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ARTICLES

Subnuclear Partitioning and Functional Regulation of the Pit-1 Transcription Factor

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Abstract Subnuclear compartmentation is postulated to play an important role in many aspects of nuclear metabolism. To directly test an application of this model to transcription factor function, we examined the subnuclear partitioning behavior of Pit-1, a tissue-specific, POU-class transactivator. Biochemical and in situ assays indicate the nuclear pool of Pit-1 is normally divided between two compartments: the majority being differentially soluble in detergent, and a significant insoluble fraction (~20%) bound to the nuclear matrix. Examination of Pit-1 deletion mutants and chimeric fusions reveal the highly conserved 66 amino acid POU-specific domain contains a necessary and sufficient nuclear matrix targeting signal. The nuclear partitioning behavior of several natural or engineered point mutations of Pit-1 was also examined. Surprisingly, the inactive point mutants were completely matrix-bound, irrespective of their ability to bind Pit-1 specific DNA. These results suggest that dynamic partitioning of Pit-1 is a component of its normal transactivator function that takes place upon the insoluble nuclear substructure where transcription occurs. J. Cell. Biochem. 72:322–338, 1999.

Key words: transcription; pituitary; protein-protein interaction; nuclear matrix; RNA metabolism

The mammalian nucleus is organized into compartments that partition different biochemical processes [Clemson and Lawrence, 1996; Nickerson et al., 1995; Spector, 1993]. Partitioning of the nucleus can be viewed in terms of both spatial organization and solubility of its molecular constituents. Evidence of subnuclear compartmentation exists for ribosomal RNA transcription and ribosome assembly, DNA replication, and messenger RNA transcription and processing [Clemson and Lawrence, 1996; Nickerson et al., 1995; Spector, 1993]. For efficient expression of protein encoding genes, it is hypothesized that the architectural relationship of key factors involved in RNA metabolism is critically important [Clemson and Lawrence, 1996; Spector, 1993; Stein et al., 1995; Xing et al., 1995].

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A related biological issue in the study of DNA-binding transcription activators involves determining how they function efficiently within the complex environment of eukaryotic nuclei. In a background of 6×10^9 basepairs of DNA, how do cells ensure that activators occupy cognate sites to achieve physiologically effective levels of gene expression? Nuclear sequestration of the activator, high-affinity and site-specific DNA binding, and chromatin accessibility all play a role. A model of nuclear metabolism, including transcription, purports that nuclear events may be regulated through their association with nuclear architecture, the relatively insoluble fraction termed the nuclear matrix [Berezney et al., 1995; Nickerson et al., 1995].

The nuclear matrix is defined biochemically and morphologically as the nonchromatin structure that is resistant to detergent extraction and nuclease digestion followed by either physiological- [Jackson and Cook, 1988; Nickerson et al., 1997] or high salt- [He et al., 1990] treatment. By either method, the matrix is morphologically comprised of a complex set of interwoven filaments and granular masses. Although it is widely reported that transcriptionally active genes are matrix-associated [Abulafia et al.,

1984; Barrack, 1987; Ciejek et al., 1983; Intres and Donady, 1985; Jost and Seldran, 1984; Lelievre et al., 1996; Murty et al., 1988; Nickerson et al., 1995; Robinson et al., 1983; Small et al., 1985; Stein et al., 1995], our understanding of the relationship between transcription factor association with the matrix is just emerging. Though several members of the steroid and thyroid receptor superfamily have been reported to be associated with insoluble nuclear structure [Barrack, 1987; Spelsberg et al., 1996; Tang and DeFranco, 1996; van Steensel et al., 1992], the mechanism of this interaction and its functional significance has remained obscure. Recently, a few studies have suggested distinct molecular mechanisms may exist for targeting transcription factors to the matrix. These reports describe regions of the carboxyl-termini from the androgen and glucocorticoid receptors [van Steensel et al., 1992] and a specific 30 amino acid region in the C-terminus of the AML transcription family [Zeng et al., 1997]. Recent evidence also suggests that the dynamic association of the glucocorticoid receptor with the matrix is ATP-dependent [Tang and DeFranco, 1996].

The subnuclear spatial targeting of karyophilic proteins is a related area of increased interest. Several transcription factors have been shown to occupy distinct regions of the nucleus [Grande et al., 1997]. Spatial partitioning has also been shown for v- and c-*myc* [Spector et al., 1987], GATA-1 [Elefanty et al., 1996], and WT1 [Larsson et al., 1995]. As a result of a chromosomal translocation, the aberrant subnuclear localization of the retinoic acid receptor, through an in-frame fusion with the PML gene, is associated with a leukemic cell phenotype [Dyck et al., 1994; Kakizuka et al., 1991]. It is interesting that a +KTS splicing variant of WT1 specifically targets the protein to RNA splicing speckles and -KTS isoforms are targeted to other subnuclear domains that colocalize with different transcription factors such as Sp1 and TFIIB [Larsson et al., 1995]. Some transcription factors including the Oct-1 [Van Wijnen et al., 1993], YY-1 [Guo et al., 1995], and NF-1 [Sun et al., 1994] have been shown to be partitioned in the nucleus based upon solubility. The large subunit of RNA polymerase II has recently been shown to be matrix-bound at specific subnuclear regions in certain cells [Bregman et al., 1995; Mortillaro et al., 1996; Grande et al., 1997]. In addition to transcription factors, other examples of subnuclear targeting include the arginine- and serine-rich (RS) domain that directs proteins such as the *transformer* and *suppressor of white apricot* splicing factors to RNA processing centers [Li et al., 1991]. In a related fashion, a subnuclear targeting domain has also been identified that localizes DNA methyltransferase and DNA ligase to replication "factories" during S phase [Leonhardt et al., 1992; Cardosa et al., 1997]. The combined findings to date suggest that signals for subnuclear partitioning of proteins are important for functional compartmentation.

To investigate the molecular mechanisms of solubility and spatial targeting of a transcription factor in the nucleus, we initiated a study of a POU-domain protein, Pit-1 [Rosenfeld, 1991], important in the development of the mammalian anterior pituitary (also known as GHF-1 [Bodner et al., 1988] and PUF-1 [Cao et al., 1988]). Pit-1 specifically activates only a few known genes, including prolactin [Ingraham et al., 1988, 1990; Cao et al., 1988], growth hormone [Lefevre et al., 1986; Nelson et al., 1988], TSHβ [Lin et al., 1995], and its own gene, *pit*-1 [Chen et al., 1990; McCormick et al., 1990]. Pit-1 is also crucial for the developmental appearance and maintenance of three cell types in the anterior pituitary: lactotropes, somatotropes, and thyrotropes [Lin et al., 1994]. As a cell-type-specific DNA-binding activator of transcription and a critical control protein in mammalian pituitary cell differentiation, Pit-1 and its discrete list of known target genes provide an excellent system for investigating the relationship between nuclear compartmentation and transcription.

In this study, we asked if functional subnuclear partitioning of Pit-1 is evident and, if so, what protein domain(s) is responsible? Our biochemical and immunolocalization data indicates the pool of Pit-1 is divided between two differentially soluble nuclear compartments, and an insoluble fraction bound to the nuclear matrix in a heterogeneous nucleoplasmic distribution. Genetic, biochemical, and microscopic data suggest that the POU-specific domain (PSD) in Pit-1 regulates partitioning, and is dominant in its targeting capacity relative to tested subnuclear targeting signals. We also show the PSD can act as a nuclear matrix-targeting signal for soluble proteins. Lastly, examination of functionally- and developmentally-defective point mutants of Pit-1 that lead

to dwarfism, and two newly engineered nonfunctional point mutations, reveals dramatic alterations in their subnuclear partitioning. Taken together, these data further support the notion that transcription factor activity and compartmentation are highly integrated with nuclear architecture.

MATERIALS AND METHODS

Bacterial Pit-1 Expression, Purification, and Monoclonal Antibody Production

Pit-1 cDNA containing *Nde*I and *BamH*I restriction sites produced by rtPCR using polyA$^+$ mRNA from GH3 cells was ligated directionally into an expression plasmid pET 16b (Novagen, Madison, WI). The construction, confirmed by DNA sequencing, was designed to produce a fusion Pit-1 polypeptide with an N-terminal tag of 10 histidines (Novagen). To purify the insoluble His-tagged Pit-1 protein, the pellet was denatured in urea and applied to a His Bind column (Novagen) to achieve about 95% pure protein based upon SDS-PAGE and silver staining. Renatured bPitHis functioned in gel shift and footprinting assays with the prolactin proximal Pit-1 binding site, and also recapitulated prolactin transcription in cell-free transcription assays using HeLa nuclear extract [Cao et al., 1987; Dingham et al., 1983; Mancini et al., unpublished data]. The bPitHis was then used for monoclonal antibody (mAb) production. Using immunoprecipitation, immunoblotting, and immunolabeling criteria, two polyclonal myeloma lines (5E4 and 2C11) were selected for single cell cloning. Epitope mapping (data not shown) demonstrated that these two mAbs recognize different epitopes in the ST region of the Pit-1 protein.

Pit-1 Mammalian Expression Plasmids and Mutagenesis

Full-length and truncated T7- or Flag-tagged Pit-1 genes were placed into the pCEP4 mammalian expression vector (Invitrogen, La Jolla, CA) for expression in GH3 and nonpituitary cells (Fig. 3). The insert cDNAs encoding the T7 tagged-Pit were prepared by isolation of *Nde*I/*Not*I fragments from various Pet21a (Novagen) constructions that express full-length and deletions of bacterial Pit-1 generated by PCR. bPit-1 expression plasmids, validated by DNA sequencing (Sequenase), were designed to produce N-terminally T7-tagged and carboxyl-terminal His-tagged fusion Pit-1 proteins. The *Nde*I sites (with the T7 tag ATG translation start codon) of the isolated fragments were filled-in (Klenow and reverse transcriptase) and the resulting fragments (lacking the C-terminal His tag) were ligated into the *Pvu*II/*Not*I site of the polycloning region of pCEP4. The DNA sequence of the ligation joints was verified by DNA sequencing. pCEP4 expression plasmids for Flag-tagged (Kodak, Rochester, NY) Pit-1 were generated by ligation of the various full-length and truncated fragments into pCEP4-Flag vector (a generous gift from W.-H. Lee and P.-L. Chen) which were also subsequently confirmed by DNA sequencing. For expression in nonpituitary cells, nontagged Pit-1 was also placed in pCEP4 expression vectors at the *Hin*dIII/*Bam*HI sites. The W261C and A158P dwarf mutations were introduced into Flag-tagged rat Pit-1 by PCR and confirmed by DNA sequencing. The α-1 (E133P/A136P) and α-4 (A183P/A188P) mutants were obtained through site directed PCR-mutagenesis and confirmed by sequencing. Amino acids 1–100 from the arg/ser-rich *transformer* protein (cDNA a gift from M. McKeown) were Flag-tagged by insertion into pCEP4-Flag and chimeric fusions were made with full length or portions of Pit-1 using *HindIII* and *BamHI* sites. DNAs for transfection into mammalian cells were prepared using Qiagen reagents.

Cell Transfections and Transcription Assays

Cells were grown as described [Cao et al., 1987]. Transfections of GH3 cells were performed using Lipofectin (Life Technologies, Bethesda, MD) reagents and protocols. Calcium phosphate (5' → 3'), with a 3 min 10% DMSO shock, was used in the transfection of other cell types. Transient transfection transcription assays were done as previously described [Smith et al, 1995]. Briefly, these data were obtained using CV1 or HeLa cells transiently cotransfected with the tagged-Pit-1 vectors, a luciferase reporter driven by a synthetic prolactin promoter/enhancer contained six copies of the Pit-1 binding site [Cao et al., 1987, Smith et al., 1995], and a *lacZ* control vector for normalization.

Preparation of Core Nuclear Matrix

Spatial and solubility partitioning of Pit-1 in GH3 in situ was analyzed by extracting cytocentrifuged cells according to previously published protocols [He et al., 1990; Durfee et al., 1994;

Mancini et al., 1994]. CV1 and HeLa were extracted while attached to poly-L-lysine coated substrates. The cells were washed in PBS and sequentially treated with 0.5% Triton X-100 in ice-cold cytoskeletal buffer (CSK), digestion buffer with RNAse-free DNAse I, 0.25 M $(NH_4)_2SO_4$ and then with 2 M NaCl followed by fixation in CSK using 4% paraformaldehyde. In some cases, cells were only CSK-extracted for 3 min on ice and then fixed. DNA digestion was confirmed by DAPI staining. Solubility partitioning of Pit-1 was also analyzed by extracting suspended or attached cells followed by Western analysis using 2C11, T7, or Flag mAbs and chemilluminescence detection system (ECL Kit, Amersham, Arlington Heights, IL).

Immunocytochemistry and Digital Microscopy

Cells were grown [Cao et al., 1987] on coverslips and prepared for immunolabeling. Endogenous Pit-1 in GH3 cells, or exogenous nontagged Pit-1 in nonpituitary cells, was detected with anti Pit-1 mAb 2C11 (IgG). Colocalization with SC-35 domains was performed with the B1C8 mAb (IgM) [Blencowe et al., 1994; Wan et al., 1994]. T7 (Novagen) and Flag (IBI, Kodak) mAbs (both IgG) were used to detect epitope-tagged Pit-1 full-length and truncated polypeptides. Laser scanning confocal microscopy was performed with a Zeiss LSM 310 (Ar and HeNe lasers), or a Molecular Dynamics LSM 2001 (KrAr dual-line laser). Nonconfocal images were collected with a three chip, color CCD camera (Hamamatsu) using separate filters (fluorescein, Texas Red, and/or DAPI) and merged using Adobe Photoshop. pCEP4T7Pit or pCEP4-Flag-Pit and truncated epitope-tagged (T7 and Flag) Pit-1 plasmids were transfected into GH3, HeLa, or CV1 cells. Eight to 24 h following removal of the DNA, the cells were prepared for immunolabeling by paraformaldehyde fixation (4% v/v for 30 min), Triton X-100 extraction (0.5% v/v for 10–30 min), and incubation in the primary mAbs ([diluted hybridoma supernatant [2C11 1:10; B1C8 1:30] or purified IgG [anti-Flag 1:4,000; anti-T7 1:4,000] for 1 h at 37°C]). Following washing, coverslips were exposed to fluorescein or Texas Red-conjugated goat anti-mouse secondary antibody (1:600 IgG or IgM for 30 min at room temperature; Southern Biotechnology, Birmingham, AL). All antibody and washing solutions were made in TBST blocking buffer (20 mM Tris, 137 mM NaCl pH 7.6 0.02% NaAzide, 0.1% Tween-20, pH 7.6) containing 5% dry milk and 0.02% NaAzide. In some cases, 0.5 mg/ml DAPI (10 sec) was used to counterstain DNA.

RESULTS

Subnuclear Partitioning of Pit-1

Our investigation of the subcellular distribution of rat Pit-1 began with production of mAbs against a bacterially-expressed, functional, His-Pit-1 fusion protein. Figure 1A shows an example of the specificity of anti-Pit-1 mAbs 2C11 and 5E4. Extracts from GH3 cells, a rat growth hormone- and prolactin-secreting pituitary tumor line, were immunoprecipitated and immunoblotted. Consistent with the size of Pit-1, both 2C11 and 5E4 immunoprecipitated a 33 kD protein. Epitope mapping (pTOPE, Novagen) indicated that both mAbs recognize a portion of the serine- and threonine-rich domain (ST) near the N-terminus of Pit-1 (unpublished observations).

To investigate the biochemical properties of Pit-1 in the nucleus, and to directly test whether Pit-1 partitions with differentially soluble nuclear fractions, unsynchronized GH3 cells were sequentially extracted to obtain the core filament nuclear matrix [He et al., 1990]. This procedure has been used to biochemically and morphologically characterize the association of multiple components involved in nuclear metabolism with the subnuclear architecture [Durfee et al., 1997; Mancini et al., 1994; Nickerson et al., 1995; Penman, 1995]. Briefly, immunoblotting was performed using the 2C11 antibody on whole cell lysates and several fractions of GH3 proteins, including those soluble in detergent-containing buffer, proteins solubilized by extended exposure to detergent (during the DNase digestion), those soluble in 0.25 M ammonium sulfate, and, finally, proteins that are soluble or insoluble in 2 M NaCl. Confirmation of the extraction efficiency, and retention of morphological integrity, was evidenced by the absence of DAPI-stained DNA following core extraction and routine microscopic examination (data not shown). The data (Fig. 1B) show that Pit-1 is differentially detected in both soluble (CSK, DNase) and insoluble (core matrix) fractions; approximately 20% of the Pit-1 nuclear pool is retained in the 2 M NaCl-insoluble core filament nuclear matrix fraction. The Pit-1 released during DNase treatment in digestion buffer (also containing 0.5% Triton-X

100) is not due to digestion of DNA, as a DNase-free mock digestion (Mock DNase) releases equal amounts of Pit-1 (Fig. 1B).

Given the numerous studies that have shown transcription and nascent transcripts are associated with an insoluble nuclear fraction [Clemson and Lawrence, 1996; Huang and Spector, 1991; Xing et al., 1991], we were interested in determining the spatial distribution of Pit-1 in extracted nuclei in situ. GH3 cells grown on coverslips were subjected to detergent extraction and immunostained using the 2C11 antibody, and then imaged by laser scanning confocal microscopy (LSCM). Consistent with the biochemical extractions and immunoblotting data, in situ detergent extraction also removed a portion of the immunostainable Pit-1, resulting in a heterogeneous nuclear labeling pattern (Fig. 1C,D). These data support the idea that the more diffuse nucleoplasmic staining of Pit-1 in whole cells (not shown) is relatively more detergent soluble.

Considering its role as a transcriptional activator, it is important to understand the relationship of Pit-1 localization with subnuclear domains involved in mRNA metabolism, such as many splicing factors, some active genes/mRNAs, and polyA$^+$ mRNA labeling and, in some cases, the hyperphosphorylated large subunit of RNA polymerase II [Bregman et al., 1995; Mortillaro et al., 1996; Moen et al., 1995; Spector, 1993]. As these domains are rich in the non-snRNP splicing factor SC-35, they are often referred to as SC-35 domains [Moen et al., 1995]. B1C8 is a nuclear matrix antigen [Wan et al., 1994] that interacts specifically with exon-containing RNA and localizes almost exclusively to the SC-35 domains [Blencowe et al., 1994, 1995]. The optical sections in Figure 1C,D demonstrate that detergent-resistant Pit-1 staining and SC-35 domains identified by anti-B1C8 are not usually coincident. In many cases, SC-35 domains are often sites of reduced Pit-1 staining (Fig. 1C,D).

Expression and Nuclear Partitioning of Epitope-Tagged Pit-1

To initiate an analysis of the molecular signals involved in nuclear partitioning of full-length Pit-1, the pCEP4 mammalian expression vector containing a rat Pit-1 cDNA, fused at the N-terminus to either the T7 or Flag epitopes, was used for transient and stable transfection. Monoclonal antibodies to the gene 10 (Novagen) and Flag (IBI, Kodak) epitopes permitted immunostaining of exogenously-expressed, tagged Pit-1 in GH3 cells without interference of endogenous Pit-1. Localization assays of transiently expressed T7-, Flag- or nonepitope-tagged Pit-1 in either CV1 or HeLa cells using T7, Flag M2, or 2C11 mAbs, respectively, also resulted in a similar localization pattern under conditions of low expression, indicating the small epitope-tags do not significantly alter subnuclear localization. Importantly, the tagged Pit-1 was also transcriptionally functional (see below).

Figure 2A–C illustrates the pattern of T7-Pit-1 when stably expressed in GH3 cells. Although the immunodetectable level of tagged Pit-1 varied from cell to cell in both the transient and stable transfectants, cells expressing a lower level often displayed a speckled localization pattern similar to endogenous Pit-1 (Fig. 1C,D), especially when detergent-extracted prior to fixation (Fig. 2A–C). When tagged Pit-1 is expressed in GH3 cells, the SC-35 domains were also relatively low in tagged Pit-1 (Fig. 2A, arrows). Cells expressing higher levels of tagged-Pit-1 were observed to have a more uniformly labeled nucleus, suggesting preferred sites for subnuclear accumulation were saturated. As a control, nonpituitary cell lines transfected with the vector alone, followed by immunostaining with anti-T7, anti-Flag, or anti-Pit-1, 2C11, demonstrated no specific staining (data not shown). The stably transfected GH3 cells express a tagged protein of the expected size and partitioning characteristics as demonstrated by immunoblotting with mAb 2C11 that labels both tagged and untagged Pit-1 (Fig. 2, top panel). It is also evident that the exogenous tagged Pit-1 partitions with soluble fractions and the core nuclear matrix similar to the endogenous Pit-1 (anti-Flag, Fig. 2D, bottom panel).

The spatial association of Pit-1 with the core nuclear matrix in situ was examined initially in GH3 cells. It proved to be difficult to image matrix-bound Pit-1 in these cells despite numerous attempts using several different experimental protocols, presumably due to lack of epitope availability and/or abundance. To facilitate a visual examination of Pit-1 association with the nuclear matrix in situ, we transfected CV1 or HeLa cells with pCEP4-Flag-Pit-1 plasmids and prepared them for immunolocalization. Similar to the detergent-resistant fraction of Pit-1, the

spatial distribution of matrix-bound Pit-1 was dependent upon the level of expression. Figure 2E shows an optical section of the core nuclear matrix from transiently transfected HeLa cells expressing relatively low levels of Flag-Pit-1. Once the differentially soluble Pit-1 is removed by detergent, and chromatin is extracted by nuclease and high-salt treatments, matrix-bound Pit-1 can been seen in multiple foci and lower levels of staining interspersed throughout the nucleoplasm (Fig. 2E). As with the previous examples, the matrix-bound Pit-1 foci do not overlap directly at the SC-35 domains, but can often be in close proximity.

The POU-Specific Domain in Subnuclear Partitioning

Since Pit-1 demonstrates spatial and solubility-based intranuclear partitioning, we were interested in determining the responsible molecular domain(s) of the protein compared to the functional regions identified by transcription assays [Ingraham et al., 1990; Theill et al., 1989]. A structural map of the functional domains of Pit-1 is shown in Figure 3A. As a first step toward elucidating the Pit-1 domains involved in subnuclear partitioning, T7- and Flag-tagged deletion constructs representing the previously mapped regions of Pit-1 were created and transiently expressed in GH3, CV1, and HeLa cells. An immunoblot of the expressed deletion mutants (Fig. 3B) shows an expected size protein was obtained for each construct. Additionally, untagged proteins containing the 2C11 epitope were assayed, and their cellular distributions were similar to that of the epitope-tagged proteins (data not shown). LSCM analyses of the cellular localization of these deletion mutants, summarized in Figure 3A, indicate an important contribution by the PSD in subnuclear localization of Pit-1. Interestingly, particularly in CV1 cells, the carboxyl-terminal homeodomain (HD), or homeodomain including the linker region (HDL), sometimes accumulated in the nucleolus, in agreement with an immunolocalization study with anti-homeodomain mAbs [Corsetti et al., 1995]. In all cases, the POU domain (PD) was nonnucleolar, suggesting separate *intra*molecular domains of Pit-1, e.g., PSD and HD/HDL, may compete to target subnuclear regions, with contribution of the PSD being dominant (see below). Note that the putative nuclear localization sequence of Pit-1 is found in the POUH, not in the PSD.

Differential Solubility of Epitope-Tagged Pit-1 Deletion Mutants

In order to correlate matrix-association with molecular domains of the Pit-1 molecule, CV1 and HeLa cells were transiently transfected with full-length or truncated versions of tagged Pit-1 and fractions from matrix preparations were immunoblotted with the appropriate mAb. As shown in Figure 4, the N-terminal ST-rich activation domain is entirely detergent extractable (bottom row). Expressed proteins containing the PSD (PD, STPSD, STPSDL) are retained in the insoluble pellet, suggesting this domain is important for core matrix-binding. The HD region alone, with or without the linker, maintains DNA binding capability, and is partially resistant to detergents, but is not found in the core matrix fraction. An internal deletion of the PSD also demonstrates its importance in matrix association, as Pit-1ΔPSD is also not found in the core matrix pellet. In summary, these data suggest that the POU-specific domain plays a dominant role in subnuclear partitioning of Pit-1.

