Cooperation between the bromodomain and PHD finger of p300 in nucleosome interaction

A study of the contribution of the PHD finger

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TAKK

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CONTENTS

SUMMARY

The different cells in an organism need to know which genes to be active and which to be inactive. A central mechanism that contributes to regulate gene expression is the organisation of DNA into dense and less dense forms. This form of *epigenetic gene regulation* can be brought about by the attachment of epigenetic marks to chromatin. Epigenetic marks can be copied to daughter cells. Some of these marks are also inherited, through germ cells, to a new generation. Proteins exist that can recognize the epigenetic marks. A protein domain present in several proteins implicated in chromatin mediated gene regulation is the PHD finger, but so far, no such evidence has been provided confirming that the PHD finger can actually interact with chromatin. The PHD finger often occurs next to one or more other domains, some of which have known chromosome-binding activities. In the cofactor p300, the PHD finger occurs next to a bromodomain. The region of p300 including both of these domains (p300BP) is found to bind to acetylated mononucleosomes *in vitro* and that the PHD finger is essential for the interaction. In this study, the relation between the domains was investigated to examine the contribution of the PHD finger. Recombinant proteins were made by swapping the PHD finger in p300BP with a PHD finger from a heterologous protein, but no nucleosome interaction was detected with the recombinant proteins. p300BP, and probably also the protein p300, therefore seems to be dependent on a specific interaction between its bromodomain and PHD finger to give a functional protein able to interact with nucleosomes.

POPULÆRVITENSKAPELIG SAMMENDRAG

Cellene til alle levende organismer inneholder DNA. I noen posisjoner av DNA finnes områder som kan, ved hjelp av blant annet mange ulike enzymer, utrykke et protein. Et slikt område kalles et gen. DNA kan beskrives som et alfabet som består av fire ulike bokstaver, der et ord på tre bokstaver utgjør en kode som passer til en gitt aminosyre. Rekkefølgen av bokstavene i DNA bestemmer rekkefølgen på de korresponderende aminosyrene. Et gen fungerer altså som en oppskrift på ulike typer proteiner. Når en organisme utvikler seg, blir cellene mer og mer spesialisert. Til tross for ulike funksjoner og utseende, beholder cellene det samme DNA. En av grunnene til at cellene blir forskjellige er at noen gener blir skrudd av mens andre blir skrudd på. Dette mønsteret blir deretter arvet til dattercellene.

DNA er kveilet rundt spesielle proteiner, og én slik kveil med protein i midten kalles et nukleosom. Nukleosomene ligger etter hverandre som perler på en snor, og danner kromatin, som er den formen DNA opptar i cellene. Ulike merker kan festes på kromatinet, merker som forteller om genene skal være av eller på. Flere ulike proteiner er vist å kunne lese og tolke disse merkene. Et protein kan bestå av flere områder som har særegne funksjoner. Disse områdene kalles domener. Et eksempel på et domene er PHD fingeren. Det er mulig at dette domenet deltar i å lese merkene som er festet på kromatinet, men så langt har ingen klart å bevise dette. Et protein som heter p300 inneholder en slik PHD finger. PHD fingeren sitter i dette proteinet ved siden av et annet domene, bromodomenet. Området av p300 som inneholder disse to domenene kan binde til nukleosomer. Det er mulig at de to domenene samarbeider for å få til denne bindingen. For å undersøke dette nærmere laget jeg proteiner der PHD fingeren fra p300 var byttet ut med en PHD finger fra ett av tre andre proteiner. Evnen til å binde til nukleosomer ble deretter testet for disse proteinene. Jeg fant ut at det ikke var mulig å bytte ut PHD fingeren i p300 med andre PHD fingre uten at evnen til å binde til nukleosomer forsvant. Det er derfor sannsynlig at det er en interaksjon mellom bromodomenet og PHD fingeren i p300 som er spesifikk for dette proteinet. Dette kan bety at PHD fingeren samarbeider med bromodomenet og på denne måten bidrar til å lese et merke som er festet til kromatinet.

