminal
addition of a short stretch of amino acids, which represents the
nuclear localization signal (NLS) of SV40 T-antigen, β-galacto-
sidase could, however, be directed to the cell nucleus as shown before (Fig. 3C) (58). When the POU domain of Tst-1/Oct6 was placed onto LacZ instead of the NLS of SV40 T-antigen, it was also able to target the fusion protein to the cell nucleus (Fig. 3D). In contrast, nuclear localization was not achieved by fusing the POU domain coding sequences in the opposite orientation to the lacZ gene, although a protein of the correct size was expressed in transfected cells (data not shown). This transfer experiment further corroborated the importance of the POU domain in the nuclear localization of Tst-1/Oct6.

In addition to the intact POU domain, we also transferred isolated regions of it to LacZ. The LacZ(POUHD) fusion contained the POU homeodomain of Tst-1/Oct6 as well as seven amino acids from the preceding linker region, whereas the LacZ(POUS) fusion carried the POU-specific domain of Tst-1/Oct6 and most of the adjacent linker region which joins it to the POU homeodomain. Nuclear localization was only observed for the fusion protein between the POU homeodomain and β-galactosidase (Fig. 3E). The POU-specific domain, on the contrary, proved to be incapable of directing β-galactosidase to the nucleus as the fusion between LacZ and the combination of POU-specific domain and adjacent linker remained cytosolic (Fig. 3E).

A Basic Cluster in the POU Homeodomain Is Involved in the Nuclear Localization of Tst-1/Oct6—To further define the nuclear localization signal present in the POU domain, we substituted highly conserved amino acids both in the POU-specific and the POU homeodomain (Table II). These regions were chosen because of their proven relevance for the function of the POU domain (59–62). With the exception of mutant dn which affected a glutamine and an arginine residue in helix 3 of the POU homeodomain, all other mutants had lost their ability to bind to DNA. Concomitant with their loss of DNA binding these mutants failed to stimulate transcription from an octamer-containing promoter (Table II).

All mutants that affected the POU-specific domain (PM1, PM2, PM3, and PM4) were still found to be nuclear in immunocytochemistry studies, although none of them exhibited significant binding to an octamer DNA element. This finding is particularly noteworthy for the mutation present in PM1, which not only disrupted the cluster of basic amino acids within helix 1 of the POU-specific domain that on the basis of its positive charge would have been a good candidate for a nuclear localization signal (28). In agreement with our transfer
Mutated amino acids | Localization of mutation | Activation of HSV oct-luc<sup>a</sup> | DNA binding<sup>b</sup> | Subcellular localization<sup>c</sup>
---|---|---|---|---
PM1 262<sup>a</sup>-D, 263<sup>a</sup>-S  | POU<sub>4</sub>, helix 1  | -- | -- | Nuclear
PM2 269<sup>a</sup>-M, 270<sup>a</sup>-E  | POU<sub>4</sub>, helix 2  | -- | -- | Nuclear
PM3 286<sup>a</sup>-V, 287<sup>a</sup>-D  | POU<sub>4</sub>, helix 3  | -- | -- | Nuclear
PM4 292<sup>a</sup>-K, 294<sup>a</sup>-E  | POU<sub>4</sub>, helix 3  | -- | -- | Nuclear
PM5 339<sup>a</sup>-A, 339<sup>a</sup>-A, 340<sup>a</sup>-A  | POU<sub>4</sub>, basic cluster  | -- | -- | Cytoplasmic
PM6 387<sup>a</sup>-V, 388<sup>a</sup>-D  | POU<sub>4</sub>, helix 3  | -- | -- | Nuclear
mt 383<sup>a</sup>-V, 384<sup>a</sup>-S  | POU<sub>4</sub>, helix 3  | -- | -- | Nuclear
dn 389<sup>a</sup>-G, 393<sup>a</sup>-W  | POU<sub>4</sub>, helix 3  | + | + | Nuclear

<sup>a</sup>Transcriptional activation of HSV oct-luc was determined by transient transfection in U138 cells as described previously in at least three independent experiments, each performed in duplicate.

<sup>b</sup>DNA binding was determined in standard electrophoretic mobility shift assays with the HSV octamer element as a probe and whole cell extract from transiently transfected U138 cells as a protein source.

<sup>c</sup>Cellular localization was determined in immunocytochemistry studies on CV1 cells transiently transfected with the respective Tst-1/Oct6 mutants.

experiments, these results argue against a participation of the POU-specific domain in the nuclear localization of Tst-1/Oct6. Similar to the mutations in the POU-specific domain, most homeodomain mutants remained unaffected in their nuclear localization despite being defective in their DNA binding activity and transcriptional activation function. None of the proteins with mutations in helix 3 of the POU homeodomain were impaired in their nuclear localization function, at least at least two of them (PM6 and dn) disrupted the basic cluster present in this DNA recognition helix. Therefore, it seemed unlikely that helix 3 of the POU homeodomain was involved in nuclear localization. This conclusion was also supported by the finding that helix 3 when transferred to a cytosolic form of luciferase was not able to target this protein to the nucleus (data not shown).