The POU-Specific Domain Functions as a Nuclear Matrix Targeting Signal

As the spatial and solubility partitioning data above suggest the PSD is a potential nuclear matrix targeting domain, we next examined if the PSD, alone, is sufficient to make stable contacts with elements of nuclear architecture. Figure 5 shows immunofluorescent labeling of HeLa cells transiently transfected with Flag-tagged PSD. Despite the absence of a nuclear localization sequence (NLS), distribution of the Flag-tagged PSD can be nuclear, presumably due to diffusion of the small polypeptide (~10 kD; Fig. 5A). In some intact cells highly expressing the PSD, cytoplasmic staining can also be seen (Fig. 5B). In low to moderately-expressing cells that have been pre-extracted to isolate the matrix in situ, nuclear PSD staining remains (Fig. 5C). The differential retention of the PSD on the matrix, as compared to its complete extraction from the cytoplasm, suggests that nuclear insolubility is not due to a general problem such as protein folding. Considering that the PSD has previously been shown to bind DNA poorly [Li et al., 1990], and that >95% of the DNA is removed during matrix preparation [He et al., 1990], it is probable that the associations with the nucleoskeleton in-

volve protein-protein interactions with presently unknown targets.

To fully ascribe a matrix-targeting function to the PSD, a demonstration of its ability to convey this property to a non-nuclear reporter protein is required. Initial experiments were performed with vectors encoding chimeric proteins consisting of a Flag-tagged, full-length *lacZ* gene product fused to the PSD, linker and including the putative NLS in the amino-terminal of the POU homeodomain. Transient transfection of this construct into HeLa cells showed that it was both nuclear and matrix-bound (data not shown). Next we examined the targeting potential of a smaller chimeric protein, by specifically fusing the PSD to a 60-amino acid region of *lac*Z (Flag-$lacZ_{60}$PSD). The lower molecular mass of this fusion (<20 kDa) allowed the protein to enter the nucleus without an NLS. Flag-$lacZ_{60}$ was transfected for comparison. Transiently transfected HeLa cultures demonstrated that both Flag-$lacZ_{60}$ (data

Figure 1

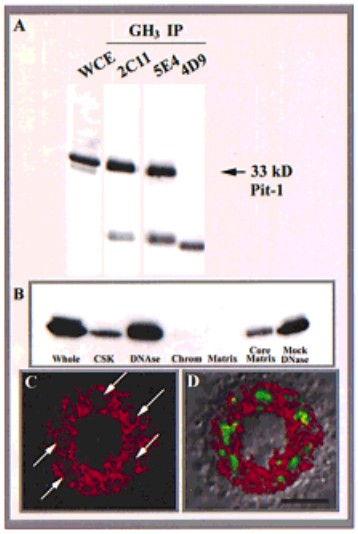


Figure 2

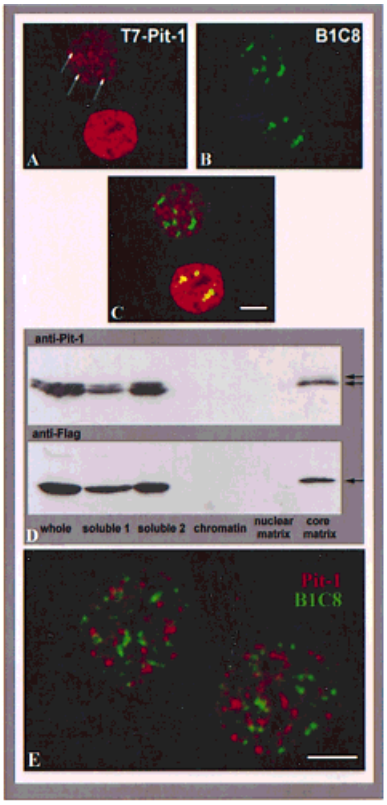


Figure 3a

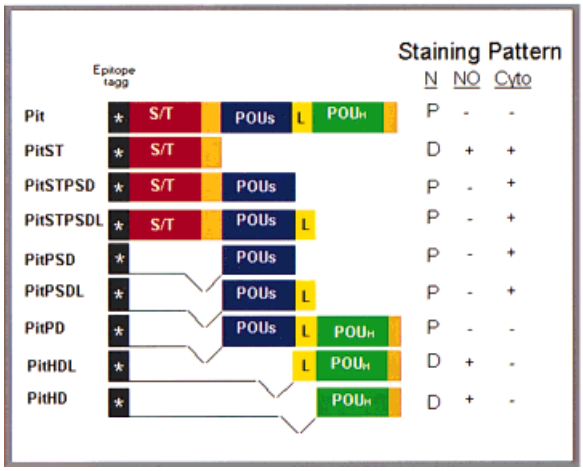
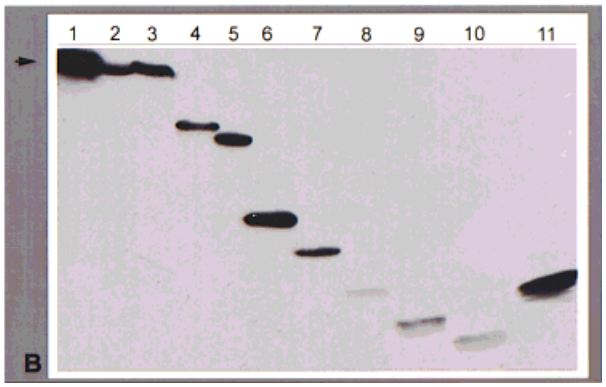


Figure 3b



Figures 1–3.

not shown) and Flag-$lacZ_{60}$ are distributed throughout the cytoplasm and nucleus (Fig. 6A). Flag-$lacZ_{60}$ is completely soluble in detergent (data not shown). In comparison, when Flag-$lacZ_{60}$ PSD was introduced into HeLa cells, extraction of soluble proteins and chromatin removed only the cytoplasmic staining, and specifically failed to displace Flag-$lacZ_{60}$ PSD from the nucleus (Fig. 6B,C). Consistent with previous findings using truncated Pit-1 proteins (e.g., STPSD, STPSDL can be cytoplasmic, and/or nuclear in whole cells; Fig. 3A), these data suggest that the Flag-$lacZ_{60}$ PSD chimera either diffuses into the nucleus or "piggy-backs" with an NLS-containing protein. The results show that the PSD can function to convey detergent- and salt-resistant tethering interactions with the nucleoskeleton to an otherwise soluble polypeptide.

The PSD is a Dominant Subnuclear Targeting Signal

To extend our investigation of the subnuclear targeting function of the PSD, we compared its targeting potential relative to a nuclear protein with a known spatial pattern of subnuclear partitioning. We showed above that the concentration of Pit-1 is usually low at SC-35 domains identified by the B1C8 antibody (Fig. 1,2). It has been demonstrated that both the non-snRNP splicing factor SC-35 [Hedley et al., 1995] and *transformer* [Li and Bingham, 1991] contain an Arg- and Ser-rich domain that can target a *lacZ* fusion to SC-35 domains [Li and Bingham, 1991]. To mechanistically investigate the propensity of Pit-1 to avoid accumulation within SC-35 domains, we translationally fused a Flag-tagged RS-rich region of the *transformer*

Fig. 1. Antibody specificity and subnuclear partitioning of Pit-1. **A:** Two IgG monoclonal antibodies (2C11 and 5E4) were used to immunoprecipitate pituitary-derived GH3 cellular proteins, and followed by Western analysis with the 2C11 mAb. Whole cell extracts (WCE) and immunoprecipitated fractions contain a prominent 33 kD band representing Pit-1. Nonspecific hybridoma supernatant 4D9 was used as a negative control. Epitope mapping (data not shown) indicated that both 2C11 and 5E4 recognize an epitope in the distal N-terminus within the ST activation domain of Pit-1. **B:** Western analysis of biochemical extractions of GH3 cells using mAb 2C11. Pit-1 partitions with both soluble and insoluble (core nuclear matrix) fractions. The various biochemical fractions described in the Materials and Methods are indicated above each lane. Note that Pit-1 is differentially soluble in detergent (CSK, DNase; mock DNase) and the remainder is core nuclear matrix bound. **C,D:** GH3 cells were extracted in CSK (Triton X-100) buffer and immunostained using 2C11 (red) and B1C8 (green) antibodies. In this typical GH3 cell, Pit-1 staining is confined to the nucleoplasm in a heterogeneous speckled pattern; also, note the diminished Pit-1 staining in the areas occupied by B1C8 staining (C, arrows). The B1C8 IgM monoclonal antibody, generously provided by J. Nickerson and S. Penman, recognizes a 180 kD nuclear antigen, related to RS proteins, that associates with splicing complexes [Wan et al., 1994; Blencowe et al., 1994, 1995] and is used here to identify SC-35 domains. Scale bar = 5 μm.