ABBREVIATIONS

A	
ADA	Adaptor
ASH1	Absent, small or homeotic discs
B	
BAH	Bromo-adjacent homology
Brm	Brahma
C	
CAF	Chromatin associated factor
CBP	CREB binding protein
CHD	Chromo-ATPase/helicase-DNA-binding domain
CHRAC	Chromatin accessibility complexes
CREB	cAMP response element
Chromo	Chromatin organisation modifier
E	
ESC	Extra sex combs
E. coli	*Escherichia coli*
E(Z)	Enhancer of zeste
F	
FYVE	Fad 1 p, YOTB, Vac 1 p, EEAI
H	
HAT	Histone acetyl transferase
HDAC	Histone deacetylase
HRX	Human trithorax
HMG	High mobility group
HP1	Heterochromatin protein 1
I	
In prep	In preparation
ISWI	Imitation switch
M	
MLL	Mixed lineage leukemia
MWS	Molecular weight standard
N	
N-CoR	Nuclear receptor Co-repressor
NoRC	Nucleolar remodelling complex
NuRD	Nucleosome remodelling deacetylase
NuRF	Nucleosome remodelling factor
P	
Pc	Polycomb
PcG	Polycomb group
Pcl	Polycomblike
PCAF	p300/CBP associated factor
PCNA	Proliferating cell nuclear antigen
Pers. comm.	Personal communication
ph	polyhomeotic
PHD	Plant homeo domain
PEV	Position effect variegation
Psc	Posterior sex comb
R	
RbAp	Retinoblastoma associated protein
S	
SET	SUV39, E(Z), TRX
Sir	Silent information regulator
SNF	sucrose non-fermenting
SUV	Suppressor of variegation
SWI	Switching mating type
T	
TIP5	TTF-I interacting protein 5
TTF-I	Trancription termination factor I
TRX	Trithorax
trxG	Trithorax group
U	
UBF	Upstream binding factor
W	
WSTF	Williams syndrome transcription factor
WD	Tryptophan (W)-aspartic acid (D)

INTRODUCTION

1.1 Differential gene expression and chromatin structure

In all living organisms, a new generation starts with a single cell, the zygote. This cell is a totipotent stem cell, meaning it has the capacity to develop into all other kinds of cells made at later stages of development. The DNA will therefore, with a few exceptions, be identical in all the cells of the organism. Despite this genetic equality, the cells in multicellular organisms have a wide range of distinct characteristics. One explanation for this diversity is that different genes are expressed in the different kinds of cells, which gives the cells distinct functions and morphology (Figure 1.1).

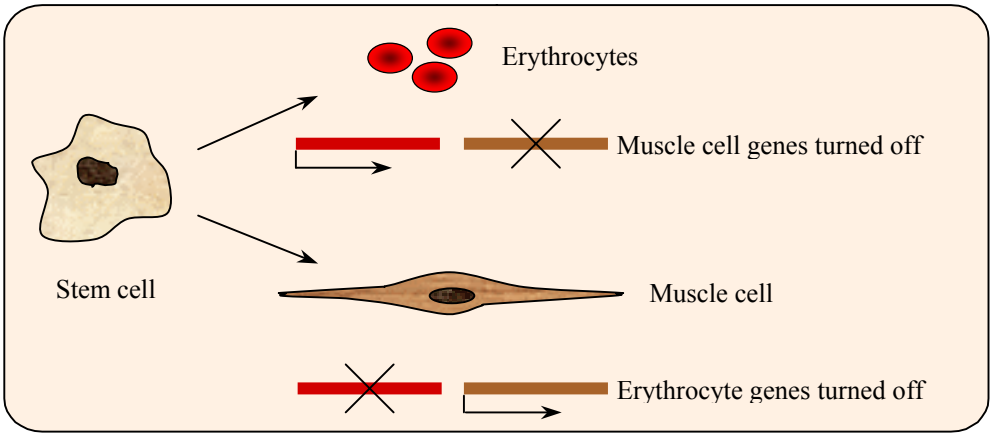


Figure 1.1 Differential gene expression and cell development. One stem cell is the origin of other cells developing in an organism. Genes are turned on and off as the cells differentiate.

What is it that determines if a gene is turned on or off, and how is the expression status for each gene maintained? Stable gene regulation is the result of a number of complex mechanisms, many which are not well understood. To understand how these mechanisms work, it is important to know the organisation of the DNA in the cell. In most organisms the DNA is packed into several chromosomes, 23 in humans, that are present in two copies in diploid cells. In eukaryotes, the chromosomes are located in the cell nucleus.

1.1.1 Nucleosome structure

It was proposed in 1974 that eukaryotic chromosomes consist of a repeating unit containing DNA and protein. This unit was named the nucleosome (Kornberg, 1974). The nucleosome consists of 147 base pairs of DNA wrapped around a core of eight proteins, the core histones; H2A, H2B, H3 and H4, each in two copies (Figure 1.2). A linker histone, such as H1, can stabilize the nucleosomes and help to establish higher-order structures characteristic of chromatin (see Section 1.1.2). Several groups contributed to solving the structure of the nucleosome (Arents et al., 1991; Luger et al., 1997; Richmond et al., 1984), revealing its molecular composition in atomic detail. Each core histone forms a helical region that constitutes the histone fold, which interacts with DNA. The histone fold comprises about 75 % of the amino acid content of the histone and forms the interior core

of the nucleosome particle. The remaining amino-terminal part of the histone protein contains a basic and flexible tail region that protrudes from the nucleosome. Both the histone fold region and the N-terminal region are highly conserved across eukaryotic species.

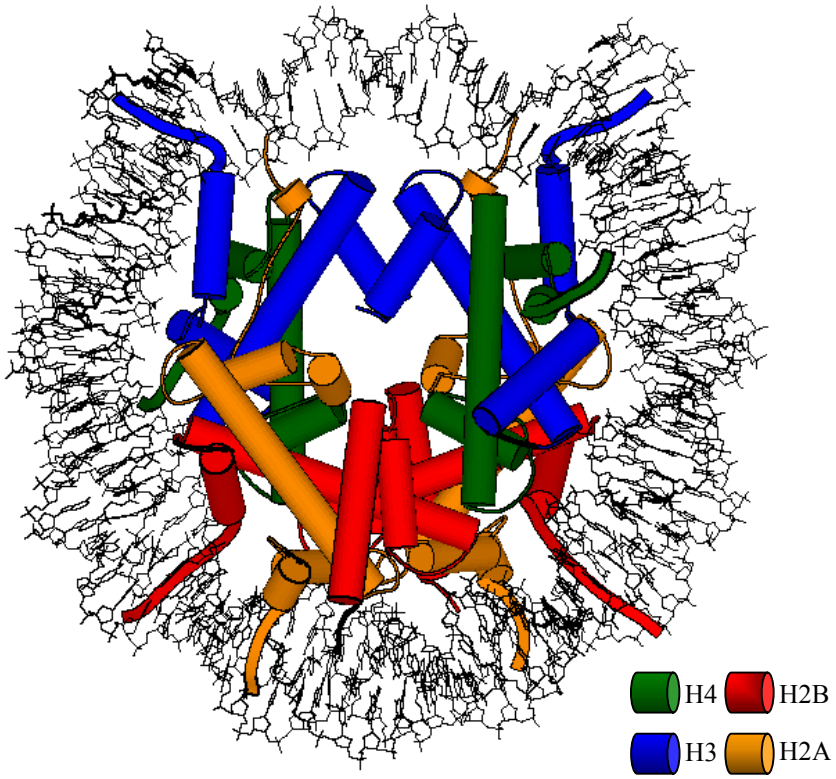


Figure 1.2 Structure of the nucleosome. Model of the molecular structure of the nucleosome (Luger et al., 1997). The different colours represent the different histones, H2A, H2B, H3 and H4, and each histone exists in two copies. The ends pointing outwards are the N-terminal tails of the histones (only parts of the tails are visible in the model). The DNA helix is coiled around the histones and is shown in black (Figure provided by Rein Aasland).

Several side chains on the N-terminal tails are subject to one or more post-translational modifications, including acetylation, methylation, ADP-ribosylation, phosphorylation and ubiquitylation. An overview of selected histone modifications is given in Table 1.1. Only the histone modifications acetylation and methylation are further discussed (Section 1.2.2).

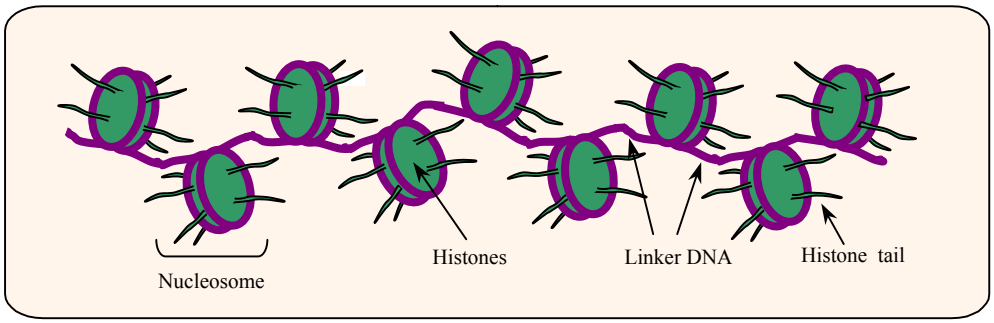
Table 1.1 Selected histone modifications [a]

Modification	**Occurs on**	**Enzyme adding**	**Enzyme removing**	**Associated protein module** [b]	**Function of the modification** [c]
Acetylation	**Lysine**	**HAT**	**HDAC**	**Bromodomain**	**Mainly activation**
Examples	H3:K9 H3:K8/16	Gcn5, p300/CBP, $TAF_{II}250$, PCAF	RPD3/HDAC1	Gcn5, p300/CBP, $TAF_{II}250$, P/CAF	
Further reading	(Kuo et al., 1996)	(Marmorstein and Roth, 2001; Roth et al., 2001)	(Thiagalingam et al., 2003)	(Haynes et al., 1992)	(Struhl, 1998)
Methylation	**Lysine Arginin**	**HMT**	**Unknown**	**Chromodomain**	**Activation and repression**
Examples	H3:K4, K9, K27 H4:K20	SUV39	-	HP1, PC, RBP1, SUV39, CHD, MOF, MSL-3, dMi2	
Further reading	(Morales and Richard-Foy, 2000)	(Peters et al., 2001)		(Eissenberg, 2001; Jones et al., 2000)	(Jenuwein and Allis, 2001; Zhang and Reinberg, 2001)
Phosphorylation	**Serin Threonin**	**Kinases**	**Phosphatases**	**Unknown**	**Activation and repression**
Examples	H3:S10 H3:T11	Dlk/ZIP The aurora family CDKs	Glc7/PP1	-	
Further reading	(Hendzel et al., 1997)	(Preuss et al., 2003)	(Hsu et al., 2000)		(Davie et al., 1999)
Ubiquitinylation	**Lysine**	**E1+E2+ligase E3**	**Ubiquitin-processing protease?**	**Unknown**	**Activation *and* repression proposed**
Examples	H2A:K120 H2B:K120	Rad6b/Ubc2p Cdc34p/Ubc3p	UbpM	-	
Further reading	(Belz et al., 2002)	(Jason et al., 2002)	(Jason et al., 2002)		(Jason et al., 2002; Moore et al., 2002)

[a] The table does not give a complete picture of histone modifications but provides some examples.
[b] The examples of proteins mentioned under the associated protein module are proteins *containing* the respective domain that do not necessarily have a nucleosome binding function.
c New functions for a certain modification is continuously being elucidated. In addition, the function can be altered when combined with one or more other modifications.

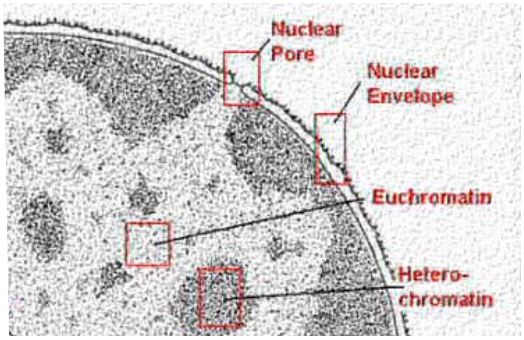
1.1.2 Chromatin structure

The nucleosomes eventually contribute to form DNA into a structure called chromatin. Chromatin is composed of nucleosomes following after one another as 'beads on a string' (Figure 1.3).



Figur 1.3 The repeating unit, the nucleosome, forms chromatin. The purple string represents DNA whereas the green core represents the histones. The N-terminal tails of the histones can be seen protruding from the nucleosomes.

Chromatin can exist in two cytologically very different forms, a condensed form and a decondensed form. The condensed form, called heterochromatin, is so compact that transcription is most likely not possible since there is no space for RNA polymerase or transcription factors to bind. The decondensed form, named euchromatin, is a form that is generally open for transcriptional activation (Figure 1.4). An interesting issue is that in regions of euchromatin, heterochromatin-like structures can be formed, making inactive genes in regions of active ones. This leads to the important question: how is chromatin modulated for the regulation of transcription of specific genes? One answer may be that certain proteins specifically modulates chromatin for gene activation, gene silencing or chromosome condensation, reviewed in (Marmorstein, 2001).



Figur 1.4 Structure of chromatin. The picture shows the organisation of DNA into euchromatin and heterochromatin in the nucleus of the cell (http://cellbio.utmb.edu/cellbio/nucleus.htm).

1.2 Epigenetic gene regulation

A dense form of chromatin makes it less accessible to transcriptional activators, and a hypothesis is that converting a part of chromatin into a dense from contributes to silencing the genes in this area (Turner, 1993). A number of phenomena that are caused by a change in the condensation state of chromatin have been discovered. Examples of such mechanisms are given in Section 1.3. A change in the condensation state can be brought about by several mechanisms. In addition to the already mentioned **histone modifications,** the most extensively studied mechanism is **DNA-methylation.** The modifications contribute to a form of gene regulation named *epigenetic gene regulation*, which is defined by (Russo et al., 1996) as; " Mitotically and / or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence." The modifications printed in chromatin can thus serve as epigenetic marks.

1.2.1 DNA methylation

In mammals, the methylation of DNA is a post replicative modification that occurs mainly on cytosines of the dinucleotide sequence CpG. DNA methylation in non-embyonic cells is distributed on about 80% of the CpG dinucleotides. An exception to the methylation of CpGs is the so-called CpG islands. These are short clusters of CpGs, proposed to function as promoters or replication origins that avoid methylation by a yet unknown mechanism. What *is* known, however, is that when the CpG islands are accidentally methylated, this can cause silencing of adjacent genes, a process that can contribute to cancer, reviewed in (Jaenisch and Bird, 2003; Jones and Takai, 2001). The pattