Structural and functional studies on the Pituitary-specific transcription factor Pit-1

Kevin Dennis Augustijn

Cover: ^{1}H-^{15}N HSQC of thioredoxin.

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Structurele en functionele studies aan de hypofyse-specifieke transcriptie factor Pit-1
(met een samenvatting in het Nederlands)

Proefschrift

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Aan opa Augustijn
Voor Esther

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"Everything should be made as simple as possible but not simpler"

Albert Einstein

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1.1.1 Introduction

Regulation of gene expression is one of the major focal points in biochemistry today. Contributions from the fields of genetics, biochemistry and structural biology have created a wealth of data on the function of the network of multi-protein "molecular machines" that is involved in transcription. The emerging picture shows tight control on gene expression by balancing activation, repression and silencing. With the recent completion of the human genome sequencing project and the advent of high throughput methods such as genome wide screening using DNA microarrays, our insight is likely to increase even further. Yet it will take the combined effort of all these disciplines to truly gain understanding of the way genetic activity is controlled.

1.1.2 Chromatin structure

The entire human genome consists of over 3 billion base pairs (bp) [1]. In order to fit this much DNA into the nucleus, it needs to be tightly packaged. To achieve this, nuclear DNA is wrapped around protein complexes, which gives the DNA an appearance like beads on a string. Each unit consists of about 200 bp of DNA, where 146 bp is wrapped around the protein complex called the histone octamer, while the remainder makes up the linker to the next nucleosome (Fig. 1). Each core complex contains two copies of the core histones (H2A, H2B, H3 and H4). A fifth histone (H1) is bound on the outside of the core. H1 can bind both DNA and core histones and is involved in forming an as yet uncharacterized higher order structure, providing further condensation the DNA. This structure is referred to as chromatin (reviewed in [2]).

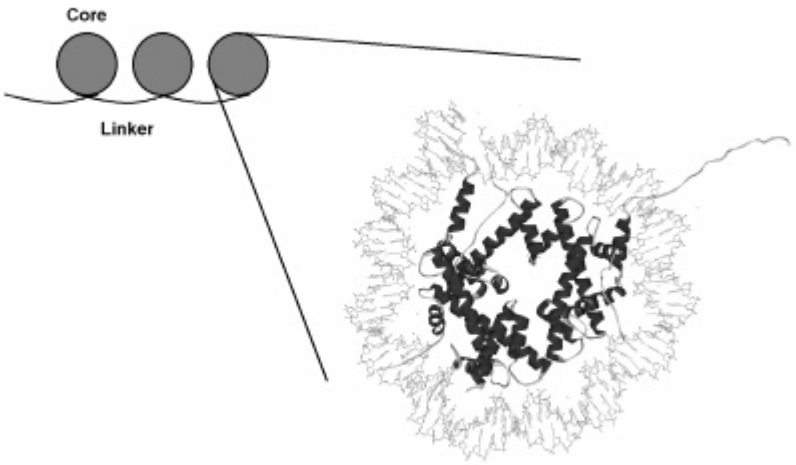

Figure 1

Beads-on-a-string structure of the core nucleosomes; the DNA is wrapped around the histone octamer, as shown in the cross-section through the core nucleosome complex crystal structure.

Packaging of DNA into chromatin has a repressive effect on transcription by limiting the accessibility of the DNA for the transcription machinery. Since the naturally occurring form of nuclear DNA is chromatin bound, this repression and its alleviation have proven to be an important regulatory mechanism of transcription. Activation of transcription from a chromatin bound template requires a "loosening" of the nucleosome structure. This can be achieved by ATP-dependent chromatin remodeling complexes (discussed below) or by covalent modifications of the histone proteins. Specifically, the N-terminal tails of the core histones H3 and H4 can be acetylated by transcription activators containing histone acetyl transferase (HAT) activity. The targeted lysine residues are believed to be involved in DNA and inter-nucleosome contacts, promoting condensation of the

DNA in their unmodified form. Acetylation of these residues neutralizes their positive charge, which serves to decrease their interaction with the negatively charged DNA (reviewed in [3]). In addition, H1 is mono-ubiquitinated by $TAF_{II}250$, which may target H1 for ubiquitin-mediated degradation. A mutant $TAF_{II}250$ lacking this activity was shown to repress transcription on two genes that require $TAF_{II}250$ for activated transcription [4]. Thus one can speculate that removal of H1 from the chromatin complex might produce a similar effect as histone acetylation.

The role of other modifications of histone proteins such as phosphorylation and methylation (reviewed in [5-7]) is less clear at present. In contrast to acetylation, methylation of histone tails (both on lysine and arginine residues) conserves the positive charge. Even though methylation would not have a similar effect as acetylation, it is definitely involved in the regulation of chromatin structure [8]. Furthermore, histone methylation can serve as a recognition marker for regulatory proteins. A recent example of this is the chromo-domain on the HP1 family of repressor proteins, which recognizes H3 methylated on lysine 9 [9]. Finally, it has been shown that transcriptional co-activator CBP/p300 interacts with a histone methyltransferase *in vivo*, suggesting a link between acetylation and methylation and a role for histone methylation in transcription activation [10]. Phosphorylation of histones has long been coupled to condensation of the genome during mitosis [11]. This condensation would obviously lead to repression of transcription, however there are studies which link an increased acetylation of histone H3 after phosphorylation [12-14]. Thus histone phosphorylation may serve both to silence gene expression by genome condensation and to activate transcription by acting in concert with histone acetylation.

Chromatin structure can also be rearranged without covalent modifications, but with the expense of ATP. For this purpose several families of ATP-dependent chromatin remodeling complexes exist in the cell, the most prominent of these being the SWI/SNF and the ISWI families (reviewed in [15-17]). These complexes are capable of rearranging higher order nucleosome structure, sliding of nucleosomes along template DNA and/or introducing negative superhelical tension on DNA. As in the case of covalent modifications of the histones, chromatin-remodeling complexes may both serve to activate and

repress transcription, depending on promoter context and the presence of transcription factors. Again, the accessibility of the DNA for the transcription machinery correlates to activation or repression.

1.1.3 Basal transcription

Transcription by RNA polymerase II (RNAPII) requires a minimal set of protein complexes known as the basal transcription factors, so termed for their requirement to reconstitute transcription activity on naked DNA templates *in vitro*. These factors cooperate to recruit RNAPII to a promoter and form the so-called preinitiation complex (PIC), which features the basal transcription factors and RNAPII bound to a partially unwound promoter region. In this PIC, the DNA may be wrapped around a complex consisting of RNAPII and the basal factors [18], reviewed in [19,20]. Key player in promoter recognition is the TATA binding protein (TBP). TBP recognizes and binds to a promoter specific sequence element, the TATA-box, which is present in the majority of RNAPII transcribed genes. *In vivo*, TBP is associated with a number of proteins of varying sizes, forming the basal transcription factor TFIID. These TATA associated factors (TAF_{II}s) may be present in different compositions in TFIID (reviewed in [21,22]). On promoters that do not contain a consensus TATA box, TAF_{II}s can interact with promoter elements to correctly position TFIID. These elements are the so-called initiator sequence (Inr), which overlaps the transcription start site, and the downstream promoter element (DPE), located around 30 bp downstream of the transcription start site [23-25]. In addition to DNA recognition, some TAF_{II}s were found to harbor enzymatic activities such as histone acetyl transferase (HAT), ATPase [26], kinase activity and, recently, ubiquitin conjugation activity [4]. TAF_{II}s are involved in mediating contacts between promoter specific transcription activators or cofactors and the basal transcription machinery.

Once bound to a promoter, TFIID is stabilized by TFIIA, which contacts TBP, TAF_{II}40 in TFIID and the DNA [27]. TFIIA is not considered a basal factor, as it is dispensable for the reconstitution of transcription *in vitro*. Additional stability is provided by TFIIB, which functions similarly to TFIIA [28]. Furthermore, TFIIB interacts with TFIIF to recruit a preformed TFIIF-RNAPII complex to the

promoter [29]. Finally TFIIE and TFIIH enter the complex. These two factors are involved in unwinding the DNA around the transcription start site [30,31]. TFIIH also contains a kinase activity, which can phosphorylate the C-terminal domain (CTD) of the catalytic subunit of RNAPII [32]. CTD phosphorylation by TFIIH is thought to be important in promoter clearance and switching RNAPII to an elongation mode. This stepwise building of the preinitiation complex is based on *in vitro* studies. In contrast, yeast studies show that a so-called holocomplex, where RNAPII is associated with several basal factors and proteins from the Mediator complex (discussed below) prior to recruitment to the promoter, exists *in vivo* [33].

1.1.4 The Mediator complex

The Mediator complex was first characterized as TRAP and DRIP, cofactors for thyroid and vitamin D hormone receptors respectively [34,35]. The Mediator complex is known under an impressive number of different names and compositions such as TRAP, DRIP, ARC, SMCC, NAT, PC2, and CRSP (reviewed in [36,37]). This variation in composition might be linked to either the method of identification or the fact that larger complexes may be prone to dissociate during purification [38]. Alternatively, it may indicate that these Mediator complexes are modular in nature and that their composition varies according to promoter requirements [37]. Like CPB/p300 (discussed below) the Mediator complex can mediate contacts between gene specific factors and the basal transcription machinery. However, unlike CBP/p300, no HAT activity has yet been positively linked to any of the Mediator complexes. Nevertheless, most Mediator complexes have been shown to be coactivators, although some are also capable of repression of transcription [35,39-43]

1.1.5 Transcription factors

Selective regulation of transcription requires the coordinated actions of the basal transcription machinery, general cofactors and gene specific transcription factors. These transcription factors generally consist of a sequence specific DNA binding domain and a

transcription activation domain (TAD). The distinction between these domains may not be as clear as it appears, since some transcription factors are still able to activate transcription after deletion of their activation domain. Their DNA binding motif might for instance act as a recruiting platform for cofactors [44-46], or, alternatively, impose conformational stress on the DNA, which might facilitate recruitment of additional DNA binding factors [47]. Transcription factors are classified by common motifs, usually their DNA binding domains. A wealth of structural data on DNA binding domains has been accumulated over the past years. Families of structurally conserved motifs have been defined including the helix-turn-helix, leucine-zipper and zinc-finger motifs [48-52].

1.1.6 Transcription activation domains

Surprisingly little is known about the exact structure-function relations in the mechanisms by which TADs may work. A complicating factor in researching TADs is their lack of clearly conserved motifs. There is little sequence conservation in TADs. Nevertheless, a classification can be made based on general amino acid content. In this way acidic, serine/threonine rich, proline rich and glutamine rich TADs can be distinguished. Homopolymeric stretches of glutamine and proline fused to a Gal4 DNA binding domain have been shown to activate transcription in HeLa cells [53]. Glutamine and proline rich TADs have been shown to transactivate promoters from proximal positions, while being unable to activate a promoter from a distal enhancer. In contrast an acidic TAD could activate the promoter from both proximal and distal positions [54,55]. Therefore, it is likely that these different TAD types function via distinct mechanisms. Furthermore, there is evidence that hydrophobic and acidic residues in TADs affect transcription at different moments in the process. In one study hydrophobic residues in the TAD of yeast heat shock factor 1 (HSF-1) were shown to be important in stimulating elongation of a paused RNAPII, while the acidic residues were more important in stimulation initiation of transcription from the hsp70 gene [56]. Furthermore, it was shown that activation domains could function by either recruiting RNAPII or TFIID to the promoter [57]. Other studies show that acidic TADs may recruit SWI/SNF chromatin remodeling

complexes to a promoter [58-60]. Thus activation domains may serve to contact the basal transcription machinery or recruit cofactors.

1.1.7 Structures of TADs

Many TADs do not fold into a stable structure in solution. This observation led to the proposal that TADs may undergo an "induced fit" when they are brought in close proximity of their target. Indeed such induced fit mechanisms were shown for interaction of VP16 and p53 with $hTAF_{II}31$ and MDM2 [61-63]. The TADs of VP16 and p53 both contain FXXΦΦ motifs (where X is any amino acid and Φ is a hydrophobic residue), which folds into an α-helix, when it is bound to $hTAF_{II}31$. This induced fit places the phenylalinine and the two remaining hydrophobic residues on one side of the α-helix, presenting a hydrophobic patch to an interaction interface on $hTAF_{II}31$, which could propagate the signal to the basal machinery. A similar motif is involved in the interaction of the activation domain of the transcription factor CREB and the KIX domain of coactivator CBP. Apart from a slight tendency to form α-helices, the phosphorylated kinase inducible domain (pKID) is unstructured in solution. Upon binding, pKID undergoes a coil to helix folding transition, wrapping into two α-helices on a hydrophobic surface on the KIX domain [64,65]. This KIX domain on CBP interacts with the TADs of a number of other transcription factors, which contain hydrophobic patches similar to the FXXΦΦ motif. For one of these, the interaction with c-Myb, studies indicate that its TAD employs a similar mechanism of binding as the pKID-KIX interaction [66].

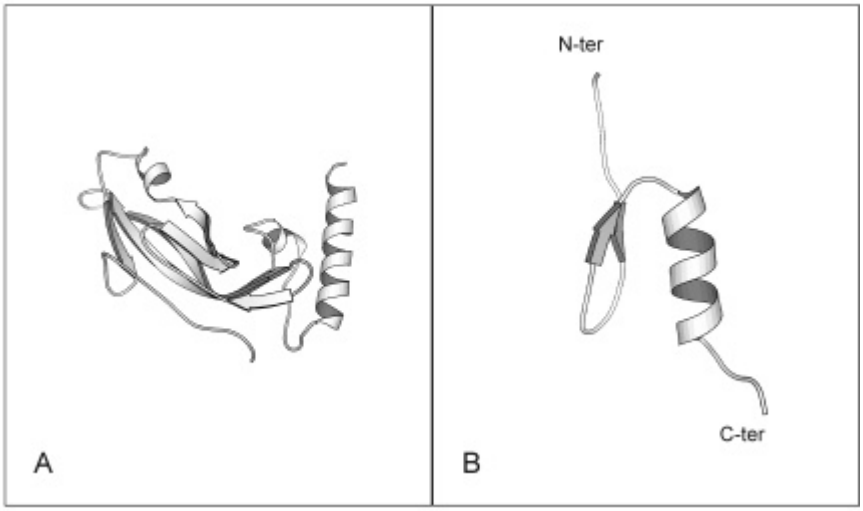

Figure 2

A) Crystal structure of HPV-E2 TAD.
B) Solution structure of the Zn-finger like TAD of ATF-2.

It is not true that all TADs are structurally undefined. The crystal structure of the proteolytic core of the E2 activation domain of the human papillomavirus (HPV) has recently been determined [67]. The structure revealed a cashew shaped β-sheet framework, on which a slightly less well-defined glutamine-rich α-helix was docked (Fig. 2a). This potentially flexible glutamine-rich α-helix may well function as a "classical" glutamine rich type TAD in contacting the basal machinery. A rationale for the high level of structure of this domain may be the dual function of the E2 activation domain in both transcription activation and stimulation of HPV DNA replication. A later study, which covered the intact full length E2 TAD, showed essentially the same fold, with the exception that the additional N-terminal residues proved to be involved in dimer formation of the TAD [68]. This dimer formation may be important in regulating the switch between the E2 TAD's function in transcription activation and replication. Another example of a structurally well-defined element in a TAD is the solution structure of ATF-2 [69]. The structure of this TAD showed a completely unfolded C-terminal part, which does

contain FXXΦΦ like patches, whiles the N-terminus folds into a typical zinc-finger structure (Fig. 2b, reviewed in [70]). This zinc-finger may mediate protein-protein contacts with other transcription factors, which could mask or unmask the function of the remaining TAD. Further structural research of TADs in complex with their targets will be required to elucidate their exact functional mechanisms.

1.1.8 Coactivators and corepressors

Sequence specific transcription factors act in concert with cofactors. These factors either do not bind DNA themselves or do so in an aspecific manner, therefore, they need to be recruited to a promoter by transcription factors already bound to DNA. Cofactors act as bridging elements between transcription factors and the chromatin or the basal machinery. Many cofactors contain enzymatic activities such as HAT activity for coactivators and the reverse reaction (histone deacetylase (HDAC)) for corepressors. One such HAT containing coactivator is the CREB binding protein CBP, which is often referred to in combination with closely related p300. Even though these two factors are highly similar, there is evidence that they are not completely functionally interchangeable. For instance, p300 lacks a PKC consensus site that is present in CBP, which needs to be phosphorylated to mediate AP-1 responsiveness [71]. Furthermore, genetic studies in mice indicate subtle differences between CPB and p300 knockouts [72,73], reviewed in [74]. CBP/p300 binds a variety of transcription factors and mediates contacts between these factors and the general transcription machinery [75-77]. In addition to histones, the acetyl transferase activity of CBP/p300 can also target other transcription factors such as p53 and Pit-1, which can modulate their DNA binding affinity [78,79]. Finally, CBP/p300 can mediate contacts with the Mediator complex.

Analogous to stimulation by coactivators, corepressors can be recruited to a given promoter to silence its gene expression. Examples of corepressors are the nuclear receptor corepressor (NCoR) and the NuRD and Sin3 complexes. NCoR is found associated with unliganded or antagonist-bound nuclear hormone receptors [80], but can also associate with other types of transcription factors such as Pit-1 [46], Rpx2 and Pbx [81], and CBF-1 [82]. Both NCoR and closely

related SMRT (silencing mediator for retinoid and thyroid receptors) function by recruiting and activating HDACs to target promoters [83], and there is evidence that they also interact with the basal transcription machinery [84,85]. These HDACs are either recruited directly, or as part of the Sin3 corepressor complex, which is involved in both NCoR and Mad-Max mediated repression [86,87]. The NuRD (nucleosome remodeling and deacetylase) corepressor complex contains both ATP-dependent chromatin remodeling and HDAC activities, which appear to complement each other [88]. Furthermore, NuRD has recently been shown to contain a subunit that specifically recognizes methylated DNA, which would link epigenetic signaling to gene silencing [89].

Part II: Transcription regulation by Pit-1.

1.2.1 Introduction

The mammalian pituitary gland provides an excellent model system for the study of organogenesis and transcription regulation. The reason for this is that it features differentiation of six distinct cell types, in addition to highly restricted gene expression in these cell lineages. The pituitary gland is a composite organ consisting of two main lobes and a smaller intermediate lobe. These lobes are derived from two distinct embryonic tissues. The anterior and intermediate lobes, which make up the anterior pituitary or adenohypophysis, originate from an invagination in the oral ectoderm called Rathke's pouch. The posterior lobe or neurohypophysis is derived from the neural ectoderm. In the mature pituitary gland the neurohypophysis secretes anti-diuretic hormone (ADH) and oxytocin. The mature adenohypophysis produces a number of hormones from six different cell lineages. These are from dorsal to ventral: melanotropes secreting melanin-stimulating hormone (MSH); corticotropes, adrenocorticotropin (ACTH); lactotropes, prolactin (PRL); somatotropes, growth hormone (GH); thyrotropes, thyroid stimulating hormone (TSH); and finally gonadotropes, which secrete luteinizing hormone (LH) and follicle-stimulating hormone (FSH). A complex network of transcription factors tightly controls the embryonic development, differentiation of cell lineages and hormone production (reviewed in [90-93]). One of these transcription factors, called Pituitary transcription factor 1 (Pit-1), plays a central part in this network. The structural and functional aspects of Pit-1, as well as some transcription factors cooperating with Pit-1 will be discussed in detail.

1.2.2 Pituitary-specific transcription factor Pit-1

Pit-1 (previously also known as Growth Hormone Factor 1 (GHF-1); now officially referred to as POU1F1) is specifically expressed in the pituitary gland [94]. Two alternative translation start sites cause the protein to be expressed in both a 31 and 33 kD form [95]. The protein consists of a N-terminal transcription activation

domain and a C-terminal DNA binding domain (Fig. 3) [96,97]. The DNA-binding domain of Pit-1 [98] is one of the founding members of the POU domain (POU is an acronym derived from the mammalian Pit-1, Oct-1 and Oct-2 and C. elegans Unc-86 factors) family of transcription factors ([99] reviewed in [100-103]), which will be discussed in detail below.

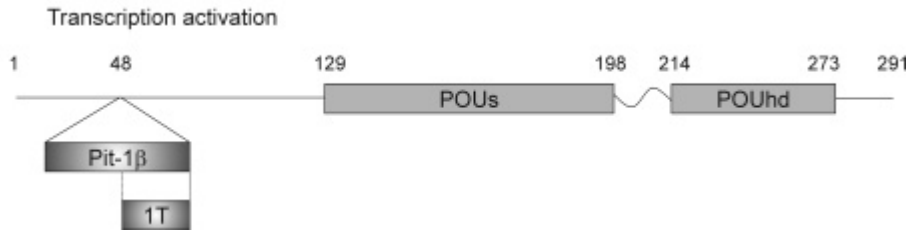

Figure 3

Pit-1 contains two DNA binding domains: the POU-specific and POU-homeodomain separated by a flexible linker. The N-terminal TAD can be alternatively spliced, giving rise to Pit-1β, which has a 26 amino acids insert at position 48 and Pit-1T, which only has the C-terminal 14 amino acids of the β-insert at the same position.

The Pit-1 gene was initially identified for its ability to bind and activate GH and PRL transcription [104,105]. Later it was found that Pit-1 also regulates the expression of the β-subunit of the thyroid-stimulating hormone (TSHβ) [106,107]. All three promoters contain a number of Pit-1 binding sites (Fig. 4). Extra-cellular signaling through both PKA and the Ras signaling pathways has been mapped to some of these binding sites [108,109].

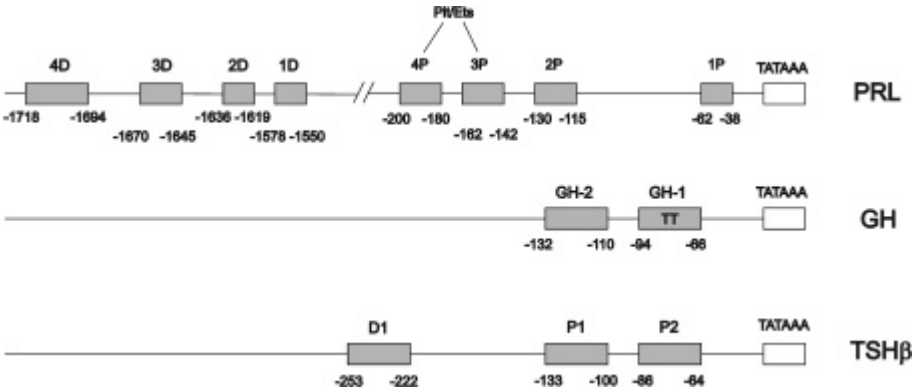

Figure 4

Promoter structure of the rat PRL and GH and the human TSHβ genes. Boxed areas indicate regions protected by Pit-1 in a DNaseI footprint. The PRL promoter contains a distal (D) as well as a proximal (P) promoter element for Pit-1. Both the 3P and 4P sites contain composite Pit-1/Ets-1 binding elements. The double T base insertion in GH-1 is marked. Data for PRL and GH promoters was taken from [95, 97] and for TSHβ from [97]

Two dwarf mouse strains have been described containing defects in the Pit-1 gene: the Snell and Jackson mouse strain, which have a substitution (W261C) in the DNA binding POU homeodomain and a deletion of a major part of the Pit-1 gene due to a genetic rearrangement, respectively [110]. A third dwarf mouse strain, the Ames mouse, contained an intact Pit-1 gene, but lacked Pit-1 expression due to a substitution (S83P) in the "Paired-like" DNA binding domain of the Prophet of Pit-1 (Prop-1) gene. Prop-1 expression precedes and is required for Pit-1 expression, but the exact mechanism involved remains unknown [111,112]. Studies of the Snell, Jackson and Ames mice showed that the secretion of GH, PRL and TSH is deficient in the absence of a functional Pit-1 gene product. Furthermore, these mice all showed hypoplastic pituitary glands, devoid of somatotropes, lactotropes and thyrotropes. Thus Pit-1 is an essential factor for both the differentiation of these three cell-types and the expression of the hormones they produce. Moreover, Pit-1 regulates its own expression by binding to sites in the promoter region

of the Pit-1 gene [113,114]. In humans, mutations in Pit-1 are associated with Combined Pituitary Hormone Deficiency (CPHD) [115].

1.2.3 TAD and splice variants of Pit-1

The TAD of Pit-1 does not seem to belong to any particular class of activation domains as mentioned above, although there is a high content of hydroxylated and proline residues. Dissection of the TAD shows an exon dependent functionality, with exon 1 (residues 1-47) acting as a "generic" activation domain [96]. Exon 2 (residues 48-72) contains an Y6Y6Y motif, which mediates contacts with the Estrogen receptor [116] and finally exon 3 (residues 73-147), which contains a proline rich and acidic region that is involved in synergistic with the thyroid receptor [117]. In this Y6Y6Y motif, any two of the three tyrosines are required for synergistic activation of the PRL promoter with Estrogen receptor. The exact spacing of the tyrosines does not seem to be important, as substitution with an Y5Y5Y motif from the human lymphoid specific transcription factor (hLEF-1) does not affect synergism. However, the interceding sequence also proves to be a determining factor, as substitution with an Y5Y5N motif from Ets-related SAP-1 results in a complete loss of synergism. This effect is likely due to the interceding residues in the SAP-1 Y5Y5N motif, as the Y6Y6N mutation in Pit-1 still shows wild-type synergism. Use of this Y6Y6Y required Pit-1 to bind as a monomer to the distal PRL-1D site [116].

Two alternative splice variants have been identified in the TAD of Pit-1: Pit-1β (also referred to as Pit-2) and Pit-1T. Both isoforms contain an insertion between exon 1 and 2 (Fig. 3). Pit-1T is an alternative splice variant that is exclusively expressed in the thyrotrope cell lineage [118]. The Pit-1T isoform is essentially the same as the Pit-1β splice variant, except that the Pit-1T insert corresponds to the C-terminal 14 amino acids of the β-insert (Fig. 3). Transfection analysis showed that while Pit-1, Pit-1β and Pit-1T alone were equally ineffective, a combination of Pit-1T and wild-type Pit-1 was capable of an optimal activation of the TSHβ promoter [119]. Pit-1T mRNA levels were detected at about 2 % of wild-type Pit-1 levels.

Pit-1β contains an insertion of 26 amino acids between exon 1 and 2 [120-122]. This alternative splicing of Pit-1 affects its ability to activate transcription. Pit-1β is more efficient in activating the PRL promoter when tested in non-pituitary cells, but less efficient in pituitary GH_4 cells [123]. Furthermore, Pit-1β is a more potent mediator of PKA response in combination with Oct-1 [109]. Finally, Pit-1β represses Ras responsive signaling by inhibiting Pit-1/Ets-1 synergistic activation of the PRL promoter probably by direct interaction with Ets-1 and by recruiting an NCoR/mSin3 corepressor complex [124-126]. Two patches of hydrophobic residues within the β-domain were found to be important in this repression of Ras signaling [126]. The level of Pit-1β mRNA is about 14 % of wild-type [120,121], while the expression level of this alternatively spliced Pit-1 is about 3 % of the wild-type Pit-1 [122]. Therefore, either Pit-1β is less stable, or less well transcribed than Pit-1.