In marked contrast to all other analyzed mutants, PM5 exhibited a strong cytoplasmic staining (Fig. 4A). PM5 had three positive charges removed from the basic cluster at the beginning of the POU homeodomain. Thus, while there are three basic clusters in the POU domain, only the one preceding helix 1 of the POU homeodomain was involved in nuclear localization.

A Basic Cluster from the POU Homeodomain of Tst-1/Oct6 Is Sufficient to Direct β-Galactosidase to the Cell Nucleus—To analyze whether this basic cluster alone represented the nuclear localization signal of Tst-1/Oct6 or functioned only in the context of the POU homeodomain as part of a more complex signal, we transferred the isolated basic cluster to the amino terminus of LacZ and analyzed the resulting β-galactosidase fusion for its cellular localization. As shown in Fig. 4B, this cluster was sufficient to direct β-galactosidase to the nucleus of transfected cells. Indeed, this short stretch of amino acids from Tst-1/Oct6 was as efficient in targeting a heterologous protein to the cell nucleus as the NLS from SV40 T-antigen and might be regarded as the NLS of Tst-1/Oct6.

**DISCUSSION**

Most proteins that enter the nucleus do so by an active transport through the nuclear pore that requires the presence of an NLS in the transported protein. Although there is no consensus sequence, NLS are usually short sequences that contain a high proportion of positively charged amino acids. They occur at various locations within the protein in single or multiple, functionally redundant copies. They can be contained within a contiguous stretch of amino acids or may be bi- or multipartite instead (28, 33). Furthermore, NLS can be strongly dependent in their function on the exact flanking sequences (63, 64). Deletion of an NLS from a nuclear protein leads to its redistribution to the cytoplasm, whereas its addition to a heterologous cytoplasmic protein often results in an accumulation of this protein in the nucleus.

**TABLE II**

Characteristics of Tst-1/Oct6 point mutants

| Mutated amino acids | Localization of mutation | Activation of HSV oct-luc<sup>a</sup> | DNA binding<sup>b</sup> | Subcellular localization<sup>c</sup>
|---|---|---|---|---
| PM1 262<sup>a</sup>-D, 263<sup>a</sup>-S  | POU<sub>4</sub>, helix 1  | -- | -- | Nuclear
| PM2 269<sup>a</sup>-M, 270<sup>a</sup>-E  | POU<sub>4</sub>, helix 2  | -- | -- | Nuclear
| PM3 286<sup>a</sup>-V, 287<sup>a</sup>-D  | POU<sub>4</sub>, helix 3  | -- | -- | Nuclear
| PM4 292<sup>a</sup>-K, 294<sup>a</sup>-E  | POU<sub>4</sub>, helix 3  | -- | -- | Nuclear
| PM5 339<sup>a</sup>-A, 339<sup>a</sup>-A, 340<sup>a</sup>-A  | POU<sub>4</sub>, basic cluster  | -- | -- | Cytoplasmic
| PM6 387<sup>a</sup>-V, 388<sup>a</sup>-D  | POU<sub>4</sub>, helix 3  | -- | -- | Nuclear
| mt 383<sup>a</sup>-V, 384<sup>a</sup>-S  | POU<sub>4</sub>, helix 3  | -- | -- | Nuclear
dn 389<sup>a</sup>-G, 393<sup>a</sup>-W  | POU<sub>4</sub>, helix 3  | + | + | Nuclear

<sup>a</sup>Transcriptional activation of HSV oct-luc was determined by transient transfection in U138 cells as described previously in at least three independent experiments, each performed in duplicate.

<sup>b</sup>DNA binding was determined in standard electrophoretic mobility shift assays with the HSV octamer element as a probe and whole cell extract from transiently transfected U138 cells as a protein source.

<sup>c</sup>Cellular localization was determined in immunocytochemistry studies on CV1 cells transiently transfected with the respective Tst-1/Oct6 mutants.

The present study shows that the POU domain of Tst-1/Oct6 harbors a signal that fulfills the above-mentioned criteria of an NLS. Consequently, deletion of the POU domain caused the resulting Tst-1/Oct6 mutant PM5 using rabbit anti-Tst1 antisera (diluted 1:1000) as primary and Cy<sup>3</sup>-conjugated goat anti-rabbit antibodies as secondary antibodies (diluted 1:500). B, histochemical X-Gal staining of β-galactosidase in cells transfected with Tst-1 NLS β-galactosidase.

**FIG. 4. Functional analysis of the NLS from Tst-1/Oct6.** A, immunocytochemistry on cells transiently transfected with Tst-1/Oct6 mutant PM5 using rabbit anti-Tst1 antisera (diluted 1:1000) as primary and Cy<sup>3</sup>-conjugated goat anti-rabbit antibodies as secondary antibodies (diluted 1:500). B, histochemical X-Gal staining of β-galactosidase in cells transfected with Tst-1 NLS β-galactosidase.