Fig. 2. Expression of epitope-tagged Pit-1. GH3 (A–-D) or HeLa (E) cells were transfected with a pCEP4 mammalian expression vector encoding a rat Pit-1 protein fused at the N-terminus with either a T7 or Flag epitope (see Fig. 3A for a diagram of the molecule) and were analyzed by LSCM. **A:** T7-mAb staining of tagged Pit-1. **B:** B1C8 staining. **C:** Dual overlay of (A) and (B). Arrows denote decreased T7-Pit-1 staining in areas identified by the B1C8 mAb, in the lower expressing cell. This suggests saturation of preferred sites occur in the presence of increasing amounts of the exogenous Pit-1. **D:** GH3 cells expressing Flag-Pit1 were biochemically fractionated to obtain nuclear matrices, separated by SDS-PAGE and then immunoblotted with the Flag (bottom row) or 2C11 (top row) mAbs. These data illustrate that the exogenously tagged and endogenous Pit-1 have the same solubility partitioning characteristics in vivo. **E:** Immunofluorescent localization of transiently transfected Flag-Pit-1 in HeLa cell nuclear matrix. The tagged Pit-1 (red) localizes to numerous bright foci with some lower levels diffuse in the nucleoplasm. Double labeling with the B1C8 mAb (green) indicates the Pit-1 foci on the matrix are mostly distinct from SC-35 domains. Scale bars = 5 μm.

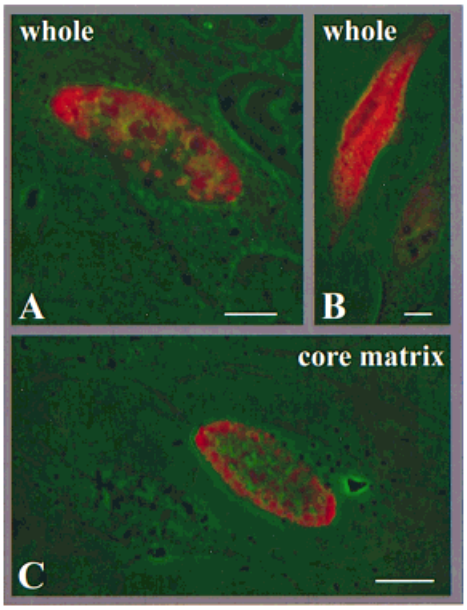
Fig. 3. Pit-1 deletion mutants and cellular compartmentation. **A:** Full-length (top line) and truncated epitope-tagged (* = T7, Novagen; and Flag, Kodak) Pit-1 polypeptides encoded by various pCEP4 (Invitrogen) plasmid constructions. S/T indicates the Ser- and Thr-rich activation region of Pit-1. POUS indicates the POU-specific domain. POUH indicates the POU-homeodomain. L denotes the linker region separating the POUS and POUH regions. The table to the right summarizes the staining results observed using the constructions. *N* refers to nuclear, *P* denotes punctate and D indicates diffuse patterns of staining. *NO* and *Cyto* refer to nucleolar and cytoplasmic localization, respectively; (+) = positive staining; (-) = no staining. **B:** Western assay of epitope-tagged Pit-1 proteins. Using the T7 and Flag mAbs, all of the truncated T7- or Flag-tagged Pit-1 polypeptides of the expected size are detected in extracts of transiently expressing CV1 cells. Each construct (see Table I) is listed above the relevant lanes. (**1**) Wild-type Pit-1 (33 kD) is indicated by the arrow. The expression of the full length point mutants, rat Pit-1 containing the Snell dwarf W261C mutation (**2**) and rat Pit-1 with the human mutation, A158P (**3**) are also shown. The expected molecular masses of the truncated proteins are: (**4**) STPSDL = 29,191 Da; (**5**) STPSD = 27,727 Da; (**6**) PD = 21,722 Da; (**7**) ST = 17,190 Da; (**8**) HDL = 14,144 Da; (**9**) PSDL = 12,011; (**10**) PSD = 10,537 Da; (**11**) HD = 12,667.

gene product (*tra)* [Li and Bingham, 1991] to full-length Pit-1 and examined its subnuclear localization in transiently transfected CV1 and HeLa cells.

As expected, the Flag-tagged *tra* RS domain, which also contains a NLS, efficiently targets to the B1C8 labeled SC-35 domains (Fig. 7A). With Flag-tagged *tra* translationally fused to full-length Pit-1, the localization of the chimeric protein in CV1 (and HeLa cells; not shown) is similar to wild-type-Pit-1 (Fig. 7B), and does not concentrate in SC-35 domains. These data suggest a spatial partitioning signal exists in full-length Pit-1 that is dominant to the RS signal in *tra*. To identify the dominant Pit-1 signal, we created several additional *tra*-fusions with subdomains of Pit-1 (summarized below in Table I). Figure 7C shows the results of a fusion between the Pit-1 activation domain (ST) and *tra*; in this case, the fusion protein specifically targeted SC-35 domains. Therefore, the N-terminal ST domain seems to play little, if any, role in the subnuclear partitioning of full-length Pit-1. Interestingly, when Flag-*tra*-ST targeted the SC-35 domains, a reproducible reorganization of these domains occurred, similar to that seen when cells are treated with transcriptional inhibitors such as actinomy-

Figure 4

Figure 5



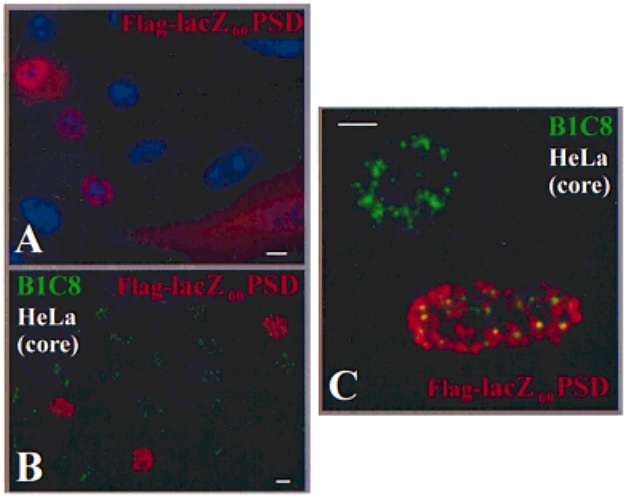


Figure 6

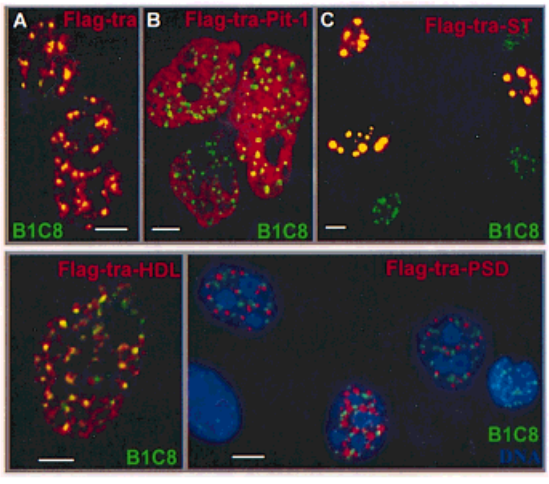


Figure 7

Figures 4–7.

cin-D or α-amanatin [Spector, 1993]. Flag-*tra*PD, containing the PSD, linker and homeodomain, exhibited a staining pattern similar to that of full-length Pit-1 (data not shown), indicating the prominent subnuclear partitioning signal of Pit-1 is contained within the POU domain (Table I).

Previous immunolocalization assays demonstrated that a Flag-tagged HDL can sometimes localize to nucleoli in CV1 cells (Fig. 3A). We also generated a *tra*-HDL construct that, when expressed in CV1, targeted to SC-35 domains; however, the targeting efficiency was clearly less than that with *tra*, or *tra*-ST (Fig. 7D; Table I). In contrast to Flag-HDL, the RS-containing chimeric protein, Flag-*tra*HDL, always showed a non-nucleolar staining pattern in CV1 cells with significant anti-Flag staining at SC-35 domains. This experiment indicates the RS signal in *tra* is dominant to the putative nucleolar signal in the POU-homeodomain.

The above data supports the notion that the RS targeting signal of *tra* is dominant to both the distal N and C termini of Pit-1. Since both full-length Pit-1 and the POU-domain (PD, containing the PSD, linker, and homeodomain) were dominant to *tra*, we directly tested if the PSD could redirect an RS domain by expressing Flag-*tra*PSD in HeLa. In this case, Flag staining reveals discrete, subnuclear foci that are clearly distinct from SC-35 domains (Fig. 7E), indicating that PSD targeting is dominant to the RS signal. At present, double labeling with several intranuclear antigens (PML, coiled body, prekinetochore/CREST) has not revealed any colocalization with the Flag-*tra*PSD pattern (data not shown). Collectively, these data suggest a hierarchy of subnuclear targeting signals may participate in establishing nuclear partitioning.

Functionally Inactive Rat Pit-1 Proteins Partition Exclusively With the Nuclear Matrix

An important advantage in using Pit-1 as model to examine functional issues of subnuclear partitioning is the availability of natural, inactive point mutations. A mutation in the PSD of human Pit-1 (A158P) results in hypopituitarism and dwarfism [Pfaffle et al., 1992]. Human Pit-1 with the A158P mutation retains Pit-1 specific DNA binding (although reproducibly reduced), but is greatly compromised in target gene activation [Pfaffle et al., 1992; Ouspenski and Mancini, unpublished]. The Snell

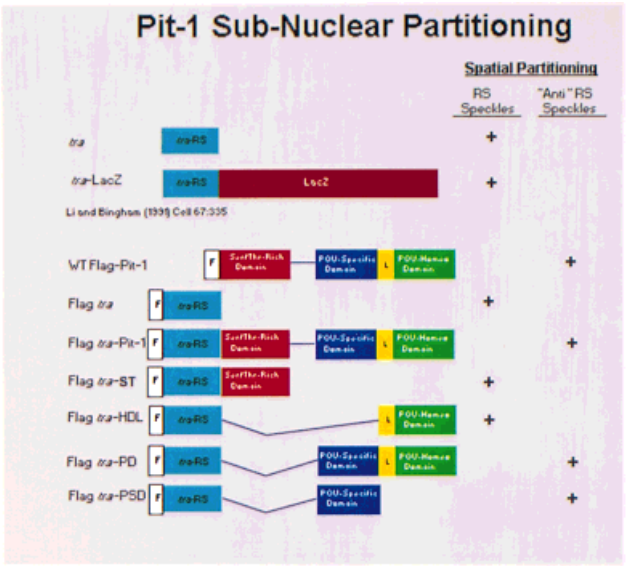
Fig. 4. Solubility partitioning of wild-type and mutant Pit-1. HeLa cells were transiently transfected with T7 or Flag expression-vectors containing cDNA coding for the stated proteins. Cultures were harvested at 24 h post-removal of DNA and extracted to obtain the core matrix. Supernatants were collected from each step and all lanes were loaded with cell number equivalents. The blots were probed with anti-Flag or anti-T7 mAbs. Wild-type and PSD-containing proteins partitioned in both the soluble fractions and the core matrix (top four rows). An interior deletion of PSD (Pit-1ΔPSD) results in total loss of the core matrix fraction. Expression of the POU-homeodomain and the adjacent flexible linker region (HDL) also fails to associate with the core matrix fraction. The N-terminal ST domain (bottom row) is completely soluble in Triton X-100.

Fig. 5. Subnuclear targeting and core matrix association of the POU-specific domain (PSD). Spatial and solubility partitioning of Flag-PSD (red) was examined in whole (**A** and **B**) or core matrix prepared HeLa cells **(C)**. Immunofluorescence was overlaid upon phase contrast images (green). These non-confocal images were obtained with a three-chip, color CCD camera. The nuclear (A, red), or cell-wide staining of whole cells (B, red), contrasts with the scattered Flag-PSD staining that is retained exclusively in the nuclei of detergent, nuclease and salt-extracted cells (C). Scale bars = 5 μm.

Fig. 6. PSD-reporter protein targeting. The first 60 amino acids of β-galactosidase were fused to the PSD and transiently expressed in HeLa cells. These nonconfocal images (A–C) show in unextracted cells **(A)** that the Flag-*lacZ*$_{60}$PSD protein is predominantly nuclear or, in some cells, distributed throughout the cell. **B,C:** Following detergent, nuclease and salt extractions to reveal the core filament matrix, Flag-*lacZ*$_{60}$PSD is retained only in the nucleus in multiple scattered foci.

Fig. 7. Competitive subnuclear targeting assay. In these *molecular tug-of-war* experiments, a Flag-tagged RS subnuclear targeting signal, *tra*, was transiently expressed in HeLa (A, C, E) or CV1 (B, D) cells. **A:** As predicted, the Flag-tagged RS rich domain of *tra* (Flag-*tra* = red) readily targets SC-35 domains marked by the B1C8 mAb (green; overlap is yellow). **B:** When fused to the full-length Pit-1 (Flag-*tra*-Pit-1) and expressed, the chimeric protein did not target to SC-35 speckles. **C:** The fusion between the Pit-1 ST activation domain (Flag-*tra*-ST) and *tra* lead to specific colocalization to the subnuclear SC-35 domains. Interestingly, this targeting frequently led to fewer, and larger, speckles, similar to the speckle pattern of actinomycin or α-amanatin treated cell, suggesting a reduction of overall transcription [Spector, 1993]. When Flag-*tra* was fused to the carboxyl terminus of Pit-1, HDL, (Table 1), the subnuclear targeting of this chimeric fusion was non-nucleolar and predominantly directed to SC-35 domains **(D)**. Identification of the PSD as a "*tra*-dominant" subnuclear targeting signal is shown in **(E)** where numerous distinct foci containing Flag-*tra*-PSD (red) do not colocalize with B1C8 (green), although they can be adjacent. DNA is counterstained blue with DAPI. Scale bars = 5 μm.

TABLE I. Hierarchy of Subnuclear Targeting Signals[a]



[a]This table summarizes competitive targeting experiments between the arg- and ser-rich region of the *tra* protein (*tra*-RS in this figure; see text) and Pit-1, or portions thereof, that were evaluated for spatial targeting by LSCM. To the right of each construct diagram, a + indicates targeting to nuclear speckles (SC-35 domains) or avoiding SC-35 domains (termed "anti-RS").

dwarf mouse phenotype is the result of a mutation in the POU-homeodomain of Pit-1 (W261C) that completely renders the molecule incapable of binding DNA and activating target promoters [Li et al., 1990]. To functionally compare thenuclear partitioning of these naturally occurring mutations, the human A158P and mouse W261C mutations were recapitulated in rat Pit-1 by PCR site-directed mutagenesis, expressed in cultured cells, and then assayed both for activator function and subnuclear partitioning. X-ray crystallography of Pit-1 [Jacobson et al., 1997] and another POU class activator, Oct-1 [Klem et al., 1994], suggest the two outer α-helices in its PSD are involved in protein-protein interactions. Therefore, we also created and tested two additional full-length PSD point mutants that disrupt the first and fourth α-helical domains with proline residues (α-1, E133P/A136P; and α-4, A183P/A188P). The α-1 and α-4 mutants are also unable to bind DNA based upon evidence from in vitro foot-print assays performed with purified bacterially expressed protein [data not shown; Liu et al., unpublished observations].

Figure 8A shows transient cotransfection assays of a synthetic prolactin promoter/enhancer luciferase reporter [Smith et al., 1995] in the presence of expressed Flag-tagged wild-type Pit-1, α-1 (E133P;A136P), α-4 (A183P;A188P), A158P Pit-1, or W261C Pit-1 in HeLa cells. As expected, the data from three different transfections (three assays per transfection) demonstrated that A158P and W261C rat Pit-1 are defective in activating the prolactin promoter relative to wild-type Pit-1; disruption of the first or fourth α-helix also results in loss of transactivation function. Figure 8B shows a series of immunoblots that illustrate the solubility partitioning of the Pit-1 point mutants. Surprisingly, in each case, inactive Pit-1 point mutants exhibited a total loss of the detergent-extractable fraction seen with wild-type Pit-1 and a complete shift to association with the matrix fraction (Fig. 8B). Therefore, a strong correlation exists between solubility of these Pit-1 molecules and their activator function.

As the inactivating point mutants partition completely to the insoluble NM fraction, it is important to determine where they localize in the cell. In each case, these mutants appear to normally translocate from the cytoplasm to the nucleus, suggesting they are not intrinsically insoluble. The nuclear distribution of Pit-A158P in CV1 or HeLa cells is similar to wild-type Pit-1, with the exception of an exaggerated avoidance of SC-35 domains (Fig. 8C). In con-

Figure 8

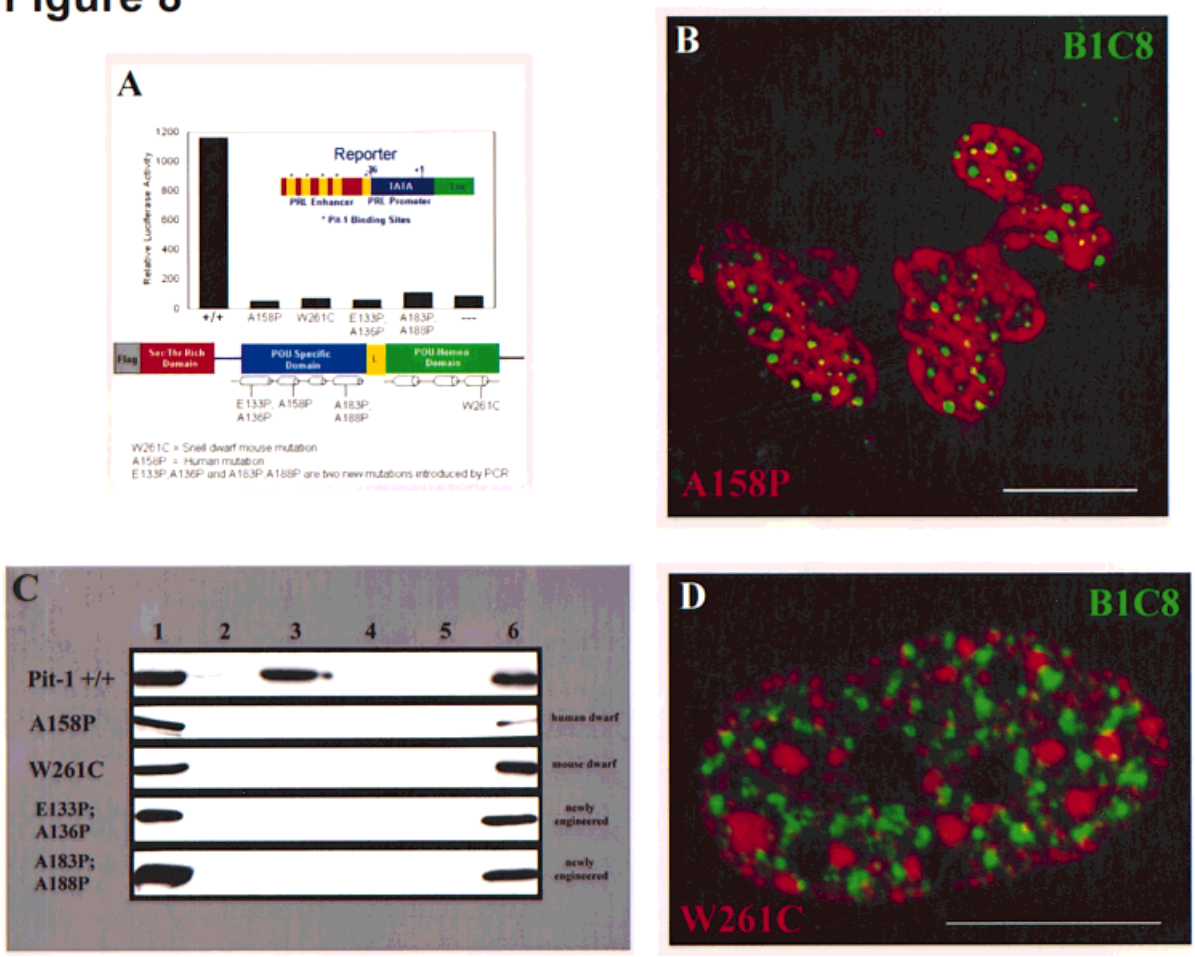


Fig. 8. Analyses of nonfunctional full-length Pit-1 mutants. **A:** Two naturally occurring Pit-1 point mutants found in the mouse (W261C) and human (A158P) were recapitulated in rat Flag-Pit-1 and assayed for prolactin promoter-specific activation function in HeLa cells (see text for further explanation). Two additional double point mutants, α-1 (E133P; A136P) and α-4 (A183P; A188P) were engineered to disrupt the first and fourth α-helices of the PSD and evaluated. The results are from HeLa cells transiently co-transfected with the Flag-Pit-1 vectors and a luciferase reporter driven by the prolactin promoter/enhancer [Smith et al., 1995; Sharp, 1995]; values are shown normalized to a cotransfected *lacZ* control vector. A schematic diagram of the relative activities of wild-type and mutated Pit-1 proteins, and the position of the mutations within Pit-1, are illustrated. As expected, relative to wild-type Flag-Pit-1 activator function on the PRL promoter, the dwarf (mouse and human) and α-mutants are inactive. **B:** Solubility partitioning of nonfunctional, full-length Pit-1. Flag-tagged point mutants (see above) and wild-type rat Pit-1 were transiently transfected into HeLa cells. Core matrix preparations were performed as described in Materials and Methods. As seen previously in Figures 1B and 2D, the distribution of epitope-tagged, wild-type Pit-1 (top row) contains a soluble and insoluble component. Following normal import into the nucleus, the solubility partitioning of both the natural Pit-1 dwarf point mutants (A158P and W261C) and the newly engineered PSD α-mutants (α-1: E133P; A136P; and α-4: A183P; A188P) is remarkably skewed to the insoluble fraction. **C:** Abnormal subnuclear compartmentation of the A158P Pit-1 dwarf mutants. These transiently transfected CV1 cells were detergent-extracted prior to immunostaining and evaluated by LSCM. The Flag-Pit-1 A158P mutant is distributed in a diffuse nucleoplasmic pattern, relative to wild-type Pit-1, with an exaggerated avoidance of the B1C8 speckles. Scale bar = 10 μm. **D:** The spatial partitioning of Flag-Pit-1 W261C in a detergent-treated CV1 cell was evaluated by LSCM. The non-DNA-binding Flag-Pit-1 W261C mutant is exclusively nuclear, avoids nucleoli, and is distributed in a hyper-speckled fashion. The Flag-W261C mutant also does not colocalize with the B1C8 speckles. Scale bar = 5 μm.

trast to the A158P protein, the W261C mutation expressed in CV1 or HeLa cells caused a markedly different nuclear staining pattern. W261C Flag-Pit-1 consistently showed an exaggerated punctate distribution that avoids SC-35 domains (Fig. 8D). The spatial distribution of the α-1 and α-4 mutants is also abnormal within the nuclei of transfected cells and will be described in detail elsewhere [Mancini et al., in preparation].

DISCUSSION

The results presented in this paper show that wild-type or epitope-tagged Pit-1 partitions within the nuclei of pituitary or nonpituitary cells, in terms of both spatial localization and solubility. While a majority of wild-type Pit-1 is differentially soluble in detergents, a substantial portion (about 20%) of endogenous or exogenous Pit-1 is associated with the nuclear matrix. Our data using deletion mutagenesis and chimeric fusions support the existence of a hierarchy of partitioning signals that regulate the functional subnuclear organization of nuclear proteins. In addition to its key role in conveying specific DNA binding to the full length Pit-1 molecule [Ingraham et al., 1990], our data attributes subnuclear partitioning as a function of the 66 amino acid PSD.

The competitive targeting experiments with the RS-rich *tra* domain showed that the PSD can function as a dominant subnuclear targeting signal, and supports the notion that even in an endogenous setting, the reduced Pit-1 staining at SC-35 domains may be PSD-mediated. Preliminary subnuclear targeting data using Flag-*tra*-PSD(A158P) suggests additional complexity in this domain as a PSD carrying the A158P mutation, that cripples activation (see below), is still dominant to *tra* (data not shown). Additional mutagenesis of the PSD will be required to resolve this issue.

Numerous reports show transcription is associated with an insoluble nuclear fraction [Abulafia et al., 1984; Barrack, 1987; Ciejek et al., 1983; Intres and Donady, 1985; Jackson and Cook, 1985; Jost and Seldran, 1984; Lelievre et al., 1996; Murty et al., 1988; Nickerson et al., 1995; Robinson et al., 1983; Small et al., 1985; Stein et al., 1995]. Recent reports using brief pulses of BrUTP to bulk label pol II transcripts [see Moen et al., 1995] have identified between several hundred to >2,000 specific transcription sites, or "transcription factories," that are firmly attached to the nucleoskeleton [Jackson et al., 1993; Wansink et al., 1993; Iborra et al., 1996]. Though the number of sites may be uncertain, it is clear that little is known about the mechanism(s) that transcription factors use to associate with the matrix. Although steroid receptors have a history as being matrix-bound [Barrack, 1987], it has been shown only recently that this property is roughly mapped to the carboxyl-terminal half of the androgen and glucocorticoid receptors [van Steensel et al., 1992]. Investigation of the AML family of transcription factors has recently defined a 30 amino acid region that acts as a nuclear matrix targeting sequence [Zeng et al., 1997]. Our data indicate the PSD is required for wild-type Pit-1 binding to the matrix, and that this molecular domain is capable of conveying matrix-targeting to either the soluble ST activation domain, the distal POU homeodomain, or to the normally soluble *lacZ* gene product. It is expected that many different subdomains of nuclear proteins will be identified that provide matrix targeting to assist the assembly of functional complexes upon the nucleoskeleton. The select target specificity of a transcription factor such as Pit-1 may require different subnuclear partitioning characteristics when compared to more general gene regulators. The more diffuse staining pattern observed in the relatively high-expressing stably-transfected GH3 cells, suggests the presence of a saturable Pit-1 docking system. A similar saturation phenomenon is also seen in transiently transfected non-pituitary cells and supports the idea that the components of these sites may be general in nature.

It is assumed that DNA-binding activators have direct or indirect protein targets in the nucleus that involves interactions with the basal RNA polymerase II transcription initiation complex. The hyperphosphorylated large subunit of RNA polymerase II (pol IIo) has recently been shown to be matrix-bound and sometimes concentrated specifically at SC-35 domains [Bregman et al., 1995; Mortillaro et al., 1996]. Combined with data that show some active genes are preferentially associated with SC-35 domains [Clemson and Lawrence, 1996; Huang and Spector, 1991; Xing et al., 1993, 1995], these regions may be the sites of active transcription as well as the storage sites for splicing factors [Huang and Spector, 1996; Spector, 1993]. In this context, the paucity of Pit-1 staining at SC-35 domains may indicate that Pit-1 target genes are random with respect to these domains, or that the active Pit-1 is only at the differential staining boundary. To address this issue, experiments are underway to determine the spatial relationship between sites of BrUTP incorporation, pol IIo and Pit1 in nuclear matrix preparations. The question remains, however, whether PSD-mediated contacts with basal components of select transcription complexes/factories are involved in generating the sub-

nuclear partitioning of Pit-1, or if there are additional interactions with architectural components of the nucleoskeleton. Moreover, what role does DNA-binding and activation have in establishing spatial and solubility partitioning of Pit-1?

In light of the foregoing questions, it was interesting to note that the endogenous staining pattern of Pit-1 in the nucleus is similar to the pattern observed in cells transfected with truncated polypeptides that included, in some cases, only the PSD. Since the PSD of Pit-1 alone is not known to effectively bind DNA (30), our working hypothesis is that subnuclear partitioning of Pit-1 has two components. One is mediated through protein-protein interactions between the PSD and a putative nuclear docking system, and the other involves both sequence-specific and nonspecific DNA-binding required for normal activation. When DNA-binding in rat Pit-1 is completely uncoupled by the α-1(E133P; A136P) or α-4 (A183P; A188P) mutations [Liu et al., unpublished observations], or in the W261C mutation, solubility partitioning is dramatically disrupted as indicated by a complete shift to the insoluble compartment (Fig. 8B). Rat Pit-1 with the A158P mutation fails to activate the PRL promoter and is also disrupted in terms of its solubility partitioning (Fig. 8A,B). Since this mutation in human Pit-1 retains some DNA-binding activity [Li et al., 1990; Ouspenski and Mancini, data not shown], our results suggest that activation potential, *per se*, may be an important component of its solubility partitioning. Taken together, the solubility and spatial data from the non-functional Pit-1 mutants point to the intriguing possibility that DNA-binding transactivators, such as Pit-1, exist in a state of dynamic equilibrium between the compartments identified by spatial and solubility partitioning.

The observed spatial and solubility partitioning of Pit-1 in the nucleus immediately prompts obvious questions: why is it partitioned and in which compartment is the functioning Pit-1? Here we highlight the distinction between compartments containing *functional* versus *functioning* Pit-1. Based on standard biochemical preparation of nuclear transcription extracts [Cao et al., 1987; Dingham et al., 1983], it is presumed that most of the cellular Pit-1 can be functional in cell-free transcription assays. In contrast, the *de facto* fraction of Pit-1 engaged in activation of target genes in the living nucleus is the functional Pit-1. The assertion of many reports is that transcription is associated with insoluble nuclear structure [Barrack, 1987; Bidwell et al., 1993; Ciejek et al., 1983; Dworetzky et al., 1992; Iborn et al., 1996; Jackson et al., 1993; Jost and Seldran, 1984; Lauber et al., 1995; Nickerson et al., 1995; Robinson et al., 1983; Schaack et al., 1990; Stein et al., 1995, 1996; Tang and DeFranco, 1996; Xing et al., 1991). If true, the candidate biochemical fraction containing the *functioning* Pit-1 pool would thus be matrix-associated. In support of this, solubilized Pit-1 and other transcription factors derived from nuclear matrix pellets retain DNA binding activity in vitro [Mancini and Sharp, unpublished observations; van Wijnin, et al., 1993].

It is interesting that all four non-functional Pit-1 proteins (A158P, W261C, α-1, and α-4) partition only to the insoluble compartment. One interpretation of these data is that the defective proteins "accumulate" in this compartment by virtue of the fact that they either cannot bind chromatin effectively or cannot make appropriate contacts with unidentified molecules that might be involved ATP-dependent shuttling [Tang and DeFranco, 1996] on and off the nucleoskeleton. In this vein, it is tantalizing to consider the possibility that the functional defects in the A158P Pit-1 mutant protein may involve a non-DNA-dependent disruption of its solubility partitioning, as is evident in Figure 8.

Preliminary results from saturation mutagenesis of the PSD region have identified another partitioning mutant that retains specific DNA-binding in vitro but is inactive in vivo [Mancini et al., in preparation].

Although it is believed that there are many sites throughout the nucleoskeleton engaged in transcription [Iborra et al., 1996; Jackson et al., 1993; Wansink et al., 1993], there do not appear to be that many Pit-1 foci on the matrix. Also, there are many more Pit-1 foci on the matrix than the number of known target genes. At present, it seems likely that most of these foci may represent storage and/or processing sites for Pit-1. In general, however, partitioning of the "excess" Pit-1 may play a role in scanning accessible chromatin in search of its few target promoters. As shown for Oct-1 [van Leeuwen et al., 1995, 1997; Kim et al., 1996], there may also be other functions for Pit-1, such as in

replication. Is the functional role of Pit-1 in cellular physiology purely related to its activation capability or could it have a "structural" role as well? Functional studies in vitro with differentially soluble Pit-1, and the precise determination of the spatial relationship between Pit-1 and its few target genes, as well as matrix-bound, bulk-labeled pol II transcription sites, will help address these questions. Nonetheless, we believe that the abundance of Pit-1, coupled with its spatial and solubility-based partitioning in the nucleus, has a key role in its function as an activator and, therefore, in maintaining the differentiated phenotype of pituitary cells. These questions and the identity of the matrix binding partners of the PSD are currently under investigation.

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