1.2.4 POU domains

The POU domain factors are modular in nature, containing two independent helix-turn-helix folds separated by a flexible linker, which is of variable length. The N-terminal POU-specific (POUs) is a four-helix bundle, while the C-terminal POU-homeodomain (POUhd) is a three-helix bundle (Fig. 5). Intriguingly, the in solution NMR structure of the Oct-1 POUs domain revealed that its fold is highly similar to the bacteriophage 434 and λ repressors, even though the these domains share little sequence homology [127,128]. This structural conservation and modular nature of the POU domain family are clear examples of complexity arising from relatively simple precursor systems. Both the POUs and POUhd are independently capable of recognizing their target sites, albeit with reduced affinity compared to the full-length POU domain [129]. A mixture of untethered subdomains still shows a certain amount of cooperativity in binding its target site. However, tethering the two domains together via the flexible linker serves to increase the local concentration of either domain, once a promoter sequence is bound by one subdomain [130]. This allows one single POU domain to act as a heterodimer and increases the cooperative effect in binding the target site.

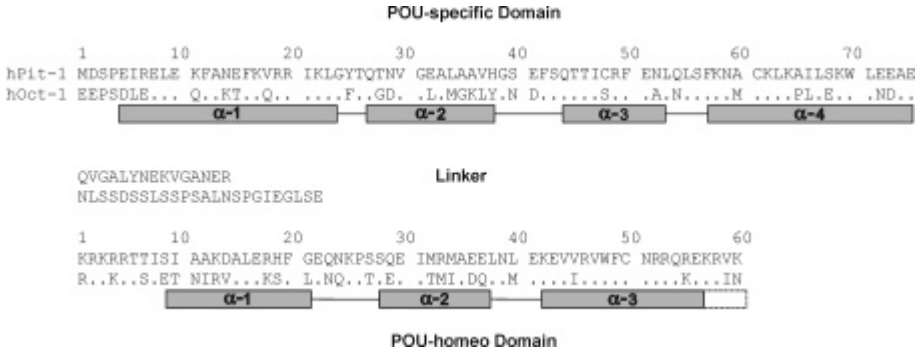

Figure 5

Comparison of the human Pit-1 and Oct-1 sequences. Structural elements as they were encountered in the crystal structure are indicated below the sequences. The numbering corresponds to the constructs used in the crystal structure; the first residues of Pit-1 and Oct-1 correspond to residue 124 and 303 respectively in the full length protein. The dashed box at the end of helix 3 in the POUhd indicates the unfolded helix at the dimerization interface of Pit-1.

A striking consequence of the modular structure and the variable linker lengths in the POU-domain transcription factors is the enormous flexibility in both sequence recognition and binding conformations. In the case of Pit-1 the recognition sequence varies around a loose consensus: (A/T)(A/T)TATNCAT (where N is any base). Pit-1 preferentially binds as a dimer, though it can also bind as a monomer [97,116,131]. In contrast, Oct-1, which is an ubiquitously expressed gene involved in activation of H2B [132], small nucleotide RNA [133] genes and viral replication (reviewed in [134]), primarily binds as a monomer, but can also act as a dimer [135,136]. Furthermore, even when binding as a monomer, Oct-1 can adopt several arrangements (Fig. 6) of the POUs and POUhd on the DNA, as was shown by photocrosslinking experiments [137].

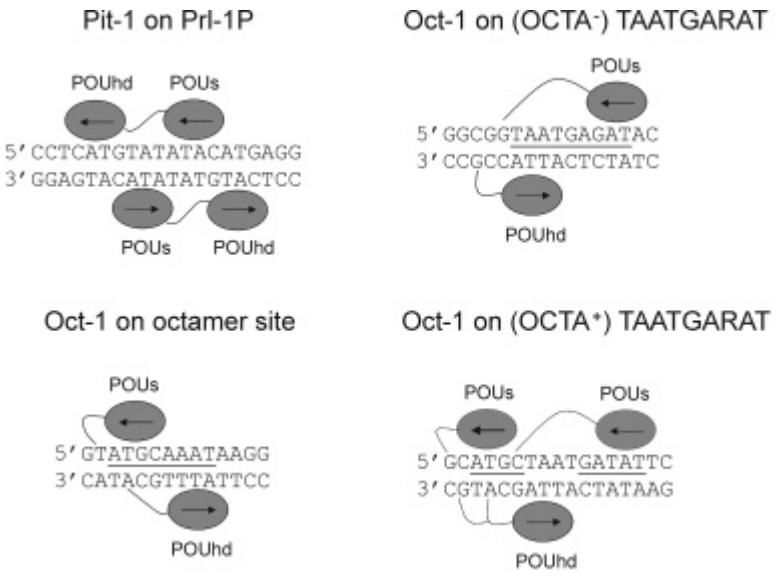

Figure 6

Flexibility in arrangement of POUs+hd. The orientation of the subdomains is dictated by the DNA binding site. Pit-1 binds as a dimer to the palindromic Prl-1P site, while Oct-1 binds as a highly extended monomer to the Octamer sequence. Different orientations were shown by photocrosslinking Oct-1 to different DNA sites.

1.2.5 Pit-1 and Oct-1 structure

The structures of Oct-1 and Pit-1 bound to DNA have both been solved by X-ray crystallography (Fig. 7a) [131,135]. In the Oct-1 cocrystal structure the POUs and POUhd bind to opposite sides of the DNA as a highly extended monomer. The third α-helix of the POUs docks in the major groove of the ATGC part of the octamer (5'-ATGCAAAT-3') DNA element. The POUs makes several base specific hydrogen bonds: Gln 44 forms a double hydrogen bond to A1, Thr 45 binds T2 and the complementary base to G3 (C'3) and finally, Arg 49 contacts G3 and G'4. One hydrophobic residue (Leu 55) contacts the methyl group of T5 (Fig. 7b). Sequence specific contacts are complemented by a number of contacts to the phosphate-backbone by residues throughout the rest of the POUs. Likewise, the third α-helix of the POUhd docks in the major groove of the AAAT part of the octamer, where it makes one hydrogen bonding (Asn 51 to A7)

and two hydrophobic (Val 47 makes a van der Waals contact to T8 and Cys 50 docks between T'9 and T'10) contacts. The flexible linker is disordered and therefore not visible in the cocrystal structure. However, the C-terminal part of the linker, towards the POUhd is located in the minor groove between the ATGC and AAAT parts of the octamer sequence, where Arg 5 of the POUhd contacts A5 of the AAAT half site. Interestingly, Leu 55 of the POUs contacts the complementary T (T'5) to this base. Such dual contacts might explain the cooperative effect of binding of the two subdomains to DNA.

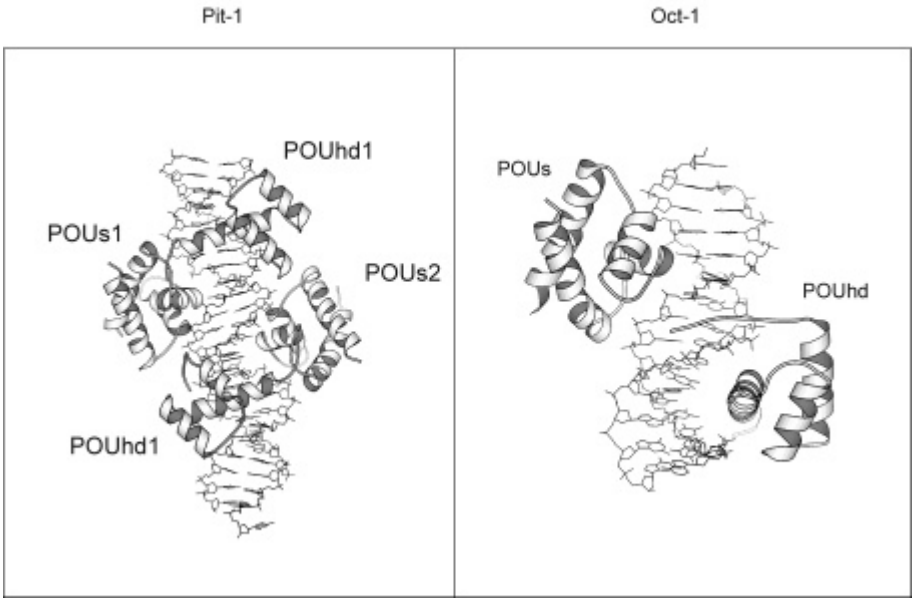

Figure 7a

Crystal structures of Pit-1 and Oct-1. Pit-1 binds as a dimer to the palindromic Prl-1P site. The flexible linkers between the POUs and POUhd are not visible.

The Pit-1 cocrystal structure shows Pit-1 bound as a dimer to a palindromic site (5'-CCTCATGTATATACATGAGG-3') derived from the PRL-1P promoter element. The structure reveals a dramatic rearrangement of the POUs and POUhd subunits. Instead of binding opposite sides of the DNA, the POUs and POUhd now bind perpendicular to each other on the DNA. In addition, the POUs have

flipped their orientation compared to the Oct-1 cocrystal structure (see also Fig. 7a). In spite of these differences, the DNA contacts of the subdomains are very similar to those observed in the Oct-1 structure (Fig. 7b). The POUs in this structure recognize an ATAC half site, while the POUhd bind to a TCAT half site. Again, the POUs contribute base specific contacts through Gln 44, Thr 45 and Arg 49, but now additional hydrogen bonds are seen with Ser 43 and Arg 49 to T4/T'9 and G3/G'10, respectively. The POUhd also shows two new contacts (Arg 46 to T-2/T'14 and Gln 54 to A1/A'12) in addition to those that were also seen in the Oct-1 structure. It is possible that the slightly higher resolution (2.3 Å vs. 3.0 Å) of the Pit-1 cocrystal allowed for the identification of these extra contacts.

The fact that Pit-1 is bound as a dimer in the cocrystal structure and the absence of the flexible linker in these studies, allows for two possible combinations of linkages. One of these would put the two subdomains of one monomer on opposite sites of the DNA, much like the arrangement in the Oct-1 cocrystal structure. The other combination would put the two monomers on more or less the same face of the DNA. The fist arrangement would require a highly extended (and thus more rigid) linker structure, while the second arrangement would allow for a more relaxed conformation of the linker. To discriminate between these two possibilities, Jacobson *et al* assayed the dimidiation ability of several linker deletion mutants of Pit-1. These studies showed that Pit-1 dimerization was tolerant to 3 and 5 amino acid deletions from the linker, confirming the relaxed linker conformation in the second monomer arrangement [131].

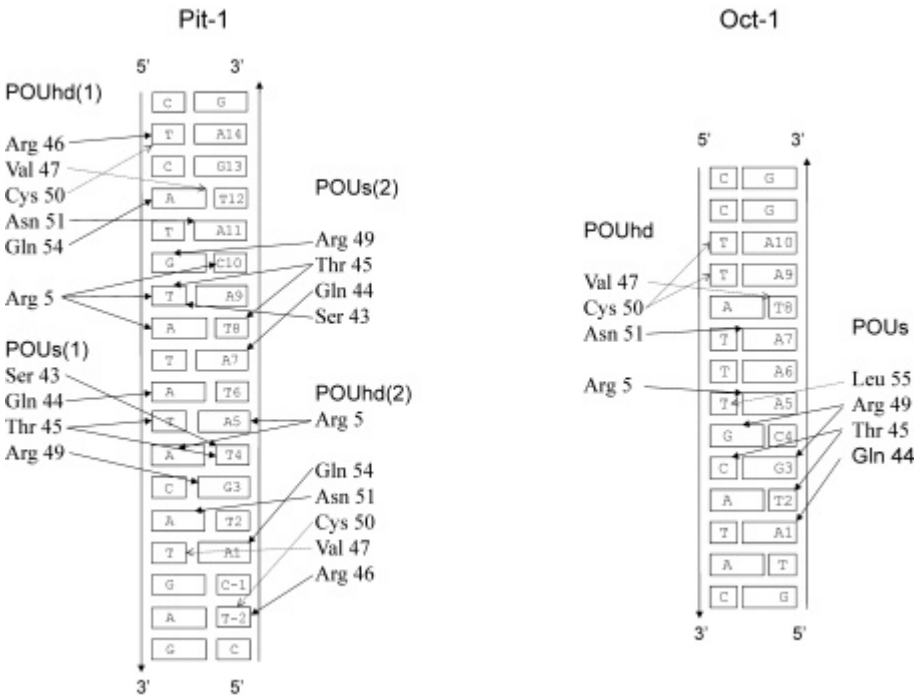

Figure 7b

Protein-DNA contacts as they occur in both the Pit-1 and the Oct-1 crystal structures. Numbering corresponds to the one used in Fig. 7. Dashed arrows depict non-polar contacts.

The dimer interface is located at the C-terminal part of the POUhd, which docks on the surface formed by the loop between the second and third α-helix and the N-terminal part of the first α-helix of the POUs of the adjacent monomer (Fig. 8). Direct hydrogen bonds are formed between the backbone amide protons from the POUhd residues Val 59 and Lys 60 with the carboxyl groups from Leu 53 and 55 on the POUs. Two additional hydrogen bonds are formed between the side chain of POUhd Arg 58 and the backbone and side chain carboxyl groups of POUs Gln 54. Furthermore, POUhd Val 59 docks in a hydrophobic pocket formed by the POUs residues Met 6, Leu 9, Leu 53 and Phe 57. Finally, POUhd Lys 60 might form a salt bridge with Glu 10 of the POUs. Intriguingly, one of the most frequent Pit-1

mutations associated with CPHD maps to the dimer interface of the POUhd. This mutation (R271W) is likely to affect dimerization of Pit-1 on DNA, as Jacobson *et al* showed deletion of the last three residues (Arg 58, Val 59 and Lys 60) from the POUhd impaired dimerization on three separate probes. The R271W mutation results in a dominant negative phenotype [138,139], which implies that dimerization of Pit-1 is essential for its *in vivo* function.

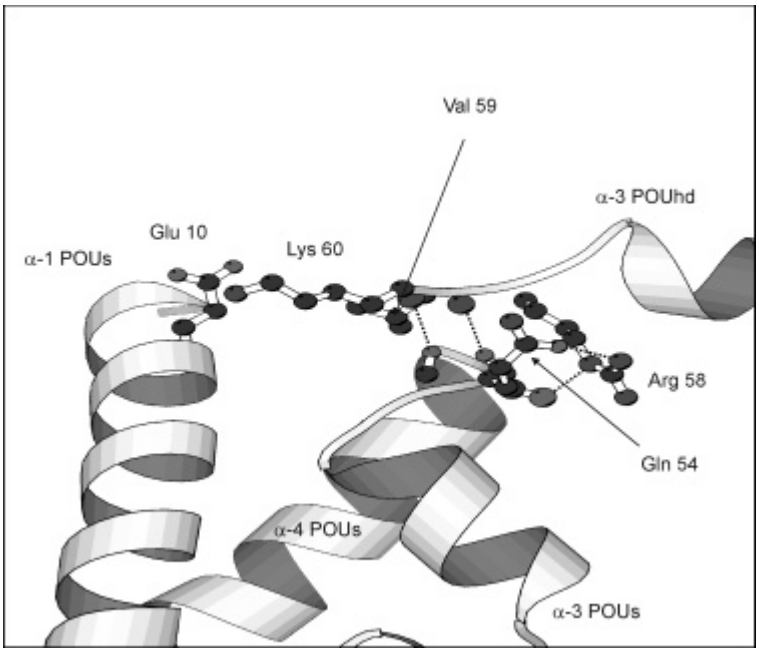

Figure 8

The dimerization interface between the POUs of one monomer and the C-terminus of the POUhd of the other monomer. Arg 58 of the POUhd makes a double hydrogen bond to the backbone and side chain carboxyl group of Gln 54. The backbone amide protons from POUhd Val 59 and Lys 60 hydrogen bond to the backbone carboxyl group from POUs Leu 53 and 55. The side chain of POUhd Val 59 docks in a hydrophobic pocket on the POUs surface. Finally, POUhd Lys 60 may form a salt bridge with POUs Glu 10.

1.2.6 Allosteric effects

The high level of flexibility in DNA binding of POU transcription factors allows for an intriguing level of promoter control. In a recent study by Scully *et al*, the differential activation of the PRL and GH genes in somatotropes and lactotropes was investigated [140]. It was found that substitution of the most proximal Pit-1 binding site in the GH promoter (GH-1) with the corresponding site from the PRL promoter (Prl-1P) resulted in expression from the mutant GH promoter in lactotropes. The finding that a single Pit-1 binding site was responsible for repression of GH expression in lactotropes prompted Scully *et al* to cocrystallize Pit-1 using the GH-1 or Prl-1P site. The resulting cocrystal structures showed Pit-1 bound as a dimer to both sites, with the same contacts and overall arrangement as was found on the palindromic site (discussed above). However, due to a two base pair insertion between the half sites of the GH-1 element, the spacing between the POUs and POUhd of each monomer was increased from 4 bp to 6 bp. This resulted in binding of each Pit-1 monomer to the same side, rather than to perpendicular faces of the DNA (Fig. 9).

The authors show, by chromatin immunoprecipitations (ChIP) and transgenic mouse studies, that this alternative conformation of the Pit-1 dimer preferentially recruits a NCoR containing corepressor complex to the GH promoter in lactotropes. Thus, a model is proposed wherein PRL and GH gene control in somatotropes and lactotropes is regulated by the balance between coactivator (CBP) and corepressor (NCoR) complex recruitment by Pit-1. A previous study, in which a Pit-1 mutant (E254A; which corresponds to E41A in Fig. 5) showed an increased affinity for NCoR, while DNA and CBP binding were unaffected, corroborates this model [46]. This point mutant proves that CBP and NCoR use different interaction surfaces on the POU domain, which can therefore be masked or unmasked by the orientation of the Pit-1 POU domain on the DNA. What the study of Scully *et al* does not show is why the same GH-1 binding site leads to activation of GH in somatotropes. Most likely, a cell-specific factor either overrides NCoR mediated repression in somatotropes or enhances this repression in lactotropes. Such an enhancer of repression might exist in lactotropes, as mutation of the -161/-146 promoter element of the

rat PRL promoter resulted in GH expression in lactotropes [140]. Similar allosteric effects were show for Oct-1 bound as a dimer to PORE (5'-ATTTGAAATGCAAAT-3') or MORE (5'-ATGCATATGCAT-3'). The PORE-bound Oct-1 dimer was able to recruit coactivator OBF-1, while in the MORE-bound dimer the interaction domain for OBF-1 was buried in the dimerization domain [136].

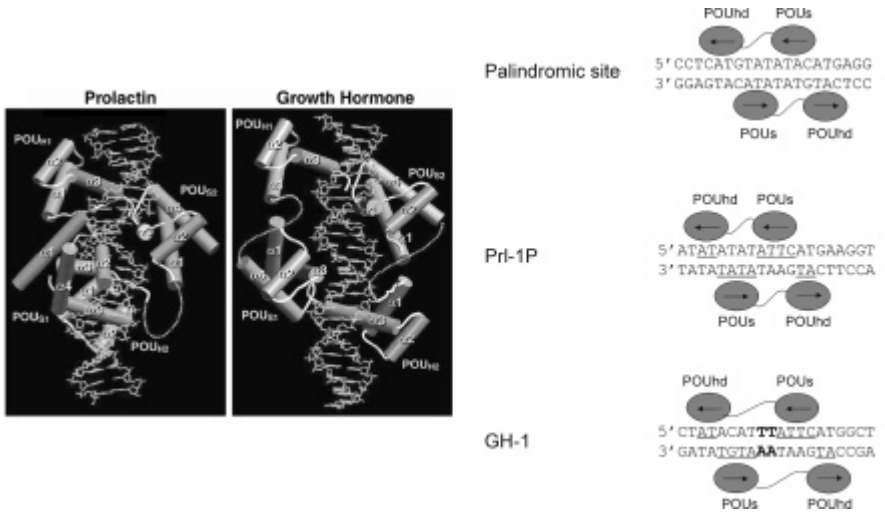

Figure 9

Allosteric effects in Pit-1 binding. The insertion of two T bases results in a different arrangement of the Pit-1 dimer on DNA (adapted from ref. 140). Both crystal structures were aligned to show the same orientation of the POUhd from monomer 1 (top right). To the right a schematic representation of the orientation of each dimer on its DNA sequence.

1.2.7 Pit-1 phosphorylation

In response to the cell cycle or homeostatic signaling, Pit-1 can be phosphorylated at two positions: S115 and T220 [141,142]. Phosphorylation of T220 seems to have a negative effect on DNA binding in general, though the effects may differ from minimal to a 10 fold decrease in affinity, depending on the exact sequence of each

binding site [141-143]. The corresponding residue in Oct-1 (S385) is also phosphorylated in a cell cycle dependent manner, which strongly inhibits its DNA binding affinity [144]. A structural rationale for the less dramatic, site-dependent effects of phosphorylation at T220 in Pit-1 can be found in the crystal structure of Pit-1 bound to the palindromic site. Contrary to S385 in the Oct-1 crystal structure, T220 is not in close proximity (3.6 vs. 6,7 Å) to any phosphate group. Therefore phosphorylation at T220 is likely to have less dramatic or more context dependent effects on DNA binding than the corresponding modification in Oct-1 [131,135]. The *in vivo* relevance of Pit-1 phosphorylation is as yet unclear. Mutating S115, T220 and a minor phosphorylation site at T219 to alanines did not affect Pit-1 transactivation capabilities on the GH and PRL promoters [145] or its ability to cooperate with Ets-1 [125]. Furthermore, while phosphorylation of Pit-1 was highly increased by Thyrotropin releasing hormone (THR), this was not the cause of an increased PRL expression, since the same stimulation by THR was observed in the presence of agents that blocked Pit-1 phosphorylation [146]. Further research is required to assess the role of phosphorylation of Pit-1 *in vivo*.

1.2.8 Pit-1 interacting factors

Pit-1 acts in concert with a number of other transcription factors to confer gene specific activation or repression. This may involve direct protein-protein interactions, or DNA mediated contacts. Figure 10 shows an overview of all protein-protein interactions with Pit-1 reported thus far. Recruitment of coactivator and corepressor complexes [46,140], the interaction with the Estrogen receptor (ER) [116] and Thyroid receptor (TR) [117] have been discussed above. A third nuclear receptor, the vitamin D receptor (VDR) was shown to interact with Pit-1 through the POUhd [147]. P-OTX or Pituitary homeobox 1 (Ptx-1) was identified in a yeast two-hybrid screen using the Pit-1 TAD as bait [148]. Further research showed that P-OTX / Ptx-1 is involved in the activation of several pituitary-specific promoters and that it can act in synergy with Pit-1 [149,150]. P-Lim is a homeobox containing protein that is expressed in the pituitary and can also act in synergy with Pit-1. Direct protein-protein interaction

involves the DNA-binding homeobox of both proteins [151]. A similar interaction exists with Oct-1: both a weak in solution protein-protein interaction and a heterodimerization through the POU domains of both proteins on the PRL-1P promoter element [152]. Heterodimerization with Oct-1 on one DNA site would allow the simultaneous use of functionally distinct pathways of transcription activation, such as recruitment of CBP by Pit-1 and OBF-1 and VP-16 by Oct-1. The interactions of Pit-1 with GATA-2 and Ets-1 will be discussed in detail below, as they are studied in this thesis.

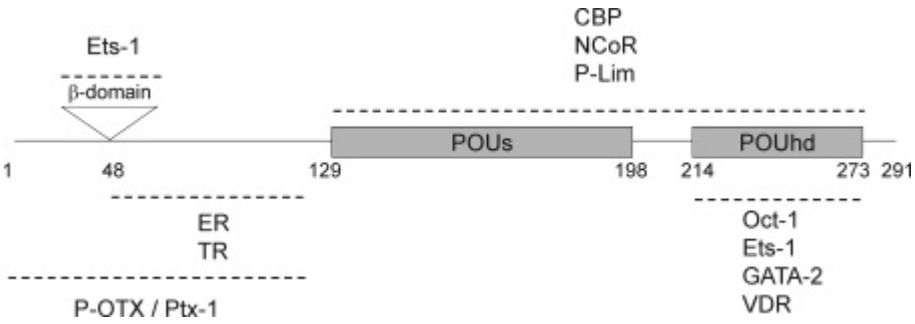

Figure 10

Overview of proteins with a reported physical interaction with Pit-1. The dashed lines indicate the mapped region of each interaction.

1.2.9 GATA-2

Zinc-finger protein GATA-2 is a member of the GATA-binding family of transcription regulators. The GATA factors are required for a number of developmental processes, such as the normal hematopoietic development [153-156]). Two CX_2C-X_{17}-CNAC Zn-finger motifs regulate DNA binding in the GATA proteins. Both Zn-fingers will recognize the consensus (A/T)GATA(A/G) motif [157], but DNA binding affinity depends on the presence or absence of clusters of basic amino acids adjacent to each finger [158]. Thus in GATA-1, where the N-terminal Zn-finger lacks this basic region, the

C-terminal finger provides most of the binding affinity, while the N-terminal finger stabilizes the DNA binding [159,160].

A factor binding along with Pit-1 to the P1 region of the TSHβ promoter was identified as GATA-2. Further investigation showed that Pit-1 and GATA-2 could synergistically activate the TSHβ promoter and that the intact binding sites of both factors were required for this response [161]. A detailed study by Dasen *et al* revealed that, along with Pit-1, GATA-2 is an essential player in the cell-fate determination in the anterior pituitary [162]. Dasen *et al* showed that GATA-2 is present in a ventral to dorsal gradient and that at the highest level of this gradient, Pit-1 expression is restricted. Furthermore, Pit-1 could bind GATA-2 in a DNA-independent fashion, thus preventing DNA binding by GATA-2. This protein-protein interaction required the POUhd of Pit-1 and the C-terminal Zn-finger, along with a stretch of basic residues, of GATA-2. Pit-1 mediated restriction of DNA binding was effective, unless a promoter contained both a Pit-1 and a GATA-2 site, such as in the case in the TSHβ promoter. Thus, in the absence of Pit-1, regulation of transcription by GATA-2 causes cells to terminally differentiate into gonadotropes. When both Pit-1 and GATA-2 are present, restriction of DNA binding by Pit-1 allows only for Pit-1 / GATA-2 composite promoter elements to be activated, causing differentiation into thyrotropes and not gonadotropes. Finally, in the absence of GATA-2, Pit-1 regulation causes precursor cells to differentiate into somatotropes or lactotropes.

1.2.10 Ets-1

Ets-1 was originally identified as v-ets, a transduced part of a fusion protein with v-myb, which was expressed by the E26 (Ets is derived from E-twenty-six specific) acute avian leukemia retrovirus [163]. The cellular counterpart turned out to be a prototypical member of an entire family of transcription factors containing the Ets domain (reviewed in [164,165]). The Ets domain in Ets-1 recognizes a GGA(A/T) motif [166]. Both the free and DNA bound structures of the Ets-1 domain, which is defined as a "winged helix-turn-helix" DNA binding domain, have been solved by NMR (Fig. 11) [167,168]. DNA binding of Ets-1 is regulated by sequences both N- and C-

terminal of the DNA binding domain, which fold into auto-inhibitory modules [167,169]. Upon DNA binding, conformational changes are observed in the auto-inhibitory modules, as indicated by altered protease sensitivity [170,171]. Inhibition of DNA binding can be relieved by cooperative binding with another factor [172,173] or reinforced by phosphorylation of sequences directly upstream of the N-terminal auto-inhibitory domain [174].

The N-terminal region of Ets-1 contains a separately folding α-helical domain called the Pointed (PNT) domain. The structure of this domain, which is shared by a number of Ets family members, was solved by NMR spectroscopy [175]. Located at the N-terminus of the PNT domain is a threonine (T38), which is targeted by MAP kinase upon Ras activation (reviewed in [176]). The exact mechanism by which Ras-mediated phosphorylation enhances transcription activation by Ets-1 remains enigmatic. The NMR study by Slupsky *et al* did not reveal any structural rearrangements or changes in protein dynamics upon phosphorylation [175]. Therefore, enhanced activity of MAP kinase phosphorylated Ets-1 is likely due to increased binding of a secondary factor.