The present study shows that the POU domain of Tst-1/Oct6 harbors a signal that fulfills the above-mentioned criteria of an NLS. Consequently, deletion of the POU domain caused the resulting Tst-1/Oct6 mutant PM5 using rabbit anti-Tst1 antisera (diluted 1:1000) as primary and Cy<sup>3</sup>-conjugated goat anti-rabbit antibodies as secondary antibodies (diluted 1:500). B, histochemical X-Gal staining of β-galactosidase in cells transfected with Tst-1 NLS β-galactosidase.

Mapping of the NLS was refined in consecutive studies to the POU homeodomain. Transfer of the POU-specific domain to β-galactosidase, on the other hand, did not cause the fusion protein to become nuclear. The absence of a nuclear localization function from the POU-specific domain was emphasized by the fact that none of the amino acid changes introduced into the four α-helices of the POU-specific domain (59, 60) disrupted nuclear localization, although severely impeding other functions of Tst-1/Oct6. Not even the removal of two positive charges from the basic cluster within helix 1 of the POU-specific domain in PM1 prevented Tst-1/Oct6 from becoming nuclear. Our conclusions are also supported by the fact that a naturally occurring splice variant of the POU domain protein Pit-1 which did not contain a POU-specific domain was found to enter the nucleus and serve as a dominant repressor of Pit-1 function (70, 71).

Taking the accumulation of positively charged amino acids within nuclear localization signals into account, the POU ho-
Table III
Comparison of the NLS from Tst-1/Oct6 with corresponding regions of other POU domain proteins

| POU class | POU protein | Amino acid sequence |
|-----------|-------------|---------------------|
| I         | Pit-1       | ERKKRKRTT           |
| II        | Oct-1       | SRRRKKRTS           |
|           | Oct-2       | GRRRKKRTS           |
|           | Skn-1       | GRKKRTS             |
| III       | Brn-1       | GRKKRTS             |
|           | Brn-2       | GRKKRTS             |
|           | Tst-1/Oct6  | GRKKRTS             |
|           | Brn-4       | GRKKRTS             |
| IV        | Brn-3.0     | GKKKRRTS            |
|           | Brn-3.1     | SRRRKKRTS           |
|           | Brn-3.2     | AEKKKRTS            |
| V         | Oct-3/4     | QRRRKKRTS           |
|           | Spri-1      | QARKRRSAS           |
| VI        | Emt/Brn-5   | SKKKRTS             |
| Homeo     | En          | NDEKRPRTA           |
|           | Mat α2      | STKPYRGHR           |

We expect our results for Tst-1/Oct6 to be prototypic for other members of the whole family of POU domain proteins.

Interestingly, the high conservation of this basic region within the POU domain family contrasts sharply with its apparent absence in several classic homeodomain proteins. The yeast Mata2 protein, for instance, has two NLS sequences that function independently of each other and are both localized at positions different from the NLS of Tst-1/Oct6 (29, 30). Thus, the NLS seems to be a recent acquisition of POU domain proteins during evolution. It deserves to be noticed that the presence of an NLS in the DNA-binding domain of the protein is a principle also realized in other classes of transcription factors, including HMG box, HLH, and bZip proteins (37–39). Although DNA binding and nuclear localization are functionally separable, colocalization of both functions might have evolved as a consequence of exon shuffling in higher eukaryotes.

The NLS of Tst-1/Oct6 is structurally very similar to the NLS of SV40 T-antigen (PKKKRKV) (31). Unlike the majority of nuclear proteins that contain bipartite NLS (28), Tst-1/Oct6 and T-antigen have single contiguous stretches of positively charged residues that serve as their NLS. The analogy reaches even further. The NLS of SV40 T-antigen is in close proximity to phosphorylation sites for casein kinase II and cdk/cdc2. Phosphorylation of these sites has been shown to increase or reduce the rate of nuclear import, respectively (73, 74). Interestingly, the NLS of Tst-1/Oct6 overlaps with a potential phosphorylation site for protein kinase A and an as yet unidentified M-phase-specific kinase, which similar to the NLS itself is strongly conserved among POU proteins and has been shown to be the target of phosphorylation in the related POU domain proteins Pit-1 (75, 76) and Oct-1 (77). Although not observed so far, it is intriguing to speculate that phosphorylation at this site could regulate nucleocytoplasmic transport of POU domain proteins in a manner similar to other proteins such as T-antigen (for review see Ref. 78). This would provide yet another means for regulation of POU domain protein activity.

As mentioned above, the POU domain is also the main region for interactions between members of this class of transcription factors and other cellular proteins (49, 65–69, 79). It is easily conceivable that such interactions also influence the accessibility of the NLS of Tst-1/Oct6. The POU domain might be an excellent target for interactions with cytosolic retention factors similar to the ones identified in other classes of transcription factors (80). Our characterization of the NLS of Tst-1/Oct6 should therefore open new directions in the functional analysis of POU domain proteins.

Acknowledgments—We thank Dr. G. DiMattia for the gift of pCMV- 

lacZ and Dr. S. Subramani for providing pRSVlacZ and rabbit polyclonal antisera directed against luciferase. We are especially grateful to Dr. S. Rhodes for his help with the production of the polyclonal antibodies against Tst-1/Oct6.

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