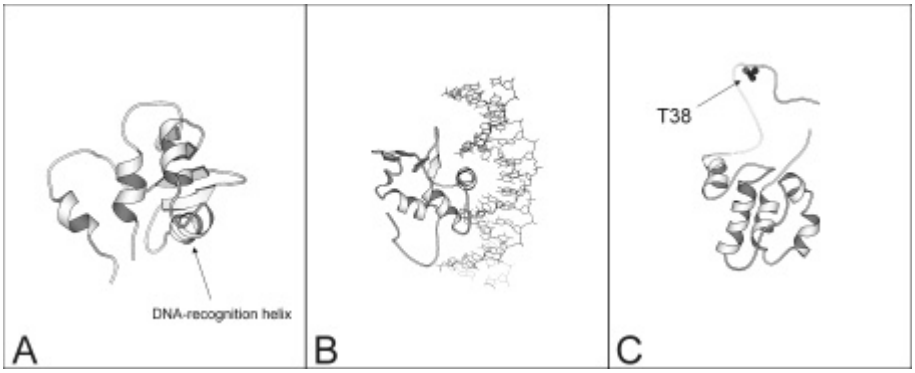

Figure 11

A) Solution structure of the DNA binding domain of Ets-1. The "winged helix-turn-helix" domain feature three α-helices scaffolded by four β-sheets. The DNA recognition helix is indicated.
B) Solution structure of the Ets-domain bound to DNA.
C) Solution structure of the N-terminal Pointed domain of Ets-1. Threonine 38, which is targeted by Ras-mediated MAP kinase phosphorylation, is indicated.

Ets-1 is involved in T-cell differentiation [177-179] and is widely expressed during mouse embryogenesis [180]. In addition Ets-1 was implicated as a cellular integrator of Ras activation of the PRL promoter [181]. Further research showed that Ras activation mapped to a composite element in the footprint-4 (FP4) region at -212/-189 of the PRL promoter. This composite site contains binding sites for Pit-1 and Ets-1. Contrary to Ets-1, Pit-1 is not a target for Ras induced phosphorylation. Nevertheless, the presence of Pit-1 is required to elicit full response to Ras activation of the composite element [108,182]. In HeLa non-pituitary cells and independent of Ras signaling, Ets-1 and Pit-1 still functionally cooperate to achieve a 6 fold synergistic activation of the PRL promoter. The highly related GH promoter does not exhibit this response, since it does not contain any Pit-1/Ets-1 composite elements. Hence, the functional cooperation of Ets-1 and Pit-1 is a mechanism to confer promoter specificity on the PRL promoter. Aside from their functional interaction, Pit-1 and Ets-1 also physically interact in solution. This interaction requires the POUhd of Pit-1 and a part of the activation domain (located between the PNT and the Ets domain), which is specific for Ets-1 [124,125]. The interaction of Ets-1 with the alternative splice variant Pit-1β has been discussed above. A detailed study on the interaction of the POUhd of Pit-1 and Ets-1 is presented in chapter 3 of this thesis.

1.3 Outline of this thesis

This thesis focuses on the structural and functional aspects of the pituitary specific transcription factor Pit-1. Pit-1 is an essential regulator for the expression of pituitary hormones such as growth hormone. Mutations in Pit-1 can therefore lead to combined pituitary hormone deficiency (CPHD) in humans. Chapter II describes a clinical case study of a patient with CPHD caused by two novel mutations in the Pit-1 gene. Biochemical characterization showed that both mutations result in a non-functional Pit-1 protein by abrogating its DNA binding activity. Pit-1 is involved in the activation of transcription of several different promoters. Regulation of the specificity of Pit-1 transactivation on these promoters requires interaction with other cellular transcription factors. One such factor is the transcription factor Ets-1, which interacts with Pit-1 in solution and regulates transcription on the PRL promoter through a composite Pit-1/Ets-1 binding site. In chapter III, the interacting region on Ets-1 is mapped to a stretch of about 70 amino acids. This Ets-1 region is used in NMR experiments to define the exact residues on the Pit-1 POUhd required for the interaction. Functional implications on the regulation of Pit-1/Ets-1 association are discussed. Chapter IV describes NMR studies on the DNA binding ability of the Pit-1 POUhd both alone and in complex with the interacting region of Ets-1. Finally, chapter V investigates a putative *in vivo* function of an interaction that was found between the Pit-1 TAD and the CR3 region of adenoviral transcription regulator E1A 13S splice variant.

Chapter 2

Combined pituitary hormone deficiency caused by compound heterozygosity for two novel mutations in the POU-domain of the PIT1/POU1F1 gene.

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[*]B.I.H-S and K.D.A. contributed equally to this work.

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2.1 Abstract

The POU-homeodomain containing transcriptional activator POU1F1, formerly called Pit1 or GHF-1 (here referred to as Pit-1), is required for the embryologic determination and postnatal secretory function of the growth hormone (GH), prolactin (PRL) and thyroid-stimulating hormone (TSH) producing cells in the anterior pituitary. Several mutations in the gene encoding Pit-1 have been described, resulting in a syndrome of combined pituitary hormone deficiency (CPHD) involving these three hormones. Most of the patients with this phenotype have either a dominant negative mutation in codon 271 (R271W) or are homozygous for a recessive mutation in the Pit-1 gene; so far only one case has been reported with compound heterozygosity for two point mutations. Here, we describe a boy with severe deficiencies of GH, PRL and TSH who turned out to have compound heterozygosity for two novel point mutations in the Pit-1 gene: a one-base pair deletion frame shift mutation (747delA), the first one described thus far in this gene, which leads to a nonfunctional truncated protein lacking the entire DNA-recognition helix of the POU-homeodomain, and a missense mutation in the C-terminal end of the fourth α-helix of the POU-specific domain (W193R) which causes a 500-fold reduction in the ability to bind to DNA and to activate transcription.

2.2 Introduction

The anterior pituitary-specific transcription factor POU1F1, as the human homologue of Pit1/GHF1 is now officially called, belongs to the family of POU-domain proteins and plays a critical role in the embryonic differentiation and survival of the somatotrope, lactotrope and thyrotrope cell lineages [183,184]. Its expression is required for the transcriptional activation of, among others, the growth hormone (GH), prolactin (PRL) and thyrotropin (TSHâ) genes [185].

The DNA-binding POU-domain of Pit-1 is located in the C-terminal part of the molecule (amino acids 119-273). It consists of a 60 amino acids (aa) POU-homeodomain (POUhd) and a 75 aa POU-specific (POUs) domain, connected by a 15 aa flexible linker [99].

Both domains contribute to the specific and high-affinity binding of the Pit-1 molecule to its recognition sequence, ($^{A}/_{T}$)($^{A}/_{T}$)TTATNCAT [97]. The crystal structure of the Pit-1 POU-domain bound to DNA shows that both subdomains contain helix-turn-helix motifs and form a dimer [131]. DNA-binding by Pit-1, as well as interaction with other nuclear proteins, are required for specific transactivation of its target genes [46].

Two strains of dwarf mice have been shown to harbor structural defects in the Pit-1 gene resulting in combined pituitary hormone deficiency (CPHD) with pituitary hypoplasia and absence of somatotropes, lactotropes and thyrotropes. The Snell dwarf mouse carries a G→T missense mutation at nucleotide 783 of the POUhd, which replaces a tryptophan residue at position 261 by a cysteine (W261C). The Jackson dwarf mice, on the other hand, have a genomic rearrangement resulting in a truncated Pit-1 protein, which has lost its DNA-binding capacity [110]. To date, twelve mutations (9 missense, 2 nonsense and 1 deletion) have been described in the human Pit-1 gene [138,139,186-196]. Two of these are located in the transactivation domain, six in POUs and four in POUhd. They result in a partial or total deficiency of GH and PRL and, to a variable degree, of TSH. In some patients anterior pituitary hypoplasia is evident on radiographic imaging of the hypothalamic-pituitary area. Dependent on their localization in the Pit-1 coding sequence, the mutations may either interfere with DNA-binding or with the transactivation process. Most are transmitted as an autosomal recessive trait, but four of them, two in the transactivation domain (P14L, P24L) and the other two located at the borders of the POUhd (K216E, R271W), appear to result in a dominant-negative phenotype with a highly variable level of penetrance.

In this report we describe a boy with CPHD who turned out to be a compound heterozygote for two novel mutations in the Pit-1 gene. Both parents, who have a normal phenotype, harbor these mutations in the heterozygous state. The maternal allele carries a missense mutation in the POUs domain resulting in complete abolishment of DNA-binding. The paternal allele harbors a one base pair deletion frame shift mutation, the first described thus far in the Pit-1 gene, resulting in a truncated Pit-1 molecule lacking helix 3 of the POUhd.

2.3 Materials and Methods

2.3.1 Mutational analysis of the Pit-1 gene

mRNA was isolated from Epstein-Barr virus transformed lymphocytic cell lines from the proband, his parents and his brother. This mRNA was reverse transcribed and the Pit-1 POU-domain amplified by nested PCR, essentially using the procedure and the oligonucleotide primers as described by Pfäffle et al.[186]. The PCR-products were sequenced bidirectionally using the Amplicycle sequencing kit (Perkin-Elmer). Genomic DNA was isolated from the lymphocytic cell lines and all six Pit-1 exons were amplified separately by PCR, using the pairs of oligonucleotide primers corresponding to the intron/exon boundaries as described by Ohta et al.[189], with modifications essentially as described by Pellegrini-Bouiller et al.[194]. The PCR-products were analyzed by SSCP (Genephor, Amersham-Pharmacia Biotech) and used for direct sequencing.

2.3.2 Plasmids

For the construction of Pit-1 POU-domain expression vectors, wild-type and mutant cDNA carrying the W193R mutation in the Pit-1 POUs-domain were obtained by RT-PCR as described above. The PCR-products were ligated into the original TA Cloning vector, pCR 2.1 (Invitrogen) and transformed to E. coli DH5α. Subsequently, both cDNAs were cloned into an NdeI/BamHI digested pET15b expression vector (Novagen) yielding His_6-tagged Pit-1 POU-domain open reading frames (ORFs). The DNA sequence was checked by dideoxy-chain termination sequencing (Invitrogen).

The GH320-luc and PRL DE/P-luc reporter/luciferase constructs, containing the rGH promoter sequence and the rPRL distal enhancer and promoter sequence, respectively, were reported previously [186] and were a kind gift of Dr. Rosenfeld, Howard Hughes Medical Institute, University of California, San Diego, La Jolla, California. For construction of a TSHβ-luc reporter/luciferase construct the GH320 insert was replaced by a 280 bp insert derived from the human TSHβ promoter (nt -206 to +74, ref. [197]) containing three putative Pit-1 binding sites [143]. This fragment was obtained by one round of PCR (36 cycles of 45 sec at 95°C, 1 min at 57°C and 2

min at 72°C) on 100 ng of genomic DNA as template, using 200 pmol each of the oligonucleotide primers 5'-GAGAGGAAAATGCATGC TTT-3' and 5'-TATCATTTCACAGAGCCTTC-3'. The fragment was cloned into the pCRII-TOPO TA cloning vector (Invitrogen) and sequenced.

For use in the transfection assays, pCMV-Pit-1 expression plasmids were constructed as follows. Pit-1 wild-type and mutant cDNAs, encompassing the entire Pit-1 coding sequence, were obtained by a one-side nested RT-PCR using 2 different upstream primers (5'-TGATTTGGGGAGCAGCGGTT-3' and 5'-CTACTCTCTTGTGGG AATGAG-3', respectively) and one downstream primer (5'-ATACA ATAGAAAACTTTATCTGCACTC-3') in two consecutive rounds of PCR, each consisting of 36 cycles of 30 sec at 95°C, 1 min at 58°C, 2 min at 72°C. We constructed a total of four different pCMV constructs: pCMV-Pit-1 *wt* cDNA, pCMV-Pit-1 cDNA containing the W193R mutation, pCMV-Pit-1 cDNA containing the 747delA mutation, and a pCMV vector without insert to be used as a control effector plasmid. All constructs were cloned into pTargeT (Promega) and sequenced bidirectionally. An RSV-βGAL construct was used as an internal control for transfection efficiency in transient transfection experiments, as has been described elsewhere [198].

2.3.3 Protein expression and purification

Wild-type and W193R-mutant Pit-1 POU-domain expression vectors were transformed to strain BL21 (pLYS). Strains containing the wild-type and mutant vectors were grown in 1 liter cultures at 37°C and room temperature, respectively. At $O.D._{600}$ = 0.5 expression was induced by adding 1 ml of 1 M IPTG. Wild-type Pit-1 POU-domain expression was continued for 3 h at 37°C, while W193R $Pit\text{-}1_{POU}$ was expressed overnight at room temperature. Cells were pelleted, resuspended in 20 ml sonification buffer (50 mM $NaPO_4$, 300 mM NaCl, 0.5 mM PMSF, 1 μg/ml aprotinin and 10 mM β-mercaptoethanol) and lysed by freeze-thawing and mild sonification. Insoluble components were removed by centrifugation in a SW41 rotor at 35.000 rpm for 45 min. Wild-type and W193R Pit-1 POU-domain proteins were partially purified on Ni-NTA agarose (Qiagen) columns. Samples were estimated to be approximately 70-80% pure on a Coomassie stained gel.

2.3.4 Gel retardation assay

For preparation of the probe, the GH320-luciferase construct described above was digested by HindIII and end-labeled using the Klenow fragment of DNA-polymerase I (Pharmacia) and α-^{32}P dCTP (Amersham, 10 mCi/ml, 3000 Ci/mmol), followed by XhoI digestion. The resulting 300 bp fragment containing the rGH-promoter was purified by polyacrylamide gel electrophoresis. Pit-1 dilutions were made in 50 mM Tris-HCl (pH 7.5), 1 mM EDTA (pH 8.0), 100 mM NaCl, 10% glycerol and 10 mM β-mercaptoethanol. Approximately 2 fmol DNA was incubated with Pit-1 dilutions in a reaction mixture containing 20 mM HEPES-KOH (pH 7.5), 100 mM NaCl, 1 mM EDTA (pH 8.0), 1mM dithiothreitol, 0.025% NP-40, 1 µg poly dIdC competitor DNA and 4% Ficoll for 30 min at room temperature. Prior to loading, 2 µl of 0.02% bromophenol blue and 0.02% xylene cyanol was added. The samples were run on a 6% polyacrylamide gel containing 0.01% NP-40, at 4°C for 3 h at 30 mA. The gel was dried and the DNA was visualized by autoradiography.

2.3.5 DNaseI footprint assay

Pit-1 dilutions were prepared as above. Approximately 10 fmol of DNA was incubated with Pit-1 as in the gel retardation assay, with an additional 1 µg of bovine serum albumin, 10 mM $MgCl_2$ and 3 mM $CaCl_2$ per reaction. After 30 min at room temperature, samples were incubated with 0.01 unit of DNaseI for 5 min. Reactions were quenched by adding 11 µl of 10 µg/ml herring sperm DNA, 200 mM NaAc (pH 8.0) and 75 mM EDTA (pH 8.0). The DNA was purified by phenol/chloroform extraction prior to ethanol precipitation. Dried samples were resuspended in loading buffer (80% formamide, 0.1 % bromophenol blue and 0.1% xylene cyanol) and loaded on a 6% polyacrylamide sequencing gel (8M urea). The gel was run for 1.5 hour at 30 mA, dried and the DNA was visualized by autoradiography.

2.3.6 Co-transfection assays

The adenovirus transformed human embryonic kidney (HEK) 293 cells [199] were cultured as a monolayer in DMEM containing 10% FCS, and 3.5 µmol L-Glutamine, 100 U of penicillin and 100 µg of streptomycin per ml. Cells were transfected when approximately 50% confluent using the calcium phosphate-DNA co-precipitation

technique [200] in N,N,bis[2-hydroxyethyl]-2-aminoethanesulfonic acid (BES, Sigma) buffered saline. Each 25 cm^2 flask was transfected with 3 μg of reporter plasmid and 500 ng of pRSV-lacZ in order to normalize for transfection efficiency. The optimal quantity of wt-Pit-1 effector plasmid to be used in combination with each reporter construct was determined by titration in such a way that the transfection potency was still in the linear range; for the hTSHβ and the rPRL reporter constructs a total of 500 ng of effector were used, whereas for the rGH reporter 25 ng sufficed. Four hours after adding the precipitate to the cells, the medium was changed and the cells were harvested 22 hours thereafter. Luciferase and β-galactosidase assays were performed as described [198,201]. Luciferase data were divided by the galactosidase activity to correct for transfection efficiency. All transfections were performed in duplicate in at least three separate experiments.

2.4 Results

2.4.1 Clinical evaluation

Our patient, a boy, was born in India and came to the Netherlands at the age of 4 months. He is the second child of unrelated healthy parents with normal stature; he has a healthy and normally growing brother. As dictated by the Dutch neonatal screening program for congenital hypothyroidism (CH) he was screened as yet for CH shortly after arrival in our country; a heel puncture T4 of only 8 nmol/l was found, together with an undetectable TSH and a normal TBG concentration. Physical examination at the age of 4.5 months revealed typical signs of CH, with a low nasal bridge, macroglossia, facial myxedema and wide-open fontanels. He exhibited generalized hypotonia, slight peripheral myxedema, constipation and hypothermia. His length at that point was 51 cm (-9 SD), body weight 4100 g.

The first laboratory results showed the combination of a very low FT4 (2.8 pmol/l) and TSH concentration (<0.1 mU/l), low IGF-1 (5 ng/ml) and IGFBP3 (0.3 mg/l) and undetectable prolactin concentrations (<1 μg/l). Plasma cortisol (580 nmol/l) and ACTH concentrations (53 ng/l) were normal and testosterone was appropriate for age (2.3 nmol/l). Following an intravenous injection of 30 μg of thyrotropin releasing hormone (TRH) all TSH levels remained <0.05

mU/l and prolactin remained <1.0 µg/l. ACTH and cortisol plasma concentrations rose normally after 40 µg of CRH intravenously. After reaching euthyroidism with appropriate thyroxine treatment an arginine provocation test was performed. Basal GH levels were undetectable and remained < 1 mU/l following an intravenous infusion of 0.5 mg/kg arginine. An MRI-scan of the hypothalamic-pituitary region showed a hypoplastic anterior pituitary but otherwise normal anatomy (Fig. 1). The boy was started on daily growth hormone injections to which he responded well; he is now 3.5 years of age and has attained a height of 98.3 cm (-1.1 SD). He is in good health and shows no signs of neurodevelopmental delay.

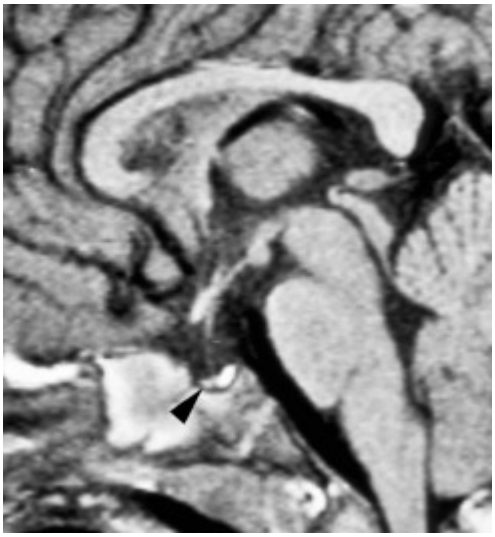

Figure 1

MRI of the proband's hypothalamic-pituitary region, showing marked hypoplasia of the anterior pituitary (arrowhead).

2.4.2 Molecular studies: Two novel mutations in the Pit-1 gene

Sequence analysis of lymphocyte-derived Pit-1 cDNA encompassing the POUs and POUhd regions [186] revealed heterozygosity in the proband and his mother for a missense mutation at position 577 in exon 4, a T→C transition that changed amino acid 193 from Trp to Arg (W193R). Since the mother had a normal phenotype as well as a normal hormonal profile, a dominant negative effect of this mutation was considered unlikely. Therefore, further investigation aimed at identifying an additional mutation in the proband's paternal Pit-1 allele. To this end, direct genomic sequencing of all six exons of the Pit-1 gene from both parents, the proband and his brother was performed, which confirmed the heterozygosity in exon 4 in the proband and his mother, and identified an additional heterozygosity in the DNA of the proband and his father for a one-basepair deletion at position 747 in exon 6, codon 249 (747delA, the A nucleotide of the ATG codon of the primary translation product being taken as position +1) (Fig. 2). The shift in the reading frame resulting from this deletion changes the subsequent codon from Glu to Asn and introduces a translational stop codon immediately thereafter.

Thus, our patient is heterozygous for a missense mutation in exon 4 (W193R) inherited from the mother and a one-basepair deletion frameshift mutation in exon 6 (747delA) inherited from the father. The effect of the frameshift mutation, a loss of helix 3 of POUhd, is very similar to the E250X nonsense mutation described by Irie *et al*.[195], which in the homozygous state led to CPHD in their patient. Since the W193R missense mutation had not been described before, the properties of the resulting mutant Pit-1 protein with respect to DNA-binding and transactivation were further analyzed.

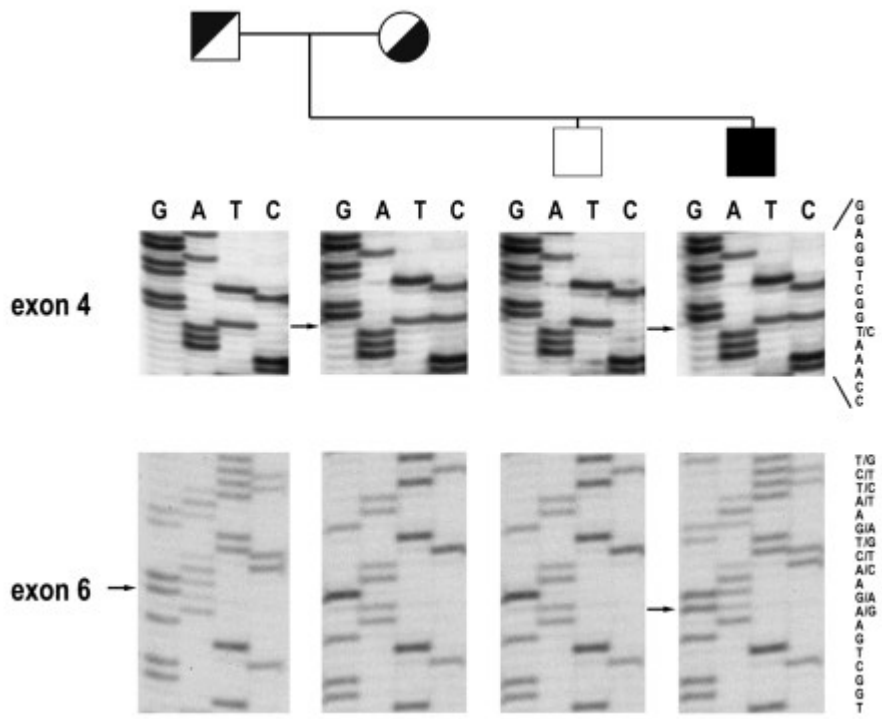

Figure 2

Genomic DNA sequence analysis of (from left to right) the father, the mother, the proband' brother and the proband. Top panel: sequence gel of exon 4, maternal missense mutation. Bottom panel: sequence gel of exon 6, paternal one base pair deletion frameshift mutation. The changes in the sequence are depicted to the right.

2.4.3 W193R binds DNA with ~500-fold reduced affinity in vitro

DNA binding affinity of the wild-type and W193R Pit-1 POU-domain were tested *in vitro*, using the Pit-1 binding site of the rGH-promoter as probe. As shown in figure 3, bacterially expressed wild-type Pit-1 POU-domain bound with high affinity, whereas binding of the W193R Pit-1 POU-domain to the same site was ~500-fold reduced. Similar results were obtained on the prolactin (PRL) proximal enhancer (data not shown). Residual DNA binding affinity can be attributed to the POUhd, which is still intact in the W193R mutant.

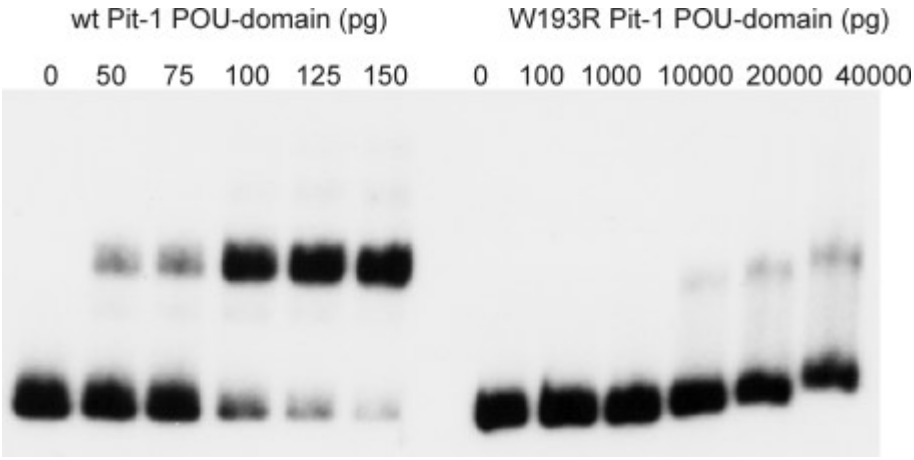

Figure 3

Bandshift assay on the GH-promoter region. Approximately 2 fmol of DNA was incubated with 50, 75, 100, 125 and 150 pg. of wild-type Pit-1 and with 100, 1000, 10000, 20000 and 40000 pg. of W193R mutant Pit-1.

2.4.4 W193R is unable to protect its site in a DNaseI footprint assay

The apparent lack of binding of the W193R mutant in the gel retardation assays might have been caused by the formation of mutant Pit-1/DNA-complexes that are too unstable to enter the gel. Moreover, the use of high concentrations of wild-type Pit-1 resulted in higher order complexes (data not shown), which might either be caused by binding of the protein to cryptic binding sites in the probe or by 'piggy-back' binding of Pit-1 to one site. To test these possibilities we performed DNaseI footprint assays. As can be seen in figure 4, the wild-type Pit-1 POU-domain was able to protect a region of 17 basepairs of the rGH-promoter, whereas the W193R mutant did not show any protection at all, even at high protein concentrations. We can therefore conclude that DNA binding of the W193R mutant is seriously impaired, which explains its lack of transactivation capability.

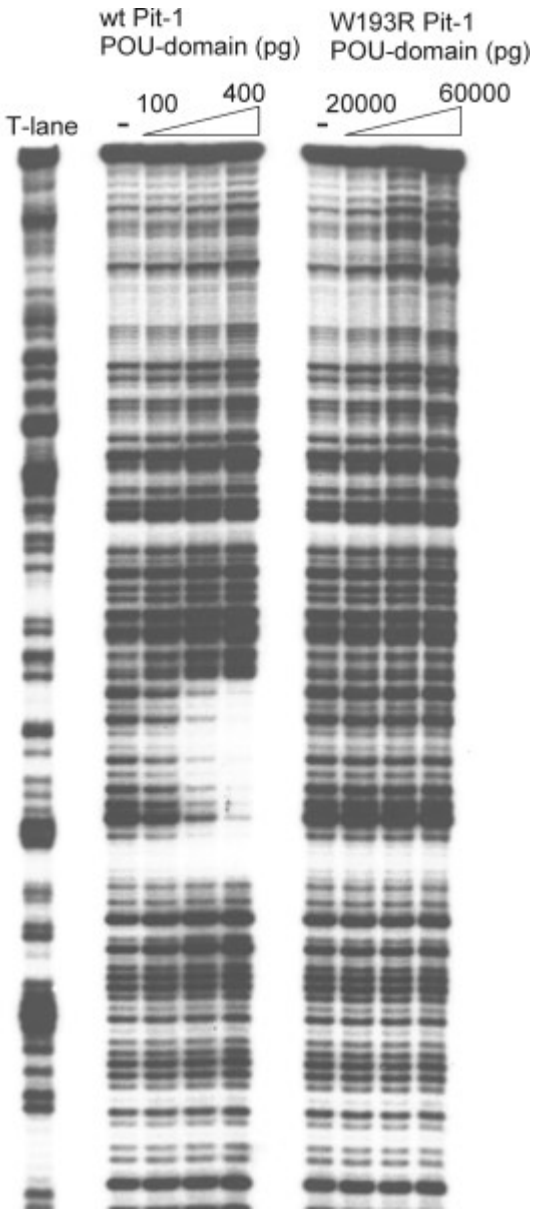

Figure 4

DNaseI footprint assay on the GH-promoter region. Approximately 10 fmol of DNA was incubated with 100, 200 and 400 pg. of wild-type Pit-1 and with 20000, 40000 and 60000 pg. of W193R mutant Pit-1. A Maxam-Gilbert T-sequencing lane was added to localize the binding site.

2.4.5 W193R and 747delA are unable to activate transcription in vivo.

To assay the *in vivo* activity of the W193R and 747delA mutants, we transfected constructs expressing various combinations of wild-type and/or mutant Pit-1 to human embryonic kidney 293 (HEK293) cells. Wild-type Pit-1 strongly activated the rPRL promoter (30x) and to a lesser extent the rGH (3x) and the hTSHβ (3x) promoter. As shown in figure 5, when either one of the mutant expression constructs was transfected together with the wild-type construct, the transactivation potency declined to approximately 60-70% of the wild-type level. This situation reflects the genetic background of the parents and the results are consistent with the fact that neither parent showed an abnormal phenotype. When both mutants were co-transfected, transactivation dropped to background level. From these results we conclude that neither mutant is able to activate transcription, which explains the minimal levels of GH, PRL and TSH found in the patient.

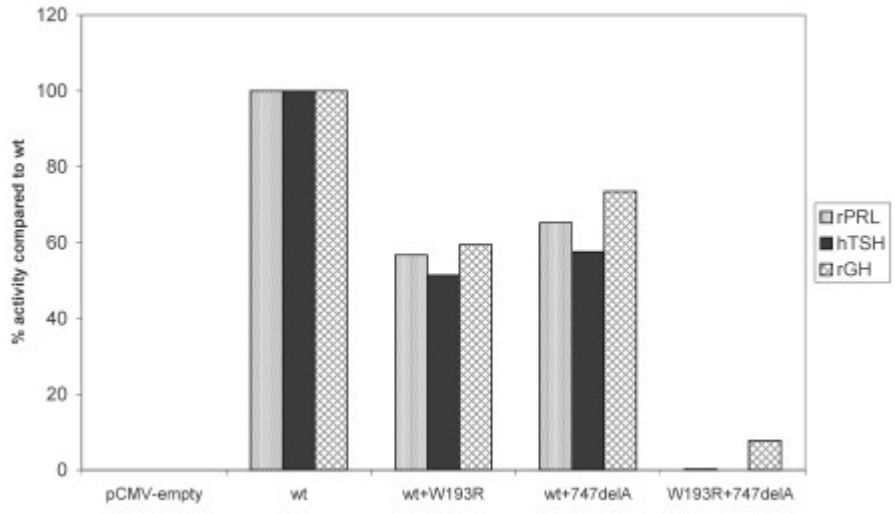

Figure 5

Stimulation of transcription on the rGH, rPRL and hTSHβ-promoter by wild-type Pit-1, W193R and 747delA mutants and combinations thereof.

2.5 Discussion

The clinical presentation in this patient is remarkable. The majority of patients with a Pit-1 defect present with growth failure and are diagnosed as having GH-deficiency, whereas less than half present with hypothyroidism as the first clinical manifestation [202]. Our patient clearly belongs to the latter category: he was detected at the age of 4.2 months by the Dutch neonatal CH-screening program. This was only possible because the CH-screening in the Netherlands is primarily a T4 screening, with a subsequent determination of TSH and TBG in heel puncture blood [203]. Furthermore, the program dictates that children under the age of six months, coming to the Netherlands and not having been screened before, be screened upon arrival in our country. The patient was found to be severely hypothyroid: his heel puncture T4 level was only 8 nmol/l (normally >100 nmol/l) and heel puncture TSH was undetectably low. However, some thyroxine production must have existed in the first months postnatally, since when he was tested psychologically at the age of 2 years no abnormalities were found. Among patients with Pit-1 deficiency, the degree of hypothyroidism, both in terms of plasma thyroid hormone and TSH levels and of response to TRH, is far more variable than that of GH and PRL deficiency [204]. Patients also show phenotypic differences with respect to the degree of anterior pituitary hypoplasia. Pfäffle [202] already speculated upon differences in the mode of interaction of Pit-1 with the GH and PRL promoters on one hand and the TSHβ promoter on the other, but how these differences relate to the nature of the mutations is still largely unknown.

Our patient turned out to be a compound heterozygote for two novel mutations in the Pit-1 gene. The one base pair deletion mutation in the POUhd at position 747 (747delA), transmitted by the paternal allele, is the first frameshift mutation in the Pit-1 gene described so far. The frameshift causes a change in the codon following E249 from Glu to Asn and introduces a translational stop codon immediately thereafter. This mutation is therefore almost identical to the E250X nonsense mutation described by Irie *et al*.[195]. In either case the translational stop codon is located at the C-terminal end of helix 2 of the POUhd. In the homozygous state, they will undoubtedly result in a severe loss of DNA binding since the entire helix 3 with the DNA-recognition domain of POUhd is deleted.

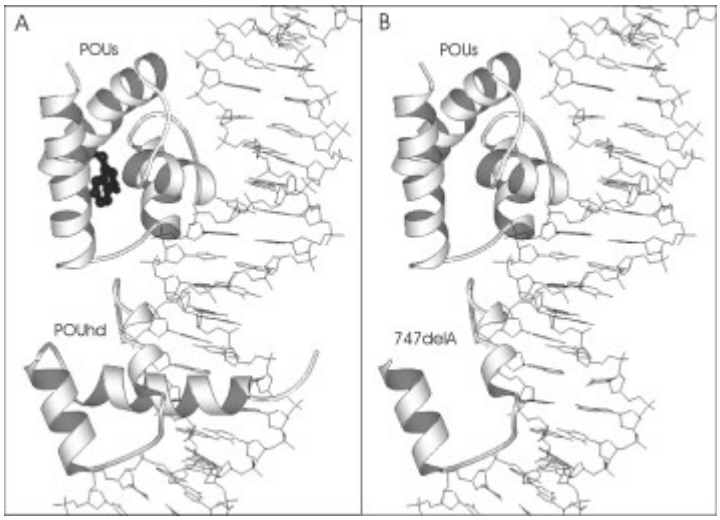

Figure 6

Crystal structure of Pit-1 complexed to DNA, as determined by Jacobsen *et al*. For the sake of clarity, only one Pit-1 molecule is shown.
A) The W193 residue, located in the hydrophobic core of the POU-specific domain is highlighted.
B) Direct result of a stop codon at position 251: the entire DNA-recognition helix of the POU homeodomain is deleted. These pictures were generated using the Molscript program [207]

The T→C missense mutation at position 577, conferred by the maternal allele, predicts a tryptophan-to-arginine substitution at codon 193 located in the C-terminal end of the fourth α-helix of the POUs domain. The natural occurrence of this W193R mutation has not been reported before, but it has been reported in a yeast *in vivo* screening model for DNA-binding negative Pit-1 mutants [205], in which it demonstrated only 2% of the DNA-binding activity of the wild-type protein. Figure 6 shows the crystal structure of Pit-1 [131], with tryptophan 193 highlighted. W193 is one of the amino acid residues that make up the hydrophobic core of the POUs domain. The α-helix harboring this codon can be considered to be a structural helix, since it does not contact the DNA directly. However, changing W193 to an arginine introduces a positively charged residue into the hydrophobic core. Our bandshift and footprint assays show that the W193R POU-domain is no longer able to bind to DNA with sufficient affinity,

probably due to improper protein folding of the POUs domain. As a result, the mutant W193R POU-domain is unable to activate transcription, as shown in our transient transfection experiments. The residual Pit-1 activity of approximately 60-70% in the heterozygous state as measured in our transient transfection assays can be expected to ensure a normal phenotype, as others have observed a clinically recessive phenotype with residual activities of approximately 50% in comparable assays [196].

In conclusion, in our patient neither allele of the Pit-1 gene yields a functional gene product, which explains his severe combined pituitary hormone deficiency and pituitary hypoplasia. These two novel recessive mutations bring the number of naturally occurring Pit-1 mutations to fourteen, among which 10 missense, 2 nonsense, 1 deletion and, finally, 1 frameshift mutation (Figure 7). Study of other patients with CPHD will undoubtedly result in a further increase of this number of mutations in Pit-1 or in related genes such as PROP-1 [112,206]. However, since a significant number of patients still turn out to have CPHD in spite of normal Pit-1 and PROP-1 coding sequences (R. Pfäffle, personal communication), the molecular unraveling of these defects remains a challenge for the future.

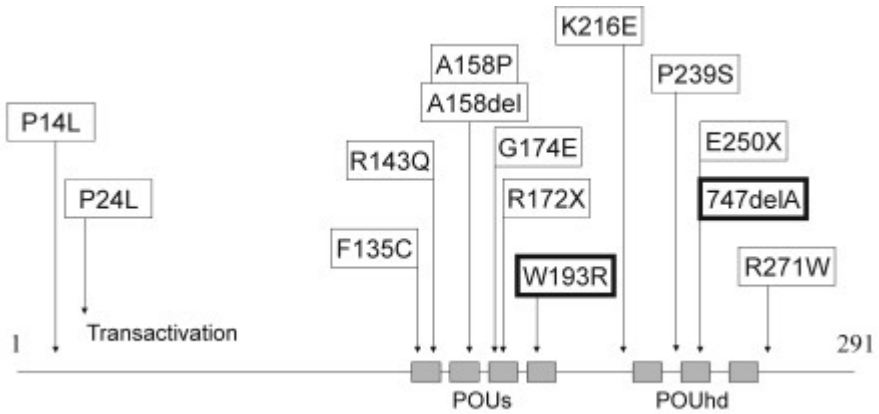

Figure 7

Presently known naturally occurring mutations in the Pit-1 molecule. The mutations described in this paper are heavily boxed.

2.6 Acknowledgements

We thank Dr. M.G. Rosenfeld and Dr. K. Scully for providing us with the rGH320-luc and rPRL DE/P-luc constructs. This work was supported in part by the Netherlands Foundation for Chemical Research (SON) with financial support from the Netherlands Organization for Scientific Research (NWO).

"There's a significant amount of voodoo involved..."

Dawn Duvall

Chapter 3

Structural characterization of the Pit-1 / Ets-1 interaction: Phosphorylation of Pit-1 homeodomain may regulate Ets-1 binding.

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3.1 Abstract

Understanding of the molecular basis of protein-protein interactions is essential in order to comprehend the molecular machinery that governs complex processes like transcription and replication. Even though a wealth of data has been generated in the fields of crystallography and NMR spectroscopy, this understanding is still limited. Here we describe a detailed NMR study of the interaction between the POU-domain protein Pit-1 and Ets-1, a member of the ETS family of transcription factors. Pit-1 and Ets-1 can associate in solution and synergistically activate the prolactin (PRL) promoter by and binding to a composite response element in the PRL promoter. We mapped the minimal region of Ets-1 required for the interaction with the Pit-1 POU-homeodomain. By using hetero nuclear single quantum coherence (HSQC) titration experiments, we were able to map the exact residues on the POU-homeodomain that are involved in the interaction with this minimal Ets-1 interaction domain. Using our NMR data, we generated point mutants in the POU-homeodomain and tested their effect on the interaction with Ets-1.

3.2 Introduction

The POU-domain transcription factor Pit-1 is one of the key regulators of gene expression in the anterior pituitary gland. The presence of Pit-1 is an essential and determining factor in the differentiation of the somatotrope, lactotrope and thyrotrope cell lineages. Furthermore, in these cell lineages, Pit-1 is required for the expression of the growth hormone (GH), prolactin (PRL) and thyrotropin (TSHβ) genes, respectively [92,184,208]. The expression of these genes is restricted to their respective cell lineages. However, since Pit-1 is present in all three cell types, cooperation with other transcription factors is required in order to achieve selective expression. Indeed, recent data show that depending on the promoter context and cell type, Pit-1 can act as either enhancer or repressor of transcription by selective recruitment of coactivator or corepressor complexes [140], reviewed in [209].

One example of a promoter-specific Pit-1 interaction is the Ras-activated expression of the PRL promoter. Ras-dependent activation of the PRL promoter is achieved through Ets-1 and requires the interaction of Pit-1 with Ets-1. Ras responsive Ets-1 is one of the founding members of the ETS family of transcription factors, which is defined by a highly conserved winged helix-turn helix DNA binding domain [167]. Previously, it was found that Ras activation of the PRL promoter required the presence of a Pit-1/Ets-1 composite element in the PRL promoter [108,210]. Furthermore, we have shown that Pit-1 and Ets-1 form a DNA-independent complex in solution and that both factors displayed strong synergy in activation of the PRL promoter in a reconstituted, Ras-independent expression system in HeLa cells [124]. The physical association of these proteins involved the POU-homeodomain of Pit-1 and a part of the region III activation domain of Ets-1 located between the N-terminal Pointed domain and the C-terminal ETS DNA binding domain [124,125].

In the present study, we further define the minimal region of Ets-1 required for the in solution interaction with the Pit-1 homeodomain. Both this minimal interacting region on Ets-1 and the Pit-1 homeodomain were capable of interacting with one another outside the context of the full-length proteins. Using NMR spectroscopy, we performed a fine mapping of the exact residues on the surface of the POU homeodomain of Pit-1 that are in contact with the minimal interacting region of Ets-1. This mapping allowed us to define a novel interaction domain that may apply for other POU homeodomain interactions. Our approach presents an interesting possibility to probe weaker protein-protein interactions.

3.3 Materials and methods

3.3.1 Plasmid Construction

The wild-type pGex Pit-1 (199-291) expression vector has been described [145]. The mutant homeodomain fragments of rat Pit-1 (T220D, K226A, K226D, E254A, W261F, R268A, K270A and L288A) were generated by overlap extension polymerase chain amplification utilizing primers purchased from Gibco Life Technologies, Inc. Mutant primers were used in combination with 5' and 3' Pit-1 (199 -291) primers that incorporate a Not I site to facilitate

subcloning. The amplified DNA was initially cloned into pCR 2.1 (Invitrogen) and then subcloned into the Not I site of pGexDFGK. The sequences of the primers are as follows: 5' Pit-1 (199-291)-GCGGCCGCCAGG TCGGAGCTTTGTACAAT, 3' Pit-1 (199-291)-GCGGCCGCTTATCTGCACTCAAGATGCTC. 5' T220D-GAGGACAGATATCAGTATCGC, 3' T220D-GATACTGATATCTGTCCTCCGT, 5' K226A-GCCGCTGCCGATGCTTTGGAGA, 3' K226A-AAAGCATCGGCAGCGGCGATAC, 5' K226D-GCCGCTGACGATGCTTTGGAG, 3' K226D-AAGCATCGTCAGCGGCGATAC, 5' E254A-TGAATCTCGCCAAAGAAGTAGTAAG, 3' E254A-TACTTCTTTGGCGAGATTCAATTC, 5' W261F-GTAAGAGTGTTCTTTTGCAACCG, 3' W261F-GTTGCAAAAGAACACTCTTACTAC, 5' R268A-AAGGCAGGCCGAAAAACGGGT, 3' R268A-CGTTTTTCGGCCTGCCTTCGG, 5' K270A-AGAGAGAAGCCCGGGTGAAAAC, 3' K270A-CACCCGGGCTTCTCTCTGCC, 3' L228A-GAGCGGCCGCTTATCTGCACTCGGCATGCTCCTTTG.

The Pit-1 expression plasmids, pRc.935Pit1wt FLAG and pRc.935Pit1A3 FLAG, encoding wild-type and a Pit-1 phosphorylation mutant, respectively [145], were provided by Dr. Fred Schaufele (University of California, San Francisco). Plasmid pSG5c-Ets-1 encodes the p68 chicken Ets-1 under control of the SV40 early promoter. This construct as well as the series of C-terminal deletions of Ets-1, pSG5c-Ets-1Δ3-1 through pSG5c-Ets-1Δ3-10 [211], and the GST- Ets-1 fusion construct, pGex p68 Ets-1 full length, were provided by Dr. Bohdan Wasylyk (I.G.B.M.C., Illkirch, France). pGex Ets-1 (190-257) was generated by combining the amino-terminus of pSG5 Ets-1Δ5-4 (AA 190-485) with the C-terminus of pSG5 Ets-1Δ3-8 (AA 1-257) using a common restriction site. The resulting construct was subcloned into pGexDFGK or pRSETA. The pSG5 Ets-1 BPV-1, BPV-2, and BPV-3 constructs contain p68 c-Ets-1 with 26 amino acid scanning block mutations of Ets-1 in the region of amino acids 190-257. These mutations were made utilizing overlap extension PCR to amplify mutated fragments of Ets-1 from nucleotides 401 to 1054. The fragments were cloned into PCR 2.1 and subcloned into pSG5 Ets-1 by digesting at internal Bgl II and Afl III sites. PCR primers utilized are listed below: BPV-1 S-CTAAATGTGCCAGCAATGTGATTCCTGCTAAAGAAGACCCTTATGCCCCCTCTGAGTTCTCTG, BPV-1 AS-TTGCTGGCACATTTAGTTGCAGGAGACTCTATATAGCGATAGGTGTCCACTCCGTTTGCTGGG, BPV-2 S-CTAAATGTGCCAGCAATGTGATTCCTGCTAAAGAAGACCCTTATGCCTCGG, BPV-2 AS-TTGCTGGCACATTTAGTTGCAGGAGACTCTATATAGCGATAGGTGTCGTGCTCGATGCCATAA, BPV-3 S-CTAAATGTGCCAGCAATGTGATTCC

TGCTAAAGAAGACCCTTATGCCGTCCAGACGGACTCCC, BPV-3 AS-TTGCTGGCACATT TAGTTGCAGGAGACTCTATATAGCGATAGGTGTCGGTCTGGTAGGACTCT, p68 Ets-1 401 S-TGAAGGGAGTGGATTTCC, p68 Ets-1 1054 AS-CATAGTCCTTGAAGGTGC.

The fragment encoding residues 213-289 of the human Pit-1 ORF was cloned into an NdeI/BamHI digested pET15b expression vector (Novagen), yielding His_6-tagged Pit-1 POU-homeodomain. The fragment encoding residues 190-257 or 235-304 of the human Ets-1 protein was cloned into an NdeI/BamHI digested pET15b-derived vector. This vector was obtained by using the primers 5'GGAAATACTTACCCATGG GCGATAAAATT3' and 5'ATATGAGATCCCATGGGTAC CTTGTCATCGTC3' purchased from Amersham Biotech (Pharmacia) to clone the ORF for thioredoxin from the pTrxFus vector (Invitrogen) into the NcoI site of pET-15b. The resulting vector codes for a N-terminal thioredoxin tag in addition to the His_6-tag. All DNA sequences were checked by cycle sequencing on an automated sequencer (ABI-Prism).

3.3.2 GST pulldown Assays

Recombinant fusion proteins were prepared from bacterial extracts and bound to glutathione- Sepharose CL-4B (Amersham Pharmacia Biotech) as previously described [125]. Ets-1 and Pit-1 fusions were also supplemented with 10 or 1 mM dithiothreitol, respectively. Protein concentration was measured by the Bio-Rad assay (Bio-Rad). Bound protein was analyzed by SDS-polyacrylamide gel electrophoresis and Coomassie Blue Staining. [^{35}S]-labeled proteins were synthesized and labeled with [^{35}S]-methionine (NEN Life Science Products), using the TNT coupled transcription-translation reticulocyte lysate system with T7 polymerase, according to the manufacturer's protocol (Promega, Madison, WI). Pull down assays were performed in dilute solution using equal amounts (5 or 20 μg) of GST fusion proteins bound to 20 μl of glutathione sepharose beads in the presence of 50 μg/ml ethidium bromide as previously described [125]. The [^{35}S]-labeled proteins were eluted from the beads by boiling in SDS sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. Bands were quantified using a Molecular Dynamics Phosphorimager with Imagequant software and normalized for input of equal amounts of fusion protein based on densitometry of the Coomassie Blue stained gels.

3.3.3 Protein purification

The Pit-1 homeodomain was expressed in BL21 (DE3) bacteria grown on synthetic medium (10.38 g/l $K_2HPO_4 \cdot 3H_2O$, 4.38 g/l KH_2PO_4, 50 mg/l $MgSO_4 \cdot 7H_2O$, 7 mg/l $(NH_4)_2FeSO_4 \cdot 6H_2O$, 10 mg/l thiamin, 0.05 μg/ml ampicillin) containing 0.5 g/l $^{15}NH_4Cl$ as the sole nitrogen source and 5 g/l ^{12}C-glucose or 1 g/l ^{13}C-glucose as the sole carbon source. Bacteria were grown to an OD_{600} of 1.0 and induced by addition of IPTG to a final concentration of 1 mM. After 2.5 hours the cells were harvested and lysed in 50 mM $Na_xH_yPO_4$ (pH 6.5), 500 mM NaCl, 10% glycerol, 0.01% NP-40, 0.5 mM PMSF, 1 μg/ml aprotinin, 10 mM β-mercaptoethanol and 10 μg/ml lysozyme. The lysate was cleared by centrifugation at 30,000 x g for 45 minutes at 4°C and loaded on a Ni-NTA column (Qiagen) equilibrated in 50 mM $Na_xH_yPO_4$ (pH 6.5), 200 mM NaCl, 10% glycerol, 20 mM imidazole and 10 mM β-mercaptoethanol. After washing, the protein was eluted in a linear gradient from 20-400 mM imidazole. Peak fractions were pooled, diluted to 100 mM NaCl and loaded on a SP-sepharose column (Pharmacia) equilibrated in 50 mM $Na_xH_yPO_4$ (pH 6.5), 100 mM NaCl, 10% glycerol and 10 mM β-mercaptoethanol. The protein was eluted in a linear gradient from 100-1000 mM NaCl. Peak fractions were pooled and concentrated to approximately 1 mM in an Amicon stirred ultrafiltration cell (Millipore) using a 3 kD cut-off filter. The buffer was exchanged to a final 50 mM $Na_xH_yPO_4$ (pH 5.5), 100 mM NaCl and 1 mM dithiotreitol (DTT). The thioredoxin-his_6-Ets-1 (190-257) or (235-304) were expressed in BL21 (DE3) bacteria grown on Luria Broth (LB) medium containing 0.05 μg/ml ampicillin. Expression and lysis were done as above, with the exception that induction took place at an OD_{600} of 0.6. The cleared lysate was bound to Ni-NTA material (Qiagen) equilibrated in 30 mM TRIS-HCL (pH 8.0), 200 mM NaCl, 10% glycerol, 20 mM imidazole and 10 mM β-mercaptoethanol by rotating for 1 hour at 4°C. The material was washed and block eluted with 400 mM imidazole. Eluted protein was further purified on a Superdex 75 gelfiltration column (Pharmacia) equilibrated in 30 mM TRIS-HCL (pH 8.0), 200 mM NaCl, 10% glycerol, 20 mM imidazole and 10 mM β-mercaptoethanol. Peak fractions were subjected to thrombin cleavage by adding 2.5 mM $CaCl_2$ and 100 units of thrombin from bovine plasma (Sigma) and incubating at RT for 2 hours. Cleaved protein was

applied to a Ni-NTA column (Qiagen). Ets-1 (190-257) or Ets-1 (235-304) were present in the flow through, while thioredoxin-his_6 remained bound to the column. After a second run on the Superdex 75 gelfiltration column (Pharmacia), the sample was concentrated and washed as described above.

3.3.4 NMR Spectroscopy

NMR experiments were carried out at 305 K (32°C) on Varian UnityInova 500 and 750 spectrometers. The protein concentration was 1 mM in all experiments, 10% D_2O was added to obtain a lock signal. Spectra were processed using NMRPipe [212] and analyzed using NMRVIEW [213]. Protein-protein titrations were carried out by repeated addition of Ets-1 (235-304) to a ^{15}N-labeled Pit-1 homeodomain and recording a 750 MHz $^{1}H^{15}N$-HSQC spectrum after each addition. The sample was concentrated in an Amicon stirred ultrafiltration cell to keep the total volume around 500 µl.

3.4 Results

3.4.1 Mapping of the minimal interacting region on Ets-1

In order to identify the minimal region of Ets-1 required for interaction with Pit-1, we used 3' deletions of p68 chicken Ets-1 and tested their ability to interact with GST-bound Pit-1 homeodomain (residues 199-291). As shown in figure 1B, ^{35}S-labeled in vitro translated full-length Ets-1 is capable of binding to the Pit-1 homeodomain (POUhd). Deletion of the C-terminal auto inhibitory region caused a 50% decrease in binding, likely caused by changes in the overall structure of the ETS domain, since subsequent deletions up to Δ3-4 increased binding affinity almost to wild-type level. The most carboxy-teminal Ets-1 deletion that retained Pit-1 binding was truncated at AA 257 (Δ3-8), indicating that AA 257-485 do not contribute to binding. However, deletion of 68 AA to position AA 189 (Δ3-9) results in almost complete loss of Pit-1 binding and further deletion to AA 71 results in complete loss of binding activity. Thus, binding affinity for Pit-1 dropped drastically with deletions in the Ets-1 RIII activation domain.

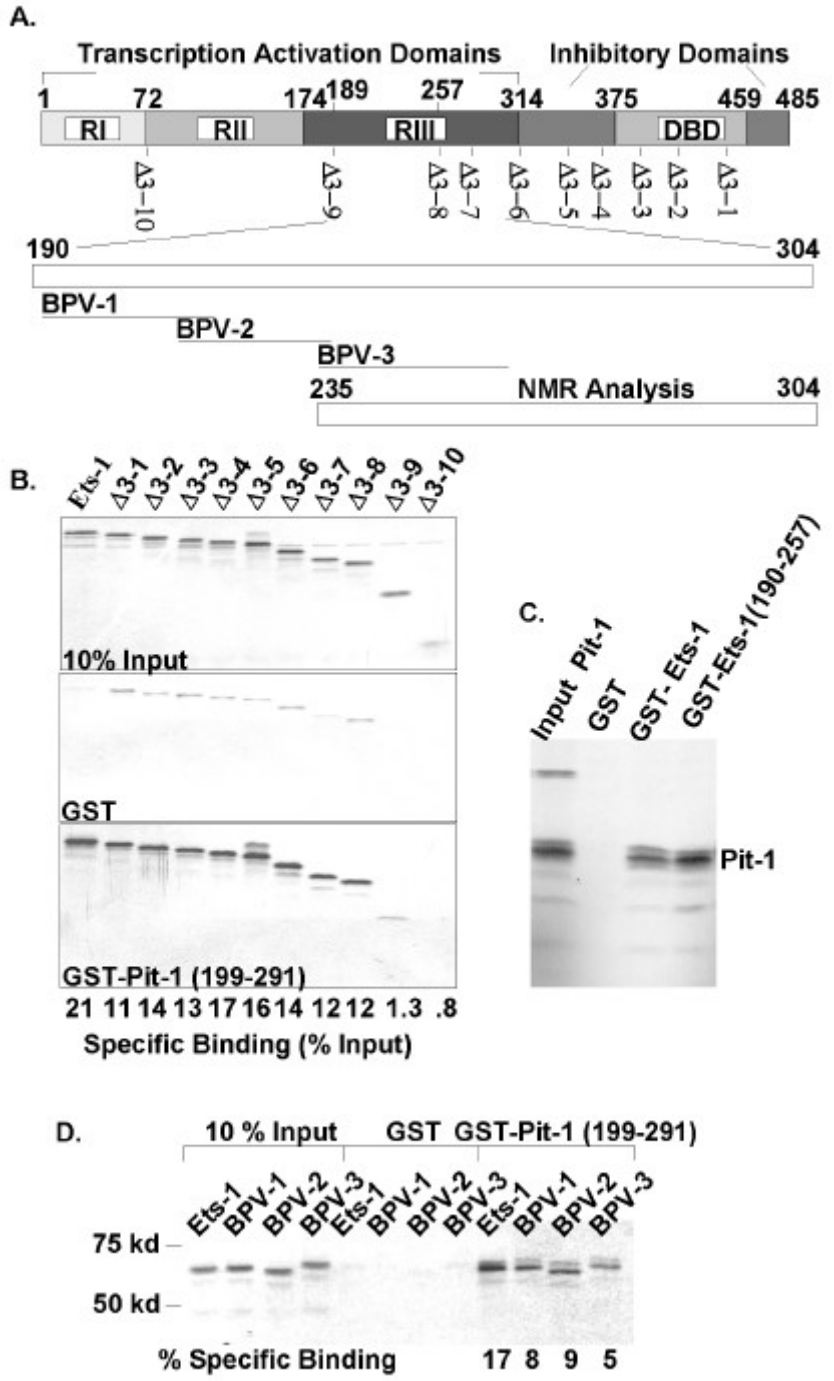

A.
Transcription Activation Domains
Inhibitory Domains
1
72
174
189
257
314
375
459
485
RI
RII
RIII
DBD
Δ3–10
Δ3–9
Δ3–8
Δ3–7
Δ3–6
Δ3–5
Δ3–4
Δ3–3
Δ3–2
Δ3–1
190
304
BPV-1
BPV-2
BPV-3
235
NMR Analysis
304
B.
Ets-1
Δ3-1
Δ3-2
Δ3-3
Δ3-4
Δ3-5
Δ3-6
Δ3-7
Δ3-8
Δ3-9
Δ3-10
10% Input
GST
GST-Pit-1 (199-291)
21 11 14 13 17 16 14 12 12 1.3 .8
Specific Binding (% Input)
C.
Input
Pit-1
GST
GST- Ets-1
GST-Ets-1(190-257)
Pit-1
D.
10 % Input
GST
GST-Pit-1 (199-291)
Ets-1
BPV-1
BPV-2
BPV-3
Ets-1
BPV-1
BPV-2
BPV-3
Ets-1
BPV-1
BPV-2
BPV-3
75 kd
50 kd
% Specific Binding
17 8 9 5

Figure 1

Residues 190-257 of the Ets-1 RIII are necessary and sufficient to bind Pit-1. Equal amounts of in vitro translated [^{35}S]- labeled proteins were bound to 5 μg of GST control or fusion proteins as described in materials and methods. Nonspecific binding to GST alone was subtracted from total binding and specific binding is expressed as % of input. Data are representative of three separate experiments.
A) Schematic representation of Ets-1 and the constructs used in this study.
B) Deletion mapping of binding of [^{35}S]-p68 Ets-1 and Ets-1 truncations Δ3-1 (AA450), Δ3-2 (AA412), Δ3-3 (AA390), Δ3-4 (AA368), Δ3-5 (AA358), Δ3-6 (AA314), Δ3-7 (AA275), Δ3-8 (AA257), Δ3-9 (AA189), and Δ3-10 (AA71) to the POUhd; 10% of input, nonspecific and specific binding of [^{35}S]-Ets-1 and deletion constructs are shown.
C) The 190-257 region of Ets-1 is sufficient to bind *in vitro* translated [^{35}S]-Pit-1.
D) Linker scanning mutations identify Ets-1 AA 232-257 as the site of the most robust interaction with the POUhd.

Combined with previous results using 5' deletions to map the interaction [124], this study defines the region of Ets-1 required for Pit-1 binding as part of the RIII activation domain, between residues 190-257. To confirm that this region is sufficient, we expressed it as a GST-fusion. As shown in figure 1D, GST-Ets (190-257) could indeed bind Pit-1. Since neither the N-terminal Pointed domain, nor the C-terminal Ets-1 DNA-binding domain are required for the interaction, we can consider the 190-257 region as an independent binding domain within Ets-1. Therefore we chose to study the interaction of the POUhd with this minimal Ets-1 region, outside the context of the full-length protein.

3.4.2 NMR studies on the interacting residues in POUhd

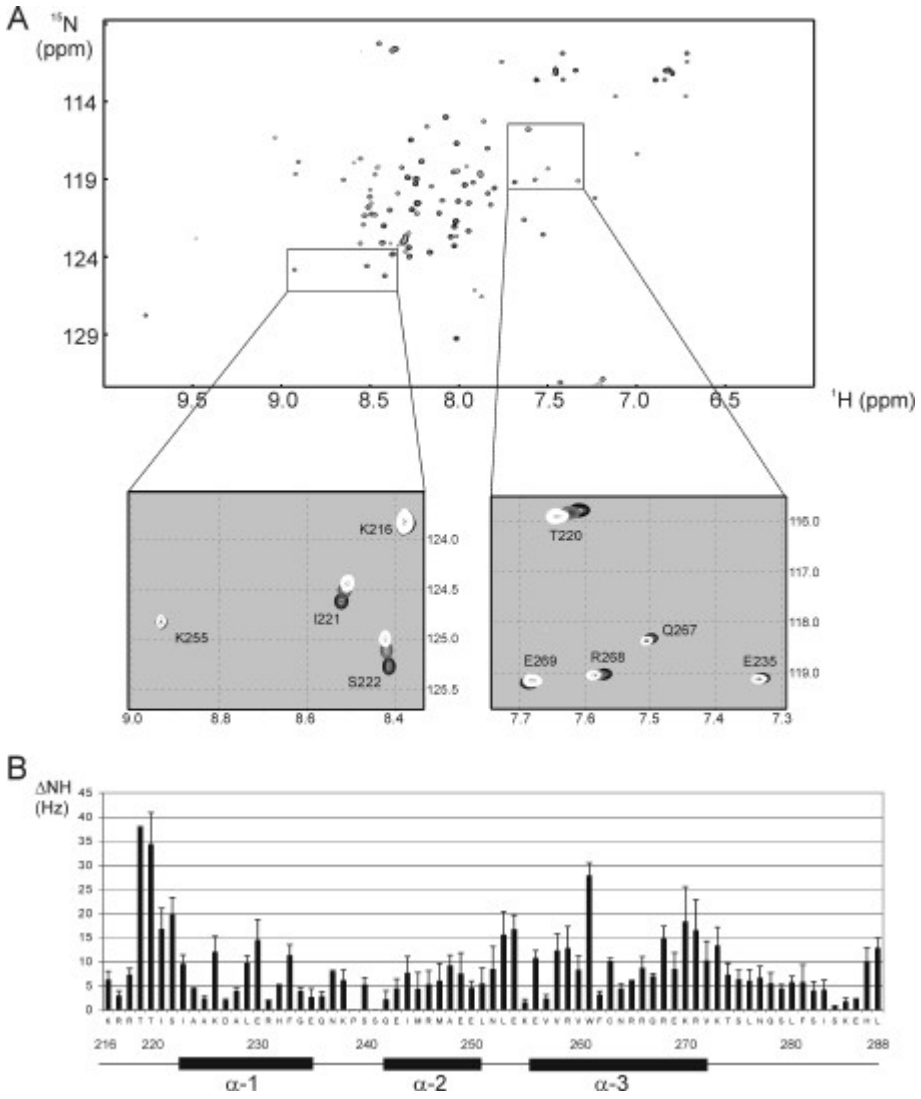
The chemical shifts of amide protons are highly sensitive to the binding of an interaction partner or ligand [214], or conformational changes in the protein [215]. Using this sensitivity, we investigated which amide protons of the ^{15}N-labeled POUhd (residues 213-289) show changes in chemical shift in a ^{1}H-^{15}N correlated HSQC (hetero-nuclear single quantum coherence) experiment upon addition of the unlabeled interaction domain of Ets-1 (residues 190-257). These

amide protons should be in close proximity to any side chain contacted by Ets-1. This approach would allow us to map which residues on POUhd are directly or indirectly involved in the interaction, without interference from the spectrum of Ets-1 (190-257).

^{15}N-labeled POUhd (residues 213-289) was expressed and purified as described in materials and methods. The ^{1}H-^{15}N-correlated HSQC spectrum recorded at 750 MHz showed well-dispersed signals, indicative of an ordered structure (figure 2A). Unfortunately, addition of any amount of Ets-1 (190-257) resulted in heavy precipitation and only minor changes in the ^{1}H-^{15}N-correlated HSQC spectrum of the POUhd. Attempts to remedy this problem by changing the pH and salt concentration did not provide satisfying results (data not shown). Linker-scanning experiments using insertions of the 26 AA sequence from the BPV L-1 capsid with an AU1 epitope along the Ets-1 (190-257) region, mapped the most robust interaction with Pit-1 to the C-terminal one third of the (190-257) region (figure 1D). Therefore we chose to use an Ets-1 construct that contained the C-terminal one third of the (190-257) region and extend it towards the C-terminus of Ets-1. The resulting (235-304) is less hydrophobic in nature and indeed did not precipitate when added to the POUhd at pH 5.5 and 100 mM NaCl.

Figure 2

NMR analysis of the Ets-1 (190-260) interaction with the POUhd. A) The ^{1}H-^{15}N-correlated HSQC spectrum of POUhd (residues 213-289) recorded at 750 MHz. The selected regions show superimposed spectra of free POUhd (black) and after the addition 0.5 (gray) and 1 (white) molar equivalent of Ets-1 (235-304). The chemical shift values of some N-H signals (like T220, I221 and S222) change drastically upon addition of Ets-1, while others are moderately (R268) or hardly (K216, K255, E269) affected. B) Combined ^{1}H-^{15}N chemical shift perturbations mapped against the secondary structure of the POUhd. The values are the mean of two independent experiments and were calculated as Euclidian distances between peaks: $\Delta\delta NH=((\Delta\delta^{15}N)^2 + (\Delta\delta^{1}H)^2)^{1/2}$, where $\Delta\delta^{15}N$ and $\Delta\delta^{1}H$ denote the nitrogen and proton shifts in Hz, respectively.



As shown in the zoomed regions in figure 2A, a number of backbone amide protons display a change in chemical shift that is dependent on the amount of Ets-1 (235-304) added, while others are unaffected. The changes in the spectra were specific for Ets-1, as an equimolar amount of the unrelated bovine serum albumin (BSA) did not result in any changes in chemical shift (data not shown). Since these backbone amides were the same as the ones displaying very

minor shifts after the addition of Ets-1 (190-257), we are confident that the majority of the interaction indeed resides in the overlapping region between our two constructs. The gradual, concentration dependent changes in chemical shift resonances in the presence of Ets-1 indicate that the two proteins are in fast exchange on the NMR timescale. We were able to add Ets-1 (235-304) up to about equimolar level to POUhd. Further addition of protein resulted in heavy precipitation without any further change in chemical shifts. The fact that the signal intensity of the HSQC spectrum was not severely affected indicates that this precipitate is probably Ets-1 (235-304). Indeed, SDS-PAGE analysis of the precipitate showed that it contained mostly Ets-1 (235-304) (data not shown). Therefore, we are unable to add more than an equimolar amount of Ets-1 (235-304). Given the moderate changes in chemical shift, the two peptides may not yet have reached saturation state of binding. This indicates that, at the conditions used to record the spectra, the K_D for the minimal domain complex is in the micro to millimolar range.

In order to identify the amide protons involved in the interaction, we expressed and purified $^{13}C/^{15}N$-double labeled POUhd. Using triple resonance experiments (HNCA, HN(CO)CA, CBCANH and CBCA(CO)NH), coupled with 3D-^{15}N-NOESY HSQC, we could assign the resonance frequencies of nearly all backbone amide protons (table I). In the 3D-^{15}N-NOESY HSQC, we were able to track the α-helices, as they were resolved in the crystal structure of the Pit-1 POU domain bound to DNA [131]. In addition, we found that the POUhd C-terminal (272-289) tail is rather unstructured in solution with few inter-residual NOEs and sharp signals in the $^{1}H-^{15}N$ HSQC. Our assignment allowed us to plot the difference in chemical shift in the absence and presence of Ets-1 (235-304) against the primary structure (fig. 2B).

Residue	H (ppm)	N (ppm)	CO (ppm)	CA (ppm)	CB (ppm)
M212	ND	ND	ND	ND	ND
R213	ND	ND	ND	ND	ND
K214	ND	ND	ND	ND	ND
R215	ND	ND	ND	ND	ND
K216	8.374	123.834	176.047	56.366	33.911
R217	8.432	123.086	179.257	55.739	31.66
R218	8.531	121.355	176.436	56.261	32.11
T219	8.36	110.62	174.905	61.588	69.024
T220	7.609	115.785	173.965	61.066	70.6
I221	8.518	124.591	173.27	61.483	39.088
S222	8.417	125.229	175.464	57.515	65.198
I223	8.511	120.795	174.203	64.93	37.737
A224	8.111	121.19	177.68	54.904	19.055
A225	7.945	122.333	180.942	54.844	19.28
K226	8.502	120.127	176.831	60.334	32.335
D227	8.006	118.467	178.175	57.41	40.889
A228	7.526	122.575	178.814	55.052	19.805
L229	8.538	121.926	180.413	58.663	38.399
E230	8.344	119.898	178.177	59.29	29.518
R231	7.821	120.647	178.947	59.708	30.073
H232	8.321	118.254	180.164	59.394	29.796
F233	9.48	122.823	177.365	61.588	39.232
G234	7.911	102.533	175.076	46.606	
E235	7.328	119.1	175.352	57.557	31.098
Q236	8.092	120.364	176.794	55.429	31.382
N237	8.555	123.127	173.082	54.154	39.319
K238	8.65	119.031	175.193	53.853	33.087
P239	ND	ND	ND	62.446	32.799
S240	8.904	117.887	176.623	57.13	65.68
S241	ND	ND	ND	ND	ND
Q242	8.39	120.995	177.172	59.469	29.114
E243	7.633	121.619	178.485	59.415	30.438
I244	8.473	121.315	179.259	65.635	42.826
M245	7.88	118.595	177.303	59.363	32.516
R246	7.837	119.914	176.24	59.484	30.567
M247	8.155	119.459	179.229	59.469	30.531
A248	8.491	121.244	174.199	56.067	18.342
E249	7.839	117.014	179.419	59.363	30.248
E250	8.233	120.542	175.336	59.256	30.531
L251	7.86	115.268	178.307	54.154	43.005
N252	7.877	118.716	175.692	54.26	37.902
L253	8.554	117.673	173.732	52.665	47.824
E254	7.919	119.208	176.571	56.492	30.531
K255	8.926	124.833	178.67	60.745	32.516
E256	8.92	118.663	178.184	58.938	30.248
V257	6.996	117.359	178.427	65.635	32.232
V258	7.234	120.217	178.882	67.101	32.516
R259	8.493	120.539	178.406	61.17	31.098
V260	8.5	119.677	179.299	66.91	33.366
W261	8.298	122.678	177.396	63.402	29.681
F262	8.592	117.943	179.919	64.04	40.17
C263	8.03	118.524	177.067	63.509	26.279
N264	8.473	118.694	177.484	55.642	38.185
R265	8.3	123.66	175.813	56.492	28.547
R266	7.957	118.182	179.174	57.662	29.397
Q267	7.499	118.323	177.259	57.874	29.114
R268	7.572	119.027	177.559	57.662	31.098
E269	7.686	119.191	177.071	57.13	30.815
K270	7.8	119.58	178.265	57.343	33.366
R271	7.943	120.539	177.177	56.811	31.382
V272	8.003	120.418	176.761	63.083	33.366
K273	8.278	123.974	174.581	56.811	33.65
T274	8.074	115.001	176.937	62.233	69.652
S275	8.21	117.856	174.654	58.609	63.982
L276	8.163	123.719	174.583	55.642	43.005
N277	8.288	118.901	177.205	53.516	39.319
Q278	8.233	120.542	174.952	56.47	29.966
S279	8.27	116.471	176.071	58.663	63.607
L280	8.026	123.29	174.596	55.635	42.84
F281	7.966	119.372	177.141	57.723	40.115
S282	8.013	116.694	175.695	58.246	63.795
I283	8.025	122.059	174.331	61.379	39.232
S284	8.244	119.288	176.234	58.246	63.933
K285	8.282	123.396	176.106	56.679	33.126
E286	8.264	120.957	176.427	56.888	30.906
H287	8.239	118.974	175.921	55.217	30.073
L288	8.015	129.252	173.352	56.783	43.118
E289	ND	ND	ND	ND	ND
W70 HNε	9.764	127.774			

Table I

Backbone assignment for the Pit-1 POUhd residues 213-289. ND: not determined.

The most drastic changes in chemical shifts of backbone amides are located around threonines 219 and 220 in the N-terminal part of the homeodomain. Interestingly, these threonines can be phosphorylated in vivo [142]. The level of chemical shift change along the first helix of the homeodomain appears to have a periodicity of three residues, indicating that only one side of this helix is in close proximity to Ets-1. This region forms a bridge between the N-terminal threonines and a less sharply defined region at the C-terminus of the DNA binding helix of the homeodomain, where the amide protons are moderately affected. The flexible C-terminus outside the homeodomain was hardly affected, except for H287 and L288 at the

very end of the protein. A surprisingly large change in chemical shift occurred on the amide proton of W261. In the crystal structure, the side chain of this residue is almost completely buried in the hydrophobic core of the homeodomain and is therefore unlikely to be involved in contacting Ets-1. There is, however, a hydrophobic pocket around the amide proton of W261, formed by V257, V258, I221 and part of the tryptophan side chain itself in which Ets-1 might dock. The only other amide protons, besides the W261 backbone amide, bordering this hydrophobic pocket are the backbone amides of V258, I221 and T219. The amide protons of all these residues shift drastically upon addition of Ets-1. Moving away from this hydrophobic pocket, on the other side of the W261 backbone amide, the shifts in the amide protons become less pronounced. Specifically, the N_{ε}-H of the tryptophan side chain is moderately affected, while the backbone amide of F262 is hardly affected.

3.4.3 Mutational analysis

In previous studies, using GST-pulldown assays, deletion of either the N- or C-terminal half of the homeodomain resulted in a 50 % decrease in pull-down efficiency [125]. This suggested that there is more than one region for the interaction with Ets-1 located on the POUhd. The NMR results suggest binding of Ets-1 might take place around T219/T220, in the hydrophobic pocket next to W261 and at the extreme C-terminus of the POUhd, possibly with stabilizing contacts along the DNA binding helix. In order to test these possibilities, we constructed several POUhd point mutants and tested their ability to interact with full-length Ets-1 in GST-pulldown assays. As shown in figure 3A, Pit-1-A_3, in which the three principal phosphorylation sites (S115, T219, T220) are mutated to alanines, showed a 1.4 fold increase in Ets-1 binding. In order to test the effect of a phosphorylated T220, we substituted it with an aspartic acid residue, which mimics a phosphorylated POUhd. Indeed, binding affinity of the T220D mutation was reduced by about one-third (Fig. 3B, lane 4). Thus, it appears that T220 represents one contact point for Ets-1 in the POUhd and that the phosphorylation status of T220 is an important regulator of the Ets-1/Pit-1 interaction.

Residue E254 showed a significant (>15 Hz) change in chemical shift upon addition of Ets-1 (235-304). This change in

chemical shift might reflect the relative flexibility of this residue, which is located in the loop preceding the DNA binding helix. However, in a previous study, mutation of this residue to alanine resulted in an increased binding affinity for N-CoR [46]. As shown in figure 3B, this mutation also increased Ets-1 binding by about two-fold.

In the crystal structure, the hydrophobic pocket next the W261 is partially covered by K226. Modifying this residue might alter the accessibility of the hydrophobic pocket. Indeed, both the K226A (which removes the positive side chain) and K226D (which switches the charge from positive to negative) mutations strongly increased Ets-1 binding. These results suggest that both the presence and charge of K226 are important in regulating the binding of Ets-1. Since the hydrophobic pocket is formed by a number of structurally important residues, it is not possible to remove it without affecting the overall structure of the POUhd. However, mutating W261 to a phenylalanine should alter the pocket to some extent, without drastically affecting the folding of the POUhd. As shown in fig 3B, the binding affinity of W261F POUhd is increased fifteen-fold.

Moderate changes in chemical shift occurred along the C-terminal part of the DNA binding helix. These may reflect stabilizing contacts with Ets-1 of the residues involved. However, mutations R268A and K270A hardly affected Ets-1 binding (Fig. 3B). Finally, we tested whether L288 at the C-terminus is involved in Ets-1 binding. Surprisingly, L288A POUhd showed an increased binding affinity (Fig. 3B), which indicates that L288 may be in close proximity to a hydrophilic interaction domain.

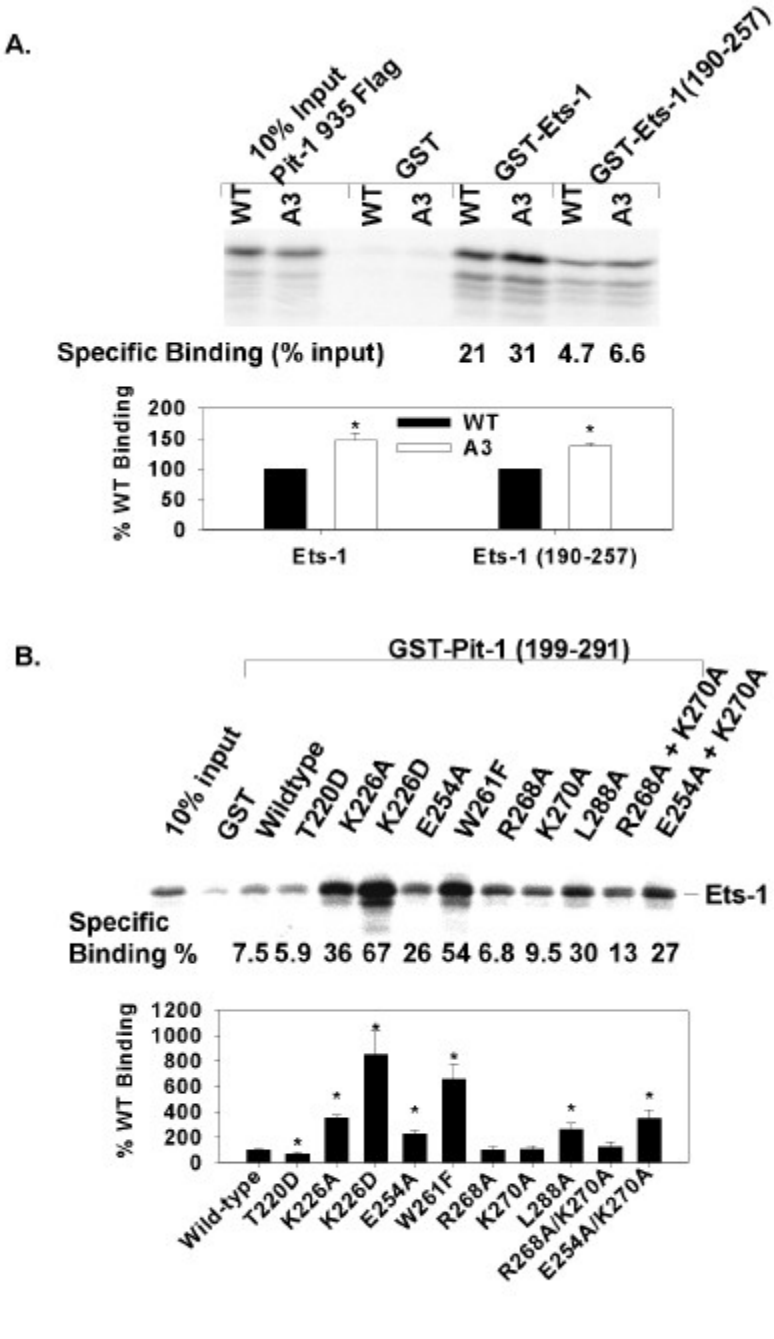

A.
10% Input
Pit-1 935 Flag
GST
GST-Ets-1
GST-Ets-1(190-257)
WT
A3
WT
A3
WT
A3
WT
A3
Specific Binding (% input)
21
31
4.7
6.6
% WT Binding
200
150
100
50
0
WT
A3
Ets-1
Ets-1 (190-257)
B.
GST-Pit-1 (199-291)
10% input
GST
Wildtype
T220D
K226A
K226D
E254A
W261F
R268A
K270A
L288A
R268A + K270A
E254A + K270A
Ets-1
Specific
Binding %
7.5
5.9
36
67
26
54
6.8
9.5
30
13
27
% WT Binding
1200
1000
800
600
400
200
0
Wild-type
T220D
K226A
K226D
E254A
W261F
R268A
K270A
L288A
R268A/K270A
E254A/K270A

Figure 3

Effect of point mutations of Pit-1 homeodomain on binding of p68 c-Ets-1. In vitro transcribed and translated [^{35}S]-labeled proteins were bound to 20 μg of GST fusion proteins as described in materials and methods. Nonspecific binding to GST alone was subtracted from total binding and specific binding is expressed as % of input normalized for equal loading of fusion constructs. The graphs contain the mean ± SEM of data from 3 experiments. Asterisks represent a change that is statistically different from wild-type binding, $p<0.05$.
A) Mutation of Pit-1 serine/threonine phosphorylation sites to alanine increases p68 Ets-1 (190-257) binding.
B) Binding of [^{35}S]-Ets-1 to GST and GST- Pit-1 (199-291) wild-type and mutants.

3.5 Discussion

There have been relatively few NMR studies on protein-protein interactions to date [64,215,216]. The interaction partners in these studies all formed high affinity complexes. Here we report a NMR study on a 75 AA polypeptide spanning the Pit-1 POU homeodomain (AA 213-289) and a 70 AA region of the Ets-1 RIII activation domain (AA235-304), which we have shown to be the essential domains for the interaction between the two full-length proteins. The full-length proteins have been shown to interact *in vivo* with sufficient affinity to be detected by fluorescence resonance electron transfer (FRET) [217]. At conditions optimal for the NMR measurements, these two protein domains did not bind each other with high affinity the K_D being in the micro to millimolar range. Even with this rather weak interaction, we were still able to show clear changes in chemical shift of the amino protons of the POUhd after addition of Ets-1 (235-304).

In the case of the POU family member Oct-1, the DNA bound crystal structure and the solution structure are highly similar [135,218], since the Oct-1 and Pit-1 homeodomain are highly homologous, we presume that this will also be the case with the POUhd solution structure. We were in fact able to track all α-helices, as they were resolved in the crystal structure by $NH_{(i, i+1)}$ contacts in the 3D-^{15}N-NOESY HSQC. Therefore we can directly correlate the amount of chemical shift difference per amide proton to the known crystal structure of the POUhd [131], which is shown in figure 4A

(page 82). All amide protons that displayed more than 10 Hz change in chemical shift are located on one side of the protein, which would put the Ets-1 interaction region on the top side of the POUhd. One limitation of this approach is that changes in chemical shift for any given backbone amide are indirect evidence that its side chain is involved in the interaction. Therefore, we verified our results by interaction assays.

Our results show large changes in chemical shift of the backbone amides around T219 and T220, which indicates that some part of Ets-1 (235-304) is in close proximity to these residues. Regulated phosphorylation of these threonines occurs *in vivo*, either as targets of PKA or of a cell cycle-dependent kinase [142]. There have been conflicting reports on the influence of phosphorylation of threonine 220 with regard to DNA binding [141,142,219]. Furthermore, Pit-1-A_3, in which the three principal phosphorylation sites (S115, T219, T220) are mutated to alanines, was unaffected or slightly less efficient than wild-type in activating transcription on the GH and PRL promoters in transient transfections [142,145]. Using Pit-1-A_3 in the HeLa reconstitution system, it was shown that removing the phosphorylation sites did not affect synergistic activation of the prolactin promoter with Ets-1 [125]. Because in transient over-expression, subtle differences in binding affinity may be lost, we tested the ability of Pit-1-A_3 to interact with Ets-1 in a GST-pulldown assay. We found that Ets-1 binding of Pit-1-A_3 was about 1.4 fold increased, while introducing a negative charge at the position of T220 reduces Ets-1 binding by about one-third. It is possible that this effect would be more pronounced with an actual phosphorylated threonine, which has a slightly different size and charge distribution than an aspartic acid. The reduction in binding affinity is not dramatic in our assay. However, given the bidentate nature of the interaction, the fact that this reduction is caused by a single point mutation seems all the more significant. Furthermore, the loss of about one-third of binding affinity could very well influence subtle *in vivo* equilibria. Therefore, even though phosphorylation of T220 does not seem to serve as an on/off switch for Ets-1 binding, it might well be an important regulatory mechanism.

Alternatively, the changes in chemical shift in the region around T219/220 might reflect secondary effects of binding of Ets-1 to

the hydrophobic pocket next to W261. The accessibility of this hydrophobic pocket seems to be modulated by the side chain of K226. In the crystal structure, Pit-1 is bound to DNA as a dimer. In one of the homeodomain structures in the crystal, K226 made a contact to the phosphate backbone of the DNA, folding over the hydrophobic pocket. However, in the other homeodomain in the protein dimer, the side chain of K226 is pointing away from the DNA, exposing the hydrophobic pocket (figure 4B). The presence of K226 is detrimental to the association of Ets-1, since truncating this side chain or switching its charge clearly increases Ets-1 binding. These findings present an interesting opportunity for an additional level of control for Pit-1/Ets-1 complex formation, as Pit-1 has been shown to be acetylated by CBP *in vitro* [79] and both Pit-1 and Ets-1 are able to bind CBP/p300 [46,220,221]. Based on our results, this acetylation could take place on K226 which would serve to increase association with Ets-1 by increasing accessibility of the hydrophobic pocket, thus decreasing the off-rate of Ets-1. Increased stability of the Pit-1/Ets-1 complex could in turn enhance synergistic activation of the prolactin promoter. Using our data, we can suggest a model for the association of Ets-1 with Pit-1 (Fig. 4C). Depending on the post-translational modification state of the POUhd, Ets-1 will dock in the hydrophobic pocket next to W261 and contact the region around T220. Additional stability will be provided by residues at the C-terminus of the DNA binding helix and by the region around L288.

Several other transcription factors, such as N-CoR, vitamin D receptor, Oct-1, and GATA-2 interact with Pit-1 through the homeodomain [46,147,152,162]. Oct-1 and Pit-1 can dimerize via the POU domain. In the absence of a cocrystal structure of an Oct-1 / Pit-1 heterodimer bound to DNA, we speculate that the regions involved in this interaction are the same as those that allow homo-dimerization of Pit-1. This would mean that the C-terminus of the POU-homeodomain would dock in a hydrophobic pocket located between α-helices 1 and 4 on the POU-specific domain. The association with the vitamin D receptor has been analyzed by deletion analysis, in which the N-terminal half of the homeodomain proved vital for the interaction, while the C-terminal half accounted for half the binding affinity. These data do not exclude a similar binding interface as the one we have proposed in this study. The interaction of Pit-1 with N-CoR is

not yet very well characterized. The fact that a point mutation in the POUhd increased the affinity of Pit-1 for N-CoR implies that the POUhd is involved in this interaction [46]. Since that same mutation (E254A) also increased the affinity for Ets-1, it is tempting to speculate that these proteins might compete for the same binding interface. The interaction with GATA-2 will be discussed in more detail below. Apart from the high sensitivity, one of the advantages of our NMR approach over random mutagenesis or deletion experiments is that in one experiment, the entire protein can be probed for interaction. This means that combinations of residues or structural motifs that may not be affected with a single point mutant can be identified. Even if the proteins of interest bind one another with moderate affinity, this approach can still produce valuable information on protein-protein interactions.

3.6 Addendum: HSQC-monitored titrations of the Pit-1 POUhd with GATA-2.

3.6.1 Introduction

Like Ets-1, GATA-2 also interacts with the Pit-1 POUhd (see chapter I). Some of the essential residues on the POUhd for binding GATA-2 have been identified by mutagenesis coupled with interaction assays [162]. These residues include R215 and K216 at the N-terminus and P239 and Q242 at the beginning of the second helix of the POUhd. None of the amide protons of these residues showed any significant change in chemical shift upon addition of Ets-1 (235-304), which indicates that GATA-2 probably employs a binding interface that is different from Ets-1. However, it is not excluded that part of the contacts may coincide with those of Ets-1. Furthermore, as mentioned above, patches of amino acids that are involved in an interaction may be missed in a screening using single point mutants.

Here we perform a similar HSQC-monitored titration as described above, using a purified peptide containing both GATA-2 zinc fingers, which should bind the POUhd based on data from Dasen *et al*. [162]. The resulting changes in the POUhd spectrum indicate that GATA-2 likely utilizes a different interaction interface on the POUhd than Ets-1.

3.6.2 Materials and Methods: *Proteins and NMR*

^{15}N-Pit-1 POUhd (213-289) was expressed and purified as described above. The human GATA-2 (290-409) peptide containing both the N- and C-terminal zinc fingers was a kind gift from Dr. David Gordon (University of Colorado Health Sciences Center, Denver). 10 mg of lyophilized peptide was dissolved in 5 ml 0.05 % trifluoroacetic acid with 1.1 equivalent of $ZnCl_2$, after which the pH was slowly increased to pH 6.0 by small additions of 0.1 M of NaOH. The GATA-2 peptide was concentrated in an Amicon stirred ultrafiltration cell (Millipore) using a 3 kD cut-off filter. The buffer of both the ^{15}N-POUhd and GATA-2 (290-409) was exchanged to a final 30 mM CD_3COOH (pH 6.0), 100 mM NaCl and 1 mM dithiotreitol (DTT). NMR experiments were carried out at described above.

3.6.3 Results / Discussion

During the reconstitution with zinc, a substantial amount of GATA-2 peptide was lost due to precipitation (final solution was 0.2 mM in 0.5 ml). Therefore, using a 0.5 mM ^{15}N-POUhd we were able to add GATA-2 only to a final 0.4 molar equivalent. Nevertheless, the HSQC spectrum of the POUhd did show changes that were dependent on the concentration of the GATA-2 peptide. Because of the low amount of GATA-2 added, the amount of chemical shift difference is smaller than with the Ets-1 titration. Figure 5 shows the level of chemical shift difference plotted against the primary structure of the POUhd.

The overall pattern differs from the one seen with the Ets-1 titration (Fig. 2B). The region that is most affected by the presence of GATA-2 is located at the N-terminus of the POUhd, which is in good agreement with previous results [162], where R215 and K216 were shown to be vital determinants for GATA-2 binding. Apart from the N-terminal region, only the amide protons of L253 and L288 were affected above background level. This change in chemical shift may reflect local flexibility of the loop between helices 2 and 3. On the other hand, L253 is located within 10Å of T220 in the tertiary structure. T220 is the last residue that is significantly (>10 Hz) affected in the N-terminal region. Thus L253 may be part of the same interaction domain that includes R215 and K216. The significance of the change in chemical shift for L288 cannot be determined at present.

The same residue did affect Ets-1 binding, which may indicate that the two factors share an interaction domain at the C-terminus. Alternatively, L288 might be more susceptible to changes for its location at the extreme C-terminus of the POUhd.

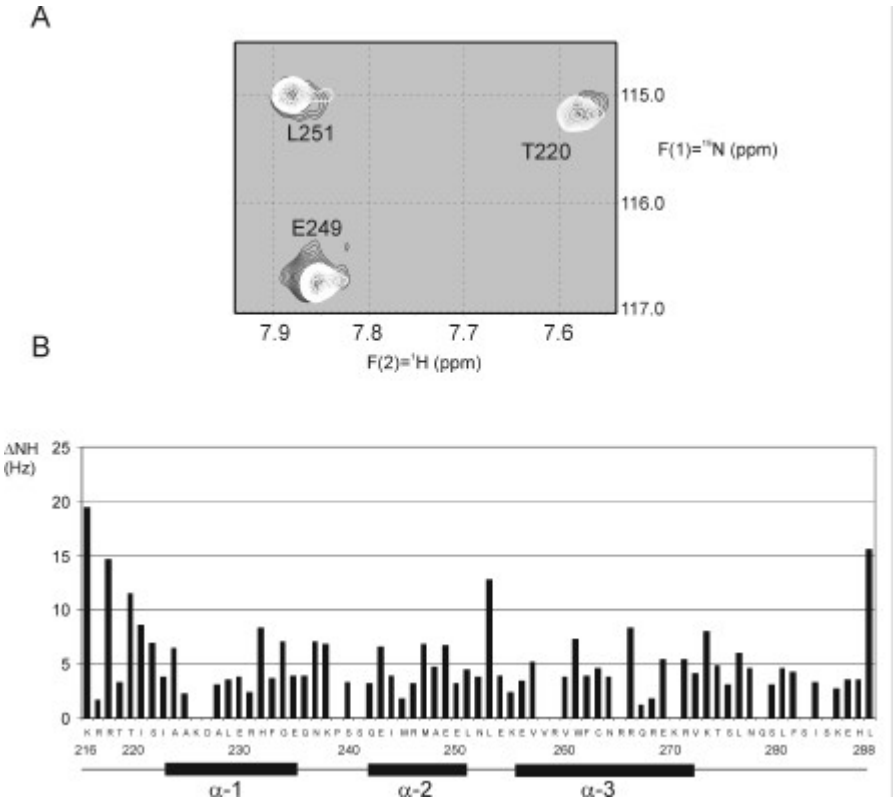


Figure 5

HSQC Monitored titration of the ^{15}N-POUhd with the Zn-finger region of GATA-2.
A) Selected region showing superimposed spectra of free POUhd (black) and after the addition of 0.4 molar equivalent of GATA-2 (290-409) (white).
B) Combined ^{1}H-^{15}N chemical shift perturbations caused by the addition of 0.4 molar equivalent of GATA-2 (290-409) mapped against the secondary structure of the POUhd. The values are calculated as Euclidian distances between peaks: $\Delta\delta NH=((\Delta\delta^{15}N)^2 + (\Delta\delta^{1}H)^2)^{1/2}$, where $\Delta\delta^{15}N$ and $\Delta\delta^{1}H$ denote the nitrogen and proton shifts in Hz, respectively.

Dasen *et al.* identified two more POUhd mutations that affected GATA-2 binding: P239S and Q242A. Of these two residues P239 cannot be studies by our approach, since it has no amide proton. The amide proton of Q242 and the surrounding residues were unaffected in our study, indicating that there are no major contact points in that region of the POUhd. This apparent contradiction could be explained by a loss of helix capping with the Q242A mutation, which could disrupt the formation of the second α-helix of the POUhd. Resulting changes in the overall structure of the POUhd might affect GATA-2 binding. An alternative explanation is the use of a GATA-2 peptide in this study instead of the full-length protein by Dasen *et al*.

In conclusion, the interaction with GATA-2 seems to involve an interaction domain at the N-terminus of the POUhd. Even though the interaction with Ets-1 also involved residues in the N-terminus of the POUhd, the overall pattern of chemical shift changes appears to be quite different. Therefore, it is likely that GATA-2 uses a different interaction interface than Ets-1. Further NMR experiments coupled with mutational analysis are required to further define this interface.

3.7 Acknowledgements

This work was supported in part by the Netherlands Organization for Scientific Research (NWO) with the financial support of the Netherlands Foundation for Chemical Research (SON) and by National Institutes of Health Grants DK46868 (to AG-H) and DK02946 (to DLD). We wish to thank A.B. Brenkman and R.N. de Jong for critical reading of the manuscript and useful discussions.

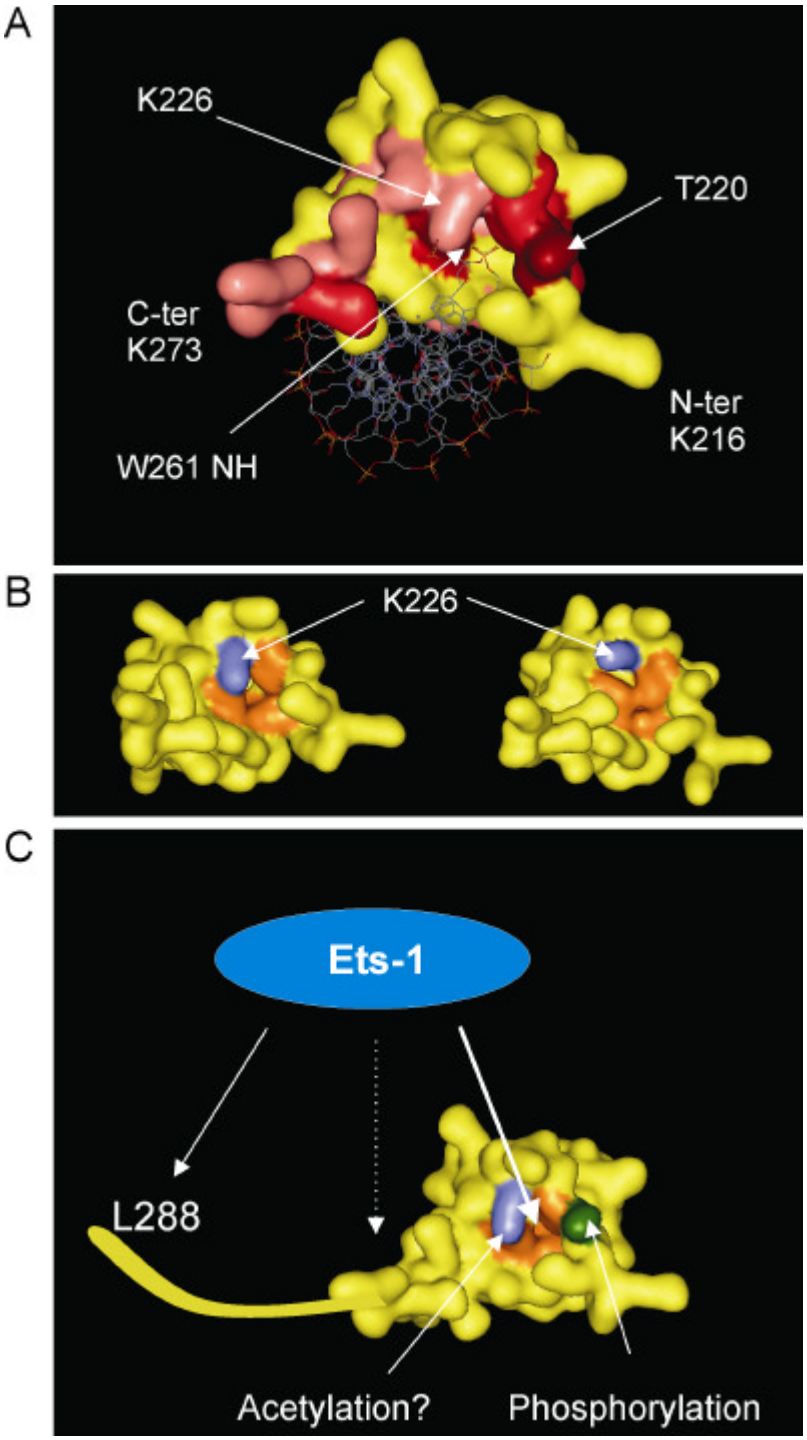

A
K226
T220
C-ter
K273
N-ter
K216
W261 NH
B
K226
C
Ets-1
L288
Acetylation?
Phosphorylation

Figure 4

Mapping of chemical shift perturbations to the POUhd structure. Surface density representations of the crystal structure of the POUhd bound to DNA. These fragments were taken from the crystal structure as resolved by Jacobson et al (1997).
A) The results from figure 2B indicated by color-coding. Chemical shift changes > 25 Hz are colored dark red, changes between 25 and 15 Hz red and between 15 and 10 pink. Unaffected residues are colored yellow.
B) Two orientations of K226 (blue) in the Pit-1 dimer with different accessibility of the hydrophobic pocket formed by V257, V258, I222 and W261 (colored in beige). The DNA has been deleted for clarity.
C. Model for association of Ets-1 to the POUhd. K226 is shown in blue, the hydrophobic pocket in beige and T220 in green. Since the crystal structure does not extend beyond residue K273, an extended tail was added to represent the C-terminus of the POUhd. Ets-1 could, depending on the post-translational state of the POUhd, dock in the hydrophobic pocket next to W261. Additional stabilizing contacts may be made at the C-terminus of the DNA recognition helix (helix 3) and at the region around L288. These pictures were generated using MSI's Weblab viewer, Lite version (www.msi.com).

"Maar...hier zit helemaal geen eiwit in!"

Rainer Wechselberger

Chapter 4

DNA titrations of the Pit-1 POUhd and ternary complex formation with the minimal Ets-1 interaction region.

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To be submitted.

4.1 Abstract

The POU-domain transcription factor Pit-1 and Ets-1, a member of the ETS family of transcription factors, can associate in solution and synergistically activate the prolactin (PRL) promoter by binding to a composite response element in the PRL promoter. We have previously mapped the minimal interaction domains for the physical interaction to the Pit-1 POU-homeodomain (POUhd) and a part of the activation domain of Ets-1. Furthermore, we have shown that the interaction domain for Ets-1 on the POUhd partially overlaps with its DNA binding interface. In this report we used mobility shift assays and HSQC-monitored protein-DNA titrations to investigate the effect of the presence of the minimal Ets-1 interaction domain on DNA binding by the POUhd. Our results show that the minimal interaction domain of Ets-1 and the POUhd do not form a ternary complex with DNA and that this Ets-1 domain does not affect DNA binding by the POUhd.

4.2 Introduction

The POU-domain transcription factor Pit-1 provides an excellent model system to study protein-protein and protein-DNA interactions. As one of the key regulators of gene expression in the anterior pituitary gland, Pit-1 is required for the expression of the growth hormone (GH), prolactin (PRL) and thyrotropin (TSHβ) genes [184,208]. However, in order to selectively stimulate these promoters, Pit-1 needs to interact with a number of other transcription factors [92]. One of these factors is ETS-family member Ets-1 [167], which interacts with Pit-1 in solution and binds to a composite Ets-1 / Pit-1 response element in the FP-4 region of the PRL promoter [108]. Together they synergistically activate transcription of the PRL promoter. We have previously investigated the interaction of the minimal interaction domains of both proteins, which comprise the POU-homeodomain (POUhd) of Pit-1 and a part of the region III activation domain of Ets-1 [125] (chapter III). However, the exact contribution of the physical interaction to the cooperative function on PRL gene activation of both proteins is as yet unclear. Thus far, efforts

to show a ternary complex with the full-length proteins and the composite DNA binding element have been unsuccessful. Therefore, we extended our studies of the minimal Pit-1 and Ets-1 interaction domain to include DNA. The minimal interaction domain of Ets-1 does not contain the ETS-DNA binding domain, but might be recruited to the DNA by the POUhd. Since the interaction domain of Ets-1 on the POUhd overlaps to some extent with the DNA binding interface, it is possible that the presence of Ets-1 might modulate the DNA binding affinity or specificity of the POUhd. Here we report HSQC-monitored protein-DNA titrations of ^{15}N-labelled Pit-1 POUhd (residues 213-289) with the Pit-1 site of the FP-4 region of the PRL promoter. In addition, we perform the same titration on a preformed Ets-1 (235-304) / POUhd complex. The NMR data, coupled with biochemical analysis, indicate that the minimal interaction domains of Ets-1 and Pit-1 are unable to bind simultaneously to DNA and that the DNA binding affinity of the POUhd is unaffected by the presence of the minimal interaction domains of Ets-1.

4.3 Materials and Methods

4.3.1 Proteins and DNA

Protein expression and purification was done as described in chapter III. The PRL-4P oligonucleotide 5'-GCC TTT GGC CTA ATT AAT CAA AAG GG-3' and its opposite strand 5'-CCC TTT TGA TTA ATT AGG CCA AAG GC-3' containing the Pit-1 binding sequence (-209 to -190) from the human PRL FP-4 region, with three CG base pairs at both the 5' and 3' ends to facilitate hybridization, were purchased from Roche. Lyophilized oligo was dissolved in H_2O, denatured at 95°C for 5 min and hybridized by cooling to room temperature. After hybridization, the oligo was loaded on a fast-flow Q-sepharose column equilibrated in 50 mM $Na_xH_yPO_4$ (pH 6.0), 100 mM NaCl and 1 mM dithiotreitol (DTT). After washing, the DNA was eluted in a linear gradient from 100-2000 mM NaCl. Peak fractions were pooled and concentrated to approximately 1.8 mM with an Amicon stirred ultrafiltration cell (Millipore) using a 1 kD cut-off filter. The buffer was exchanged to a final 50 mM $Na_xH_yPO_4$ (pH 5.5), 100 mM NaCl and 1 mM dithiotreitol (DTT). For the gel mobility shift assay, the oligo 5'-GGA GCT CGA GCT CTC ATT TCC TTT TGG CCT AAT TAA TCA

AAA TCC TTC CCT CGA GGA GG-3' and its opposite strand 5'-CCT CCT CGA GGG AAG GAT TTT GAT TAA TTA GGC CAA AAG GAA ATG AGA GCT CGA GCT CC-3' (Pharmacia) containing the -221 to -183 region from the hPRL promoter (flanked on both sides by an XhoI site) were end labeled using T4 kinase (Pharmacia), denatured at 95°C for 5 min and hybridized by cooling to room temperature. After hybridization, the oligo was purified by polyacrylamide gel electrophoresis.

4.3.2 NMR Spectroscopy

NMR experiments were carried out at 305 K (32°C) on a Bruker Avance 750 spectrometer. The protein concentration was 1 mM in all experiments, 10% D_2O was added to obtain a lock signal. Spectra were processed using the software package NMRPipe [212] and analyzed using NMRVIEW [213]. Protein-DNA titrations were carried out by repeated addition of small volumes of the PRL-4P oligo to a ^{15}N-labelled Pit-1 homeodomain sample and recording a 750 MHz $^{1}H^{15}N$-HSQC spectrum after each addition. The sample was concentrated in an Amicon stirred ultrafiltration cell to keep the total volume around 500 µl.

4.3.3 Gel mobility shift assay

Pit-1 POUhd (213-289) and Ets-1 (235-304) were purified as described in chapter III. Protein dilutions were made in 50 mM Tris-HCl (pH 7.5), 1 mM EDTA (pH 8.0), 100 mM NaCl, 10% glycerol and 10 mM β-mercaptoethanol. After 15 min. preincubation at room temperature, POUhd and Ets-1 (235-304) proteins were added to approximately 2 fmol DNA in a final reaction mixture of 20 µl containing 20 mM HEPES-KOH (pH 7.5), 100 mM NaCl, 1 mM EDTA (pH 8.0), 1mM DTT, 0.025% NP-40 and 1 µg poly dIdC competitor DNA. After another 15 min. at room temperature, 2 µl of 0.02% xylene cyanol was added and the samples were run on a 8% polyacrylamide gel containing 0.01% NP-40, at 4°C for 3 h at 30 mA. The gel was dried and the DNA was visualized by autoradiography.

4.4 Results

4.4.1 Ets-1 (235-304) does not form a ternary complex with the POUhd in EMSA.

Physical interaction of Ets-1 and Pit-1 involves a part of the RIII activation domain of Ets-1. As shown in chapter III the interaction interface for Ets-1 on the POUhd of Pit-1 overlaps to some extent with the DNA binding surface of the POUhd. This raised the question whether both the minimal domain interaction partners could simultaneously bind DNA. We addressed this question by testing whether addition of the Ets-1 interaction domain, which does not bind DNA by itself (data not shown), could supershift a POUhd / DNA complex in a bandshift (EMSA) assay. At the conditions used in the EMSA, the POUhd and Ets-1 are able to bind each other (unpublished data). As shown in figure 1A, the Pit-1 POUhd can bind to a probe containing the FP-4 region of the human PRL promoter. At higher concentration, a slower migrating complex appeared, which likely reflects the POUhd bound as a dimer. To examine whether a ternary Ets-1 / POUhd / DNA complex could be formed, an amount of POUhd that would shift about 50% of the labeled probe was incubated with increasing amounts of Ets-1 (235-304) prior to the addition of the DNA probe. As shown in figure 1B, Ets-1 (235-304) was unable to form a ternary complex with the DNA bound POUhd. In fact, even addition of an excess of Ets-1 (235-304) did not affect the POUhd / DNA complex in any way, which indicates that the POUhd has a much higher affinity for DNA, than for Ets-1 (235-304). Furthermore, we conclude that the amino acids on the POUhd that are not involved in DNA binding, but are contacted by Ets-1 (235-304) are insufficient to retain Ets-1 in this assay.

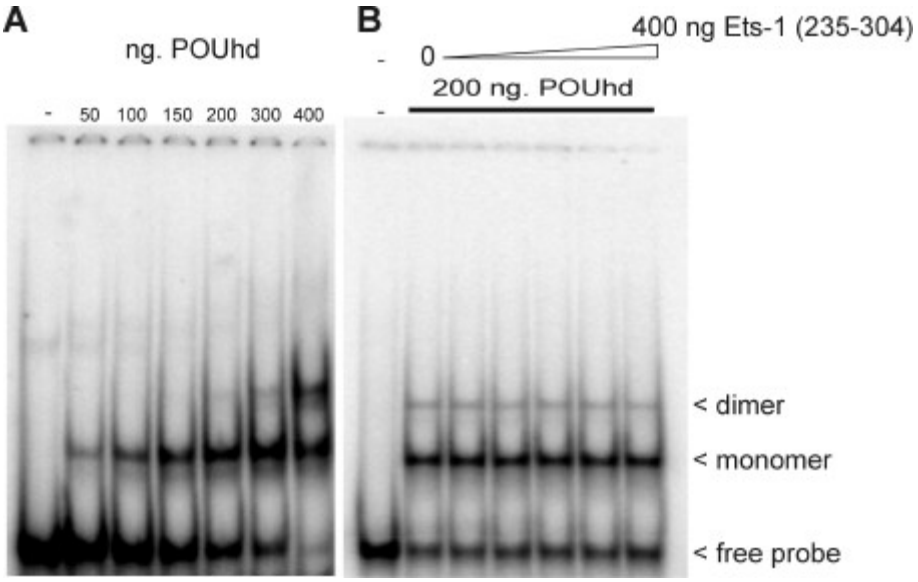


Figure 1

A) DNA binding of an increasing amount of the Pit-1 POUhd to an oligo containing the footprint 4 region from the human PRL promoter. The POUhd initially binds as a monomer, but will dimerize at higher concentration.
B) DNA binding of a constant amount of the POUhd, with the addition of an increasing amount of Ets-1 (235-304). Ets-1 (235-304) is unable to form a ternary complex with DNA-bound POUhd; neither does the presence of Ets-1 (235-304) decrease DNA binding by the POUhd.

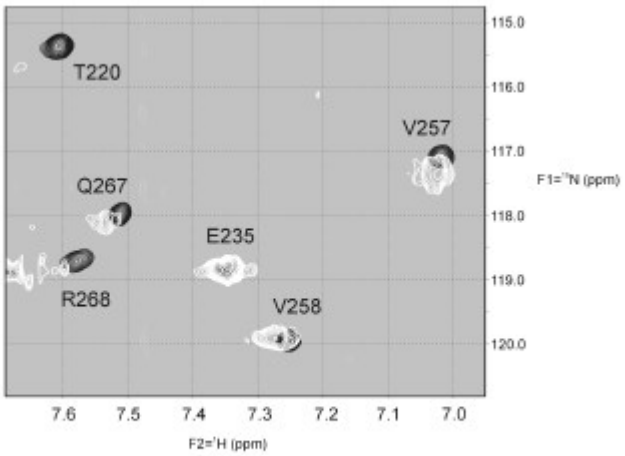
4.4.2 HSQC-monitored DNA titrations of the Pit-1 POUhd.

Since EMSA analysis is not a sensitive method to pick up weak interactions, we used HSQC-monitored titration of ^{15}N-labeled POUhd with unlabeled DNA to verify if a ternary complex could exist in solution. In order to do this, we first needed to investigate the effects of a titration of the ^{15}N-POUhd with an oligonucleotide containing the Pit-1 binding site of the FP-4 region from the PRL promoter. Addition of small volumes of the 1.8 mM solution of the PRL-4P oligo resulted in slight precipitation, which cleared after 5 minutes incubation at room temperature. ^{1}H-^{15}N HSQC spectra of the

^{15}N-POUhd recorded after the each addition of DNA showed a gradual broadening of most signals in the spectrum, indicative of a large molecular weight complex being formed. Figure 2A shows an overlay of a selected region of the free POUhd (black) spectrum and the same region after the addition of 1/6 molar equivalent of DNA to POUhd (gray). This region clearly shows that some signals (such as T220 and R268) are severely broadened and shifted, while others (such as V257, V258 and Q267) are moderately or hardly (E235) affected.

Upon reaching equimolar levels of DNA, the spectrum had deteriorated to a point where many of the signals could not reliably be determined. The differences in chemical shift per residue after the addition of 1/6 and 1/3 molar equivalent of DNA compared to the free form are shown in figure 2B. All residues showed a proportional shift to the amount of DNA added. Even though almost all backbone amides were affected to some extent, the major changes in the spectra were, as could be expected, limited to the DNA binding helix (α-3) of the POUhd. Several signals such as N264, Q267 and R268 were already severely broadened upon the lowest addition of PRL-4P, which probably indicates intermediate exchange for these residues. These residues are in direct contact with the DNA in the crystal structure. The same line broadening can be seen with the backbone amide signals from T220, I221 and S222. These residues are located in the N-terminal flexible linker, which docks into the minor groove in the crystal structure.

A


T220
V257
Q267
E235
R268
V258
115.0
116.0
117.0
118.0
119.0
120.0
F1=^{15}N (ppm)
7.6
7.5
7.4
7.3
7.2
7.1
7.0
F2=^{1}H (ppm)

B

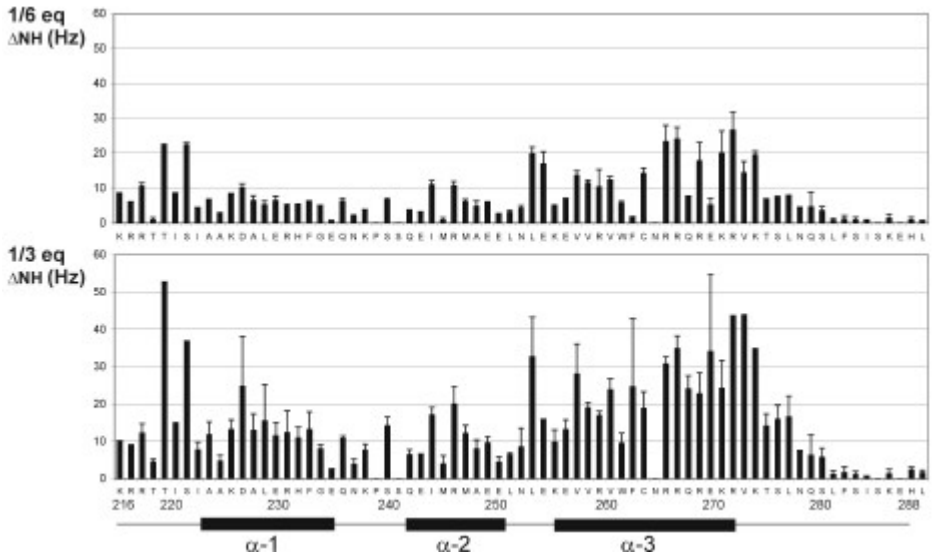

1/6 eq
ΔNH (Hz)
1/3 eq
ΔNH (Hz)
0
10
20
30
40
50
60
216
220
230
240
250
260
270
280
288
α-1
α-2
α-3

Figure 2

A) Overlay of a selected region of the $^{1}H^{15}N$-HSQC spectrum of the ^{15}N-POUhd showing the free POUhd (black) and the spectrum after the addition of 1/6 molar equivalent of PRL-4P oligo to the POUhd (gray).
B) Graphs showing the combined ^{15}N–^{1}H chemical shift perturbations, after the addition of 1/6 and 1/3 molar equivalent of PRL-4P oligo, mapped against the secondary structure of the POUhd. The values are the mean of two independent experiments and were calculated as Euclidian distances between peaks: $\Delta\delta NH=((\Delta\delta^{15}N)^2 + (\Delta\delta^{1}H)^2)^{1/2}$, where $\Delta\delta^{15}N$ and $\Delta\delta^{1}H$ denote the nitrogen and proton shifts in Hz, respectively. Major changes are restricted to the flexible N-terminus and the DNA binding helix (α-3) of the POUhd.

4.4.3 The presence of Ets-1 (235-304) does not prevent DNA binding by the POUhd.

In order to assess whether Ets-1 (235-304) could modulate the DNA binding capability of the POUhd, we performed the same titration as described above on a preformed Ets-1 (235-304) / POUhd complex. As described in chapter III, at the conditions used to record the spectra, it was not possible to add more than an equimolar amount of Ets-1 (235-304) to the POUhd without severe precipitation of Ets-1 (235-304). Therefore, in order to obtain a saturated POUhd / Ets-1 (235-304) complex, we added a 1.1 molar excess of Ets-1 (235-304) to a ^{15}N-labeled POUhd sample. After 5 minutes incubation at 32°C, the precipitate was removed by centrifugation and a $^{1}H^{15}N$-HSQC recorded. The resulting spectrum displayed the same shifts as described in chapter III (data not shown). Upon addition of the PRL-4P oligo the same line broadening and peak shifts occurred as seen with the free POUhd / DNA titration (Figure 3). The overall spectrum after the addition of 1/3 molar equivalent of DNA was highly similar to the corresponding spectrum of the free POUhd / DNA titration. Furthermore, the residues that directly contact DNA in the crystal structure were severely broadened and shifted after the lowest additions of PRL-4P, as they were with the titration of the free POUhd. Thus, in agreement with the EMSA, the NMR data indicates that the POUhd binds with higher affinity to its target DNA sequence than to Ets-1 (235-304) and that the binding of Ets-1 (235-304) is prevented by the presence of DNA.

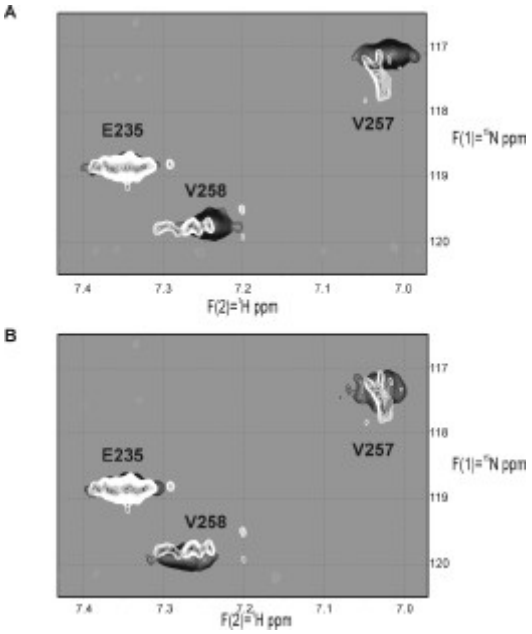


Figure 3

A) Overlay of a selected region of the $^{1}H^{15}N$-HSQC spectrum showing the ^{15}N-POUhd / Ets (235-304) preformed complex without DNA (black) and after the addition of 1/3 molar equivalent of PRL-4P oligo (white).
B) Overlay of a selected region of the $^{1}H^{15}N$-HSQC spectrum showing the POUhd in the presence of 1/3 molar equivalent of PRL-4P (black) and the POUhd / Ets (235-304) preformed complex after the addition of 1/3 equivalent of PRL-4P oligo (white).

4.5 Discussion

The crystal structure of Pit-1 bound as a dimer [131] provides a static picture of the POU domain bound to the DNA as a dimer. Here we report a NMR study of DNA binding by the POUhd, which allows for a more dynamic approach. Titration of ^{15}N-POUhd with the PRL-4P showed a rapid line broadening of the HSQC spectrum, which indicates the formation of a large complex in solution. Specific changes in chemical shift and severe line broadening occurred on the residues that directly contact DNA in the crystal structure. For example, the amide proton from residue N264 was no longer detectable after the lowest addition of PRL-4P.

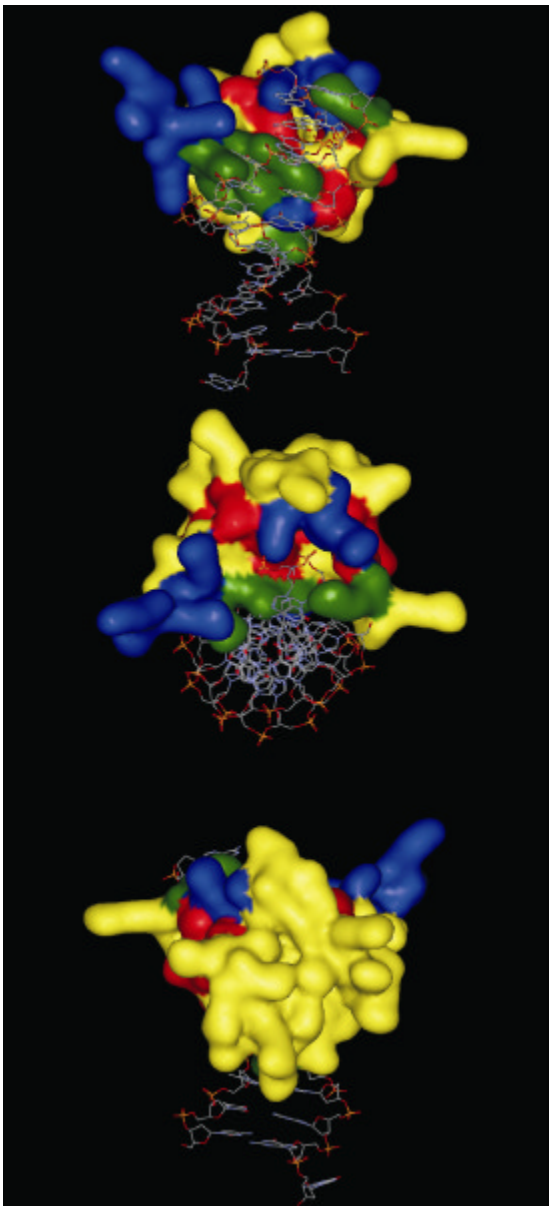


Figure 4

Three views of the cocrystal structure as determined by Jacobson *et al*. Color-coding indicates residues that are affected in our NMR study by the minimal Ets-1 interaction domain (red), DNA (green) or

both (blue). It is clear that the interaction domains overlap to a large extent, while the "back" of the protein is not affected by either Ets-1 or DNA.

Overall, the amount of chemical shift difference was remarkably proportional to the amount of PRL-4P added, which indicates that there are no sudden structural transitions in DNA binding by the POUhd. However, the residues R271, V272 and K273 at the end of the DNA binding helix (α-3) show a significant difference in chemical shift in the DNA titration. These residues do not contact DNA in the crystal structure, but instead form the dimidiation interface for the POU-specific domain of the other monomer. Dimer formation causes a slight unraveling of the C-terminus of the DNA binding helix. Given the relative concentration of POUhd and DNA in our NMR study and the EMSA analysis, it is likely that the POUhd will dimerize on the DNA. However, we presume that in the absence of a POU-specific domain, a bound POUhd monomer will not make protein-protein contacts with the other monomer. Thus, in the absence of a POU-specific domain to interface with, the residues from the dimidiation interface may extend the DNA binding helix upon the addition of DNA, which could explain the changes in chemical shift.

Almost identical results were obtained with the DNA titration of a preformed POUhd / Ets-1 (235-304) complex. The resonances from the preformed complex rapidly shifted to the DNA-bound form with the addition of PRL-4P. Small differences between the POUhd / DNA and the POUhd / Ets-1 / DNA spectra can be explained by the presence of a small population of POUhd / Ets-1 complex in the latter spectrum. The absence of any major difference between these titrations is in agreement with the EMSA data and confirms that the relative affinity of the POUhd for DNA is much higher than for Ets-1 (235-304).

Figure 4 shows the crystal structure of the POUhd, with the residues that are involved in DNA binding and Ets-1 binding color-coded. It is clear from this figure that there is significant overlap between the interaction interfaces for DNA binding and Ets-1 interaction. Our data indicates that amino acids on the POUhd that are not involved in DNA binding, but are contacted by Ets-1 (235-304)

(like for instance L288, which is not show in the crystal structure) are insufficient to retain Ets-1 in this assay.

Since the Ets-1 peptide used in this study does not contain the ETS DNA-binding domain, our study does not preclude ternary complex formation with the full-length proteins. Such a ternary complex would not necessarily use the same contacts as seen with the interaction of the minimal interacting domains. It is unknown at present whether the full-length proteins affect each other's DNA binding affinities. This is the case with the interactions between Pit-1 and GATA-2 [162], where binding of Pit-1 inhibits DNA binding by GATA-2, unless the GATA-2 recognition site has a neighboring Pit-1 binding site. DNA binding by Ets-1 can be modulated by interaction with other factors like Pax-5 [222] and AML1/CBFα2 [172,173]. The latter interaction relieves auto-inhibition of Ets-1 DNA binding by binding the auto-inhibitory region in exon VII (238-328) of Ets-1. Since this region partially overlaps with the interaction domain for Pit-1 (190-257), it is possible that Pit-1 exerts a similar effect as AML1/CBFα2, relieving auto-inhibition of Ets-1 DNA-binding. Future study could address these issues by EMSA analysis using the full-length Pit-1 and Ets-1 proteins and structure determination of the full-length proteins bound to the PRL-4P composite element by crystallography.

4.6 Acknowledgements

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“Wat wil je hier nou mee?!…”

Arjan Brenkman

Chapter 5

Study on the interaction of E1A 13S and Pit-1.

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5.1 Abstract

The POU-domain transcription factor Pit-1 is involved in the selective expression of the GH, PRL and TSH-β genes in the anterior pituitary. In order to modulate its transactivation properties, Pit-1 interacts with a number of other transcription factors. Here we report a novel interaction of the transcription activation domain of Pit-1 with the 13S splice variant of the adenoviral immediate early gene product E1A. This interaction was dependent on the structure of the zinc-finger domain of conserved region 3. Co-transfection of E1A 13S, but not E1A 12S, with Pit-1 led to a decrease in Pit-1 mediated activation of both the GH- and PRL-promoters, indicating that a functional interaction may occur *in vivo*.

5.2 Introduction

In order to regulate activation of its target promoters, Pit-1 functionally interacts with a number of other transcription regulators. As described in the general introduction, a number of factors also physically associate with Pit-1 to regulate its function. This physical interaction is used to recruit key transcriptional regulators such as CBP and N-CoR to a Pit-1 responsive promoter. The adenovirus immediate early gene product E1A is a transcription cofactor that is expressed upon infection of a host cell ([223] reviewed in [224]). Like Pit-1, E1A can also interact with CBP/p300 [76]. Additional interactions include a large number of key transcriptional regulators such as the SWI/SNF [225] and Mediator complexes [41]. Furthermore, E1A can interfere with the host cell cycle by interacting with cellular factors such as the retinoblastoma protein (RB) [226]. Through these interactions E1A is able to prepare the cell for viral replication. Two different splice variants of E1A are known (Fig. 1), which differ in the insertion of the 48-amino acid Conserved Region-3 (CR-3) domain. CR-3 contains a zinc-finger based activation domain, which can activate transcription by contacting important factors such as TBP [227,228], CBP [76,229], nuclear receptors [230,231] and several TAFs [232,233].

Here we report an *in vitro* interaction of the CR-3 activation domain with the activation domain of Pit-1. Furthermore, we show

that in contrast to E1A 12S, the presence of E1A 13S represses Pit-1 mediated activation of the GH and PRL promoters in transient transfections. This repression is shown to be at least in part dependent on the structure of the CR-3 domain of E1A 13S.

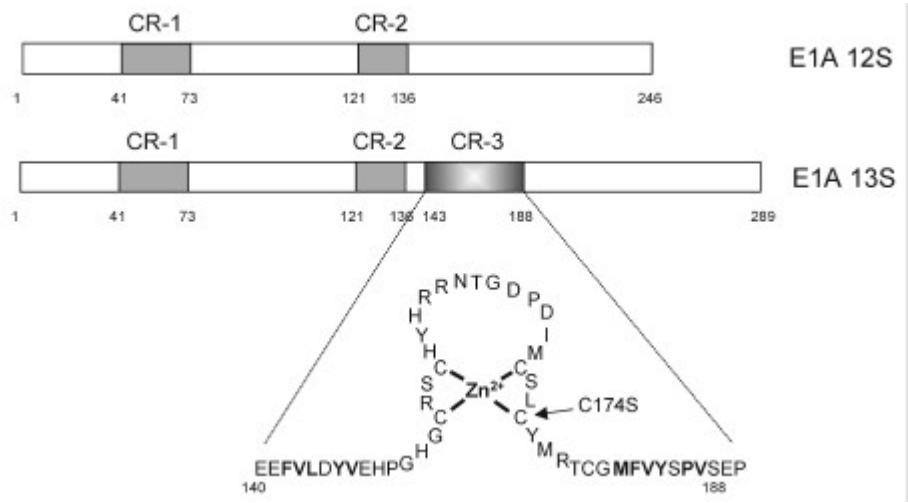

Figure 1

Two splice variants of E1A differ in the insertion of the CR-3 domain (highlighted). The CR-3 domain contains a zinc finger and two hydrophobic patches (shown in bold). The location of the C174S mutation used in this study is indicated.

5.3 Materials and Methods

5.3.1 Plasmids

The expression plasmids for the GST-fusions of the SP1 (140-250), VP16 (411-490), Oct-1 (175-269), Retinoic acid receptor AF1 (1-76) and the E1A CR-3 (142-188) activation domains were kindly provided by Dr. G. Folkers. For the transient transfections we used the previously described cytomegalovirus (CMV) promoter driven Pit-1 construct, GH and PRL-luciferase reporter constructs, Rous sarcoma virus (RSV) driven β-galactosidase and empty CMV-vector [234].

RSV driven E1A 12S, 13S and C174S E1A 13S were also kindly provided by Dr. G. Folkers. All plasmids were transformed in E.coli DH5α and grown overnight at 37°C in LB medium containing 50 μg/ml of ampicillin. Cells were harvested and plasmid DNA was extracted using a Jetstar maxiprep kit (Genomed). Concentration and purity of all DNA was determined by measuring absorption at 260/280 nm.

5.3.2 GST-pulldowns

Glutathione agarose beads equilibrated with binding buffer (50 mM TRIS pH 8.0, 100-2000 mM KCl, 20% glycerol, 1 mM DTT, 1 mM EDTA, 0.01% Triton-X100) were loaded with 0.5 ml of crude lysate (in 50 mM TRIS pH 8.0, 500 mM KCl, 10% glycerol, 1 mM DTT, 1 mM EDTA, 0.01% Triton-X100) from GST-fusion expressing E.coli BL21 by rotating for 1.5 hour at 4°C. After three washing steps with binding buffer, the beads were incubated with the target protein in 500 μl end volume for 1 hour at 4°C. After three more washing steps, bound proteins were boiled off the beads in sample buffer and detected by SDS-PAGE.

5.3.3 Cell culture and transient transfections

The human osteogenic sarcoma cells U-2 OS were grown in Dulbecco's modified Eagle medium (DMEM, Life Technologies, Enc), supplemented with 10% fetal calf serum, 3.5 μmol L-glutamine, 100 u penicillin and 100 μg/ml streptomycin. For transfection $9 \cdot 10^6$ cells were plated in six well plates at day 0. At day 1 (approximately 25% confluency) cells were transfected by calcium-phosphate co-precipitation. Each well was transfected with 3 μg reporter plasmid and 500 ng RSV-LacZ to normalize for transfection efficiency. The total amount of DNA was complemented to 4.375 μg by the addition of empty CMV vector. DNA was added to 125 μl 2X HBS pH 7.05 (40 mM HEPES, 274 mM NaCl, 10 mM KCl, 1.4 mM $Na_2HPO_4 \cdot 2H_2O$). Under vortexing 125 μl 0.25 M $CaCl_2$ was added to obtain a fine precipitate. This precipitate was added to the cells, 17 hours after which the medium was refreshed. Cells were harvested at 31 hours after this. The cells were washed with PBS and 300 μl of lysisbuffer (25 mM TRIS-PO_4, 15% glycerol, 1% Triton-X100) was added per well. After 10 min incubation on ice, the cells were scraped

from the wells and lysates were collected in eppendorf tubes. After centrifugation (3 min, 4°C, 14,000 rpm), the supernatant was stored at -80°C until luciferase and β-galactosidase assays were performed. Luciferase and β-galactosidase assays were done as described previously [234]. Luciferase values were divided by the β-galactosidase activity to correct for transfection activity. All transfections were performed in duplicate in at least two separate experiments.

5.4 Results

5.4.1 The Pit-1 TAD, but not the POU-domain, associates with the E1A-13S CR-3 Zn-finger.

Most proteins that physically associate with Pit-1 do so by contacting the POU DNA binding domain. In order to identify factors that would interact with the Pit-1 TAD, we screened a number of GST-immobilized factors for their ability to pull down purified Pit-1 TAD (residues 48-129) at different salt concentrations. During this screening, we found that the CR-3 domain of the immediate early gene product of adenovirus (E1A) interacted with the Pit-1 TAD at high salt concentration (Fig. 2a). Titration of the salt concentration showed that the interaction remained stable up to 2 M of KCl, although the optimal concentration was around 500 mM. From this we conclude that the interaction likely includes a number of hydrophobic contacts. Addition of 10 mM EDTA completely blocked the interaction (Fig. 2b). Removal of the coordinated zinc ion from the zinc finger by EDTA is likely to disrupt the overall fold of the CR-3 domain. Therefore, the structure of the CR-3 zinc finger is an important determinant for binding of the Pit-1 TAD. In contrast, GST-immobilized E1A CR-3 was unable to pull down any significant amount of the Pit-1 POU-domain at any of the salt concentrations tested (Fig. 2c).

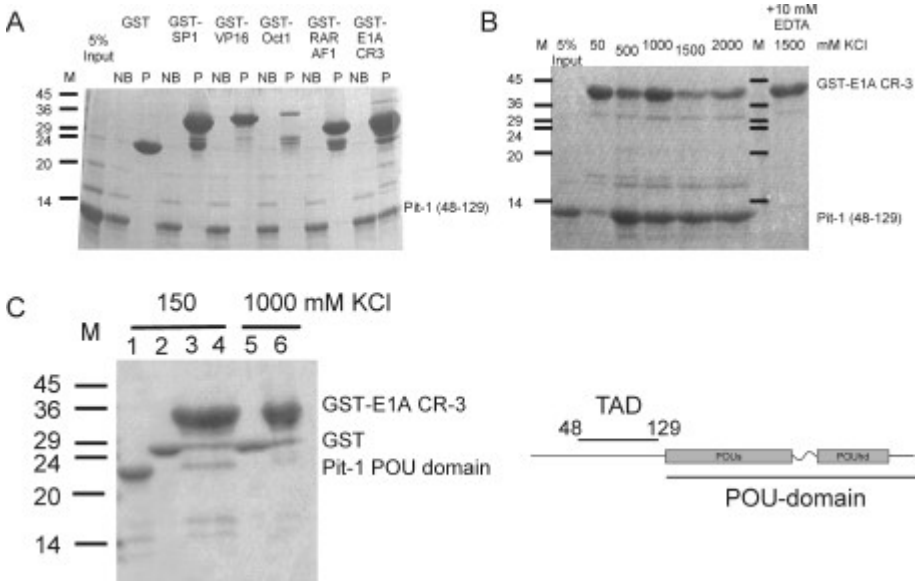

Figure 2

A) Coomassie stained gel of the screening of different activation domains for binding to the Pit-1 TAD at 1 M KCl. Molecular size marker and 5% input lanes are indicated. Not bound (NB) lanes show samples taken after the incubation of the fusion proteins with the Pit-1 TAD, before the first wash. Pellet (P) lanes show all bound proteins after the final wash step. Except GST-E1A CR-3, none of the fusions retained any Pit-1 TAD at these conditions.
B) Interaction of E1A CR-3 domain with the Pit-1 TAD required at least 500 mM KCl and remained stable up to 2 M of KCl. The addition of 10 mM EDTA completely blocked association at 1500 mM of KCl.
C) The POU domain of Pit-1 was not retained by GST-E1A CR-3. Lane 1 shows 10% of the input Pit-1 POU-domain, lane 2 shows background binding to GST loaded beads. Binding of the POU-domain by GST-E1A CR-3 at 150 mM KCl was minimal both in the absence (lane 3) and presence (lane 4) of 10 mM EDTA. Lanes 5 and 6 show background binding to GST loaded beads and binding of the POU-domain by GST-E1A CR-3 at 1000 mM KCl.

5.4.2 E1A 13S represses transcription activation by Pit-1 on GH and PRL promoters.

Since the interaction between the Pit-1 TAD and the E1A 13S CR-3 region had not been reported previously, it was not known whether association of E1A 13S would in any way affect Pit-1 function *in vivo*. In order to investigate this issue, we tested the influence of E1A 13S on Pit-1 activation of the GH and PRL promoters in co-transfection assays in U-2 OS cells, which do not express endogenous Pit-1. As shown in figure 3, Pit-1 is capable of stimulating both the GH and PRL promoters. The amount of Pit-1 expression vector (375 ng) transfected, resulted in an about 4-fold stimulation of the GH promoter. Pit-1 was much more efficient in stimulating the PRL promoter, as the same amount of expression vector resulted in a 145-fold activation. This difference in Pit-1 response likely reflects different coactivator requirements of each promoter. Adenovirus E1A 13S did not affect basal transcription levels of the GH and PRL promoters at any of the amounts transfected. However, in co-transfection with Pit-1 increasing amounts of E1A 13S resulted in a concentration dependent repression of Pit-1 promoter activation. On the GH promoter E1A 13S was able to repress Pit-1 activation to basal level when 500 ng of expression vector was co-transfected. The effect on the PRL promoter is less dramatic with a 1.4 fold repression at 500 ng of E1A 13S, again indicating the difference between the GH and PRL promoters.

5.4.3 CR-3 zinc finger is partially involved in E1A 13S mediated repression of Pit-1 activity.

In order to test whether the CR-3 domain is the part of E1A 13S that mediates the repression of Pit-1 activation, we performed co-transfection assays with E1A 12S. The 12S splice variant of E1A lacks the CR-3 domain and should therefore be affected in its ability to influence Pit-1 mediated activation. Beyond a slight repression of the basal transcription level, E1A 12S hardly affected the GH promoter. In contrast to the repression of Pit-1 activation by E1A 13S on both the PRL and GH promoters, co-transfection of the 12S variant with Pit-1 resulted in a markedly higher (about 1.7 fold at 125 ng of co-transfected E1A 12S) activation of the GH promoter. This activation was lost at higher amounts of co-transfected E1A 12S, probably due to

quenching effects. At 500 ng of co-transfected DNA, E1A 12S did not affect Pit-1 activation, while the same amount of E1A 13S repressed Pit-1 activation almost to background level (compare fig. 3 and fig. 4). Therefore, the repression of Pit-1 mediated GH activation by E1A 13S requires the presence of the CR-3 domain.

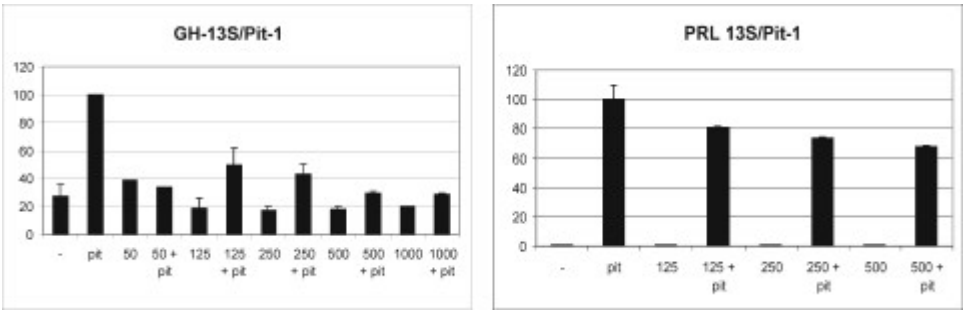

Figure 3

Effects of transfection of E1A 13S and co-transfection with Pit-1 on both GH and PRL promoters. Activation by 375 ng of transfected Pit-1 expression plasmid was normalized at 100%. The - lane shows basal activity of each promoter relative to Pit-1 stimulation. Amounts of E1A 13S are given in ng.

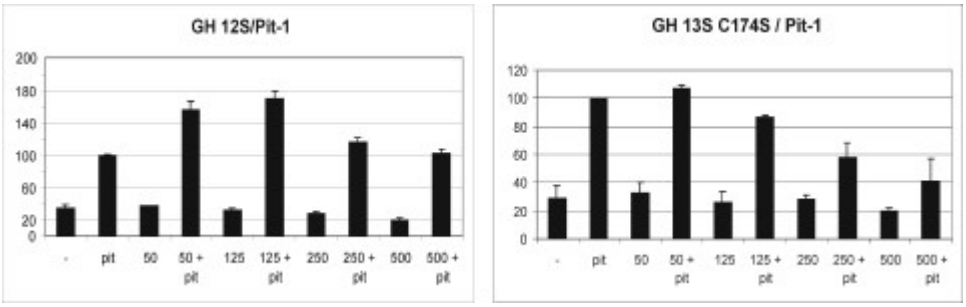

Figure 4

Effects of transfection of E1A 12S or C174S E1A 13S and co-transfection of Pit-1 on the GH promoter. Activation by 375 ng of transfected Pit-1 expression plasmid was normalized at 100%. The - lane shows basal activity of each promoter relative to Pit-1 stimulation. Amounts of E1A 12S and C174S E1A 13S are given in ng.

Our *in vitro* data suggest that the structure of the zinc finger in the CR-3 domain is an essential determinant for binding the Pit-1 TAD. Therefore we tested whether the C174S mutant E1A 13S could still repress Pit-1 activation. Mutating the C-terminal coordinating cysteine to a serine residue should disrupt the structure of the zinc finger. Once again, as shown in figure 4, C174S E1A 13S did not affect basal transcription levels of the GH promoter. Unexpectedly, the 13S mutant was still able to repress Pit-1 activation in a concentration dependent manner albeit with less efficiency than the wild type E1A 13S.

5.5 Discussion

The adenovirus immediate early gene product E1A is a multifunctional protein that can interact with a wide variety of other proteins. Depending on the promoter context and cell type, E1A can both activate and repress transcription [235-237]. Here we report an interaction of the CR-3 of E1A with the activation domain of Pit-1. Of the many physical interactions that have been reported with Pit-1 only three seem to involve the TAD. The Estrogen and Thyroid receptor target a Y6Y6Y motif and a serine/threonine rich region in the TAD respectively [116,117], while P-OTX / Ptx-1 interacts with the entire TAD [148]. These interactions with the TAD of Pit-1 all resulted in synergistic activation of the GH and PRL promoters. Here, co-transfection of E1A 13S with Pit-1 resulted in a repression of Pit-1 activation on both the GH and PRL promoters in U-2 OS cells. This repression was solely mediated by the presence of the CR-3 domain, as E1A 12S (which lacks the CR-3 domain) did not repress GH activation by Pit-1. In fact, for unknown reasons, co-transfection of low amounts of E1A 12S resulted in an increase in GH promoter activity.

The PRL promoter was much more efficiently stimulated by Pit-1 than the GH promoter. This effect was also observed previously in HeLa and 293 cells (chapter II). Since the PRL promoter contains more Pit-1 binding sites than the GH promoter (Fig 4 in chapter I), this effect might simply be related to a more efficient Pit-1 binding by the PRL promoter. Alternatively, the presence of a coactivator like Ets-1, which synergizes with Pit-1 on the PRL, but not on the GH promoter,

might explain the differential response of the GH and PRL promoters. Ets-1 likely is present in U-2 OS, as it is involved in hematopoietic development. In order to circumvent this problem of unknown cofactors, one could perform the same transfections in pituitary cell lines instead of U-2 OS cells, which do not normally express Pit-1.

The presence of a coordinated zinc ion in the CR-3 zinc finger was required for the *in vitro* interaction with the Pit-1 TAD. However, the *in vivo* effect of E1A 13S was only partially dependent on the structural integrity of the zinc finger, as the C174S mutant was also capable of repression, albeit with less efficiency than the wild type. This might reflect an incomplete unfolding of the zinc finger structure by this mutation, compared to a complete unfolding when all zinc is chelated by EDTA. Alternatively, there might be other regions within the CR-3 domain that must cooperate with the zinc finger region to obtain full effectiveness. This is the case with the interaction of E1A 13S with Drosophila $TAF_{II}110$ / human $TAF_{II}135$, where deletions in both the zinc finger and hydrophobic patches in the N- and C-terminal part of the CR-3 domain (Fig. 1) showed diminished affinity [232,233]. As the interaction with the Pit-1 TAD was picked up at high salt concentration (favoring hydrophobic interactions), these hydrophobic patches seem likely interaction surfaces. The CR-3 domain might require an intact zinc finger in order to optimally present this hydrophobic patch to the Pit-1 TAD.

Transcription activation by Pit-1 involves recruitment of the coactivator CBP [46]. Furthermore, it has been shown that CBP can acetylate Pit-1 *in vitro* and that the presence of E1A can block this acetylation [79]. This implies that these three proteins can form a complex and modulate each other's activity. Formation of such a ternary complex would provide an environment where the hydrophobic contacts, which are involved in Pit-1/CR-3 interaction, could actually take place *in vivo*. It is therefore tempting to speculate that, within the context of a ternary complex with CBP, E1A 13S causes an inhibition of the acetylation function of CBP by directly interacting with Pit-1. The 12S splice variant, which does not bind directly to Pit-1, would not be able to modulate the enzymatic activity of CBP in the same way. This model would assume that acetylation of Pit-1 positively regulates its transactivation capability, which is in keeping with the suggested effect of acetylation on Ets-1 binding in

chapter III. In order to verify this model, one could co-transfect wild type or acetylation deficient mutants of CBP, which would counter the repressive effect of E1A 13S on Pit-1 activation. Alternatively, binding of E1A 13S might physically block functional interactions of proteins other than CBP with the Pit-1 TAD. This would result in repression of Pit-1 transactivation function by E1A 13S, but not by 12S.

Since Pit-1 is almost exclusively expressed in the pituitary gland, which is not a likely tissue for adenovirus infections, it seems unlikely that the Pit-1 / E1A interaction reported here occurs in the same manner in cell types that endogenously express Pit-1. There are, however, cellular proteins that can substitute for E1A functions. Such factors were supposed to be responsible for replication of E1A deficient adenovirus in embryonic carcinoma cells [238,239]. One specific example of such an E1A-like factor is the basic helix-loop-helix factor Twist, which can interact with CBP/p300 in an analogous manner to E1A [240]. It is possible that such a factor is involved in the regulation of Pit-1 activity, for instance (given the differential effect of E1A 13S on the GH and PRL promoters) to restrict GH expression in non-somatotropes. Alternatively, the effects of E1A 13S on Pit-1 in U-2 OS cells might reflect the fact that E1A needs to be able to interact with a host of cellular proteins and therefore needs to be rather promiscuous in its contacts.

5.6 Acknowledgements

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Summary / General Discussion

Pit-1 is a pituitary specific transcription factor that plays a central role in the development and maintenance of a number of cell lineages in the anterior pituitary gland. In these cell lineages, Pit-1 is required for the selective expression of the growth hormone (GH), prolactin (PRL) and the β-subunit of the thyroid-stimulating hormone (TSHβ). Pit-1 contains a POU DNA-binding domain, which consist of two independent DNA-binding modules (the POU-specific domain (POUs) and the POU-homeodomain (POUhd)), separated by a flexible linker. Loss of Pit-1 function leads to combined pituitary hormone deficiency (CPHD) syndrome, the main feature of which is dwarfism.

Chapter II of this thesis describes a clinical case study of a CPHD patient carrying two novel mutations (each on a different allele) in both the POUs and the POUhd. One of these mutations, located in the POUs, substitutes one of the hydrophobic core residues with a charged residue, which leads to a misfolded POUs domain that is unable to bind DNA. The other mutation leads to the deletion of the entire DNA-recognition helix from the POUhd. In the context of the full-length POU-domain, neither of these mutants was capable of high-affinity DNA-binding *in vitro* or stimulation of the Pit-1 target promoters *in vivo*. Therefore, the structural integrity of both the POUs and POUhd is required for high-affinity, sequence-specific DNA binding by Pit-1 and this DNA binding is essential for the function of Pit-1 *in vivo*.

The expression of GH, PRL and TSHβ is highly restricted to their respective cell lineage, while Pit-1 is present in all three cell lineages. Therefore, Pit-1 needs to cooperate with other transcription regulators in order to silence or activate its target promoters. The interaction of Pit-1 with two such factors, Ets-1 and GATA-2, is studied in chapter III. Ras-responsive Ets-1 cooperates with Pit-1 to synergistically activate the PRL promoter, which contains composite Ets-1 / Pit-1 recognition sites. However, both proteins can also associate in solution, in absence of DNA. This physical interaction involves the POUhd of Pit-1 and a part of the region III activation domain of Ets-1. Using nuclear magnetic resonance (NMR) monitored protein-protein titrations, we were able to study which residues on the POUhd are likely contacted by Ets-1. The NMR data gathered on the

minimal interaction domains suggests that Ets-1 binds the POUhd through multiple interacting regions, which is in agreement with previous biochemical work.

One of the possible interacting regions on the POUhd mapped by the NMR analysis is the site of regulated phosphorylation. Indeed, introducing a phospho-mimicking group (an amino acid with a pronounced negatively charged side-chain, like an aspartic acid) at the phosphorylation site of the POUhd decreased the binding affinity for Ets-1 by about 30%. This is a remarkable result, as it is the first demonstrated effect of Pit-1 phosphorylation on a protein-protein interaction. For that reason it would be intriguing to study the effect of this phospho-mimicking mutation on the stimulation of not only the PRL, but also the GH and TSHβ promoters in transient transfections. The NMR and biochemical data indicate that, analogous to phosphorylation, regulated acetylation of one particular POUhd lysine residue may affect the binding affinity for Ets-1 by altering the accessibility of another interacting region on the POUhd. This is an interesting observation; given the facts that Pit-1 can be acetylated by CBP *in vitro* and that both Ets-1 and Pit-1 are able to bind CBP. Whether acetylation of the POUhd is of any consequence for Ets-1 binding remains to be determined. One approach would be *in vitro* acetylation of Pit-1, followed by interaction assays and coupled with mass spectrometry to verify whether the suggested residue is actually acetylated. Additionally, the *in vivo* acetylation state of Pit-1 needs to be determined. This may be done by creating a stably transfected pituitary cell line, expressing a HA-tagged Pit-1. Such a tagged version of Pit-1 may be immune-precipitated from a nuclear extract and analyzed by mass spectrometry.

It is as yet unclear exactly how the physical interaction contributes to the functional synergism of Ets-1 and Pit-1. In fact, substitution of the interaction region on Ets-1 with the corresponding region from Ets-2 or the VP-16 activation domain abolished the physical interaction, but not the functional synergism in transient transfections (Dawn Duvall, to be published). This effect might be explained by targeting of both factors to the PRL-promoter through their respective DNA binding domains, which would imply that the physical interaction prior to promoter binding is of no functional relevance. However, our results imply that the physical association of

Pit-1 and Ets-1 is regulated by the post-translational state of Pit-1. If this is the case, than it is likely that at a cellular level the physical interaction presents an additional layer of control for the regulation of PRL expression.

At present, it is unknown whether the physical association of Ets-1 and Pit-1 affects the DNA binding specificity or affinity of either protein. Such effects have been shown for the interactions of Pit-1 with GATA-2 (altered specificity of DNA binding by GATA-2) and Ets-1 with AML1/CBFα2 (increased affinity through alleviation of Ets-1 auto-inhibition of DNA binding). Since the interaction interface on the POUhd for both DNA and Ets-1 binding overlap to a large extent, it would not be surprising if the DNA binding affinity of the POUhd were somehow affected by Ets-1. However, the study described in chapter IV shows that, at least for the minimal interaction domain of Ets-1, this is not the case. This study did not address whether the auto-inhibition of DNA binding by Ets-1 is affected by the interaction with Pit-1. Given the location of the interaction domain on p68 Ets-1 (residues 190-257), just N-terminally one of the two auto-inhibitory modules (residues 280-331), such an effect would not be farfetched. It will be interesting to direct future study towards EMSA analysis using the full-length Pit-1 and Ets-1 proteins and structure determination of the full-length proteins bound to the PRL-4P composite element by crystallography.

Chapter V investigates the functional relevance of a novel interaction of the conserved region 3 (CR-3) of the 13S splice variant of the adenovirus immediate early gene product (E1A 13S) with the N-terminal activation domain of Pit-1. Transient co-transfection of Pit-1 with E1A 13S showed an inhibitory effect of E1A 13S on Pit-1 mediated stimulation of the GH and PRL promoters. This effect could be caused by simple masking of the activation domain of Pit-1, or alternatively might be caused by an inhibition of the acetylation function of CBP by E1A 13S. Such an inhibition of CBP mediated Pit-1 acetylation by E1A has been shown *in vitro*. Therefore, it is tempting to speculate that acetylation by CBP might not only increase Pit-1 binding affinity for Ets-1 (chapter III), but might also enhance Pit-1 transcription activation efficiency in general. Future study should address whether any residues on Pit-1 are acetylated by CBP *in vivo* and if so, to what functional effect. In addition to the approach

mentioned above, this may be done by using acetylation mutants of CBP in co-transfections with Pit-1 and E1A 13S.

Samenvatting / Algemene discussie

Pit-1 is een transcriptiefactor die specifiek in de hypofyse tot expressie komt. Hier speelt Pit-1 een centrale rol in de ontwikkeling en overleving van een aantal hormoonproducerende cellijnen. In deze cellijnen is de aanwezigheid van Pit-1 noodzakelijk voor de selectieve expressie van groeihormoon (GH), prolactine (PRL) en de β-subunit van het thyroid-stimulerend hormoon (TSHβ). Pit-1 bevat een POU DNA-bindend domein, dat bestaat uit twee onafhankelijke DNA-bindende modules (het POU-specifieke domein (POUs) en het POU-homeodomein (POUhd)), die verbonden zijn door een flexibele linker. Verlies van de functie van Pit-1 leidt tot het CPHD syndroom, dat gekenmerkt wordt door deficientie van een aantal van de in de hypofyse geproduceerde hormonen. Het belangrijkste gevolg hiervan is dwerggroei.

Hoofdstuk II van dit proefschrift beschrijft een klinische casus van een CPHD patiënt met twee nieuwe mutaties (elk op een apart allel) in zowel het POUs als het POUhd. Bij één van deze mutaties is een apolair residu dat deel uitmaakt van de hydrofobe binnenkant van het POUs, vervangen voor een geladen residu. Dit heeft een ontvouwing van het POUs tot gevolg, waardoor dit domein niet meer aan DNA kan binden. De andere mutatie leidt tot een deletie van de gehele DNA-herkennings helix van het POUhd. Geen van beide mutanten was, binnen de context van het gehele POU-domein, in staat om met enige affiniteit DNA te binden *in vitro*, of een Pit-1 afhankelijke promoter te activeren *in vivo*. Hieruit kunnen we concluderen, dat de structurele integriteit van zowel het POUs als het POUhd noodzakelijk is voor hoge affiniteit en sequentie-specifieke DNA binding van Pit-1 en dat deze DNA binding essentiëel is voor het functioneren van Pit-1 *in vivo*.

Hoewel Pit-1 in alle drie de cellijnen aanwezig is, blijft de expressie van zowel GH, PRL als TSHβ beperkt tot zijn eigen hormoon producerende cellijn. Hieruit volgt, dat Pit-1 moet samenwerken met andere transcriptieregulerende factoren om de promoters van deze hormonen ofwel te activeren, ofwel te remmen. In hoofdstuk III wordt de interactie bestudeerd tussen Pit-1 en twee van zulke factoren, Ets-1 en GATA-2. Ets-1 is een transcriptiefactor die via de Ras signaaltransductie route geactiveerd kan worden. Samen met Pit-1 zorgt Ets-1 voor een synergistische activatie van de PRL promoter, waarin zich een samengestelde Ets-1 / Pit-1 herkenningssequentie bevindt. Beide eiwitten kunnen ook onafhankelijk van DNA aan elkaar binden. Bij deze fysieke

interactie bindt het POUhd van Pit-1 aan een deel van het activatiedomein van Ets-1. Met behulp van magnetische kernspin resonantie (NMR) werd een eiwit-eiwit titratie van het POUhd met het interactie domein van Ets-1 gevolgd. Hierbij werd onderzocht welke residuen van het POUhd betrokken zijn bij de interactie met Ets-1. De NMR gegevens wijzen erop dat meer dan één gebied aan de oppervlakte van het POUhd betrokken is bij de binding van het minimale interactiedomein van Ets-1, wat overeenkomt met eerdere biochemische data.

De plaats waar één van de mogelijke contactpunten tussen Ets-1 en het POUhd zich bevindt, kan *in vivo* gefosforyleerd worden. Een Pit-1 mutant, waarbij fosforylering op die plaats wordt nabootst (door het introduceren van een aminozuur met een negatief geladen zijketen, bijvoorbeeld een aspartaat), verliest inderdaad 30% van zijn affiniteit voor Ets-1. Dit is een opzienbarend resultaat, omdat dit het eerste voorbeeld is van een eiwit-eiwit interactie van Pit-1, die waarschijnlijk gereguleerd wordt door fosforylering. Het zou daarom interessant zijn, om het effect van deze mutant op de stimulatie van niet alleen de PRL, maar ook de GH en TSHβ promoters te testen in transiënte transfecties. De NMR en biochemische resultaten wijzen erop dat, analoog aan fosforylering, ook acetylering van een lysine residu in het POUhd de bindingsaffiniteit voor Ets-1 zou kunnen reguleren. Deze acetylering zou namelijk de toegankelijkheid van één van de interactiegebieden voor Ets-1 op het POUhd kunnen verbeteren. Gezien het feit dat Pit-1 *in vitro* geacetyleerd kan worden door CBP en dat zowel Ets-1 als Pit-1 aan CBP kunnen binden, is zo'n regulatie via acetylering niet ondenkbaar. Of acetylering van het POUhd ook werkelijk invloed heeft op Ets-1 binding, moet nog worden vastgesteld. Om dit te testen zou *in vitro* acetylering van Pit-1, gevolgd door interactie assays met Ets-1, gecombineerd moeten worden met massa-spectrometrie om de acetylatie-status van gebonden Pit-1 te bepalen. Daarnaast zou de *in vivo* acetylatie-status van Pit-1 bepaald moeten worden. Een mogelijke benadering hiervoor is een stabiel getransfecteerde cellijn van hypofysecellen te creëren, die Pit-1 met bijvoorbeeld een HA-tag tot expressie brengt. Met behulp van deze 'tag' zou Pit-1 uit deze cellen geï soleerd kunnen worden, om vervolgens met behulp van massa-spectrometrie onderzocht te worden.

Het is op dit moment nog niet duidelijk wat de precieze bijdrage is van de fysieke interactie tussen Ets-1 en Pit-1 aan het functionele synergisme van de twee factoren op de PRL promoter. Sterker nog,

wanneer het gebied van Ets-1, dat verantwoordelijk is voor de binding van Pit-1, wordt vervangen voor het overeenkomstige gebied uit Ets-2 of zelfs het activatie domein van VP-16, wordt er nog wel functioneel synergisme in transfecties waargenomen, terwijl er geen fysieke interactie meer is (Dawn Duvall, nog te publiceren materiaal). Deze resultaten zijn te verklaren, met het feit dat beide factoren nog steeds afzonderlijk de PRL promoter herkennen via hun DNA-bindend domein, wat impliceert dat de fysieke interactie geen functioneel belang heeft. Onze resultaten wijzen er echter op dat de associatie van Pit-1 en Ets-1 gereguleerd wordt door de post-translationele status van Pit-1. In dit licht is het waarschijnlijk dat de fysieke associatie een extra controleniveau creëert voor de regulatie van PRL expressie.

Op dit moment is het nog niet bekend of de fysieke interactie tussen Ets-1 en Pit-1 de DNA-bindingsaffiniteit of -specificiteit van één van beide eiwitten beï nvloedt. Zulke effecten zijn bekend voor de interacties tussen Pit-1 en GATA-2 (veranderde specificiteit van GATA-2) en tussen Ets-1 en AML1/CBFα2 (verhoogde affiniteit van Ets-1, door het opheffen van de auto-inhibitie van DNA-binding van Ets-1). Omdat de interactiegebieden voor Ets-1 en het DNA-bindende deel van het POUhd elkaar grotendeels overlappen, zou het niet verassend zijn als de DNA-bindingsaffiniteit van het POUhd gemoduleerd zou worden door Ets-1. De studie die beschreven staat in hoofdstuk IV toont echter aan dat dit, voor het minimale interactiedomein van Ets-1, niet het geval is. In deze studie wordt niet gekeken naar enig effect van Pit-1 op de auto-inhibitie van DNA-binding van Ets-1. Gezien de lokatie van het Pit-1 interactiedomein op p68-Ets-1 (van residu 190-257), net N-terminaal van een van de twee auto-inhibitie modules (residu 280-331), zou zo'n effect niet vergezocht zijn. Daarom zal het interessant zijn, om bij toekomstige studies (zoals bijvoorbeeld mobiliteits proeven (EMSA) en structuuropheldering m.b.v. kristallografie) de volledige eiwitten te gebruiken.

In hoofdstuk V wordt de functionele relevantie van een nieuw gevonden interactie tussen het activatiedomein van Pit-1 en de conserved region 3 (CR-3) van de 13S splice variant van het adenovirus immediate early gen product (E1A 13S) onderzocht. Transiënte co-transfecties van Pit-1 met E1A 13S lieten een remmend effect zien van E1A 13S op de door Pit-1 geactiveerde GH en PRL promoters. Dit effect zou veroorzaakt kunnen worden door maskering van het activatiedomein van Pit-1 door de binding van E1A 13S. Anderzijds, zou de inhibitie van de

acetyleringsfunctie van CBP door E1A 13S kunnen leiden tot remming van de geteste promoters. E1A is in staat de acetylering van Pit-1 door CBP te remmen *in vitro*. Het is daarom verleidelijk om te speculeren dat acetylering van Pit-1 door CBP niet alleen de bindingsaffiniteit voor Ets-1 zou kunnen doen toenemen (hoofdstuk III), maar ook de algemene efficiëntie van transcriptieactivatie van Pit-1. Toekomstige studie zal moeten uitwijzen of er *in vivo* residuen van Pit-1 geacetyleerd worden door CBP en wat de eventuele functionele consequenties daarvan zijn. Naast de al eerder genoemde benadering, zouden hiervoor mutanten van CBP, die gestoord zijn in hun acetyleringsfunctie, gebruikt kunnen worden in co-transfecties met Pit-1 en E1A 13S.

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Curriculum vitae

Kevin Augustijn werd geboren op 3 februari 1975 te Gouda. In 1993 behaalde hij het gymnasium-β diploma aan het Chr. Lyceum te Gouda. In hetzelfde jaar werd begonnen met de studie scheikunde aan de Rijksuniversiteit Leiden. Tijdens de docteraal fase werd als hoofdvak moleculaire biologie gevolgd aan de afdeling moleculaire genetica, onder leiding van Dr. L. Zulianello, Dr. N. Goosen en Prof. Dr. P. van de Putte. Als bijvaken werden organische chemie en structuuropheldering gevolgd. In 1997 werd het docteraalexamen scheikunde, met de specialisatie biochemie, behaald. Van 1997 tot 2001 was hij werkzaam aan de vakgroep Fysiologische Chemie aan de universiteit Utrecht. Hier werd onder leiding van Prof. Dr. P.C. van der Vliet, in nauwe samenwerking met de NMR vakgroep van de universiteit Utrecht onder leiding van Prof. Dr. R. Kaptein, het in dit proefschrift beschreven onderzoek verricht.

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After presenting a seminar at the NMR facility, Lawrence McIntosh suggested I got in touch with the competition. What happened next is described in chapter three of this thesis. Arthur and Dawn, thanks for the excellent cooperation and your hospitality during my visit to Denver.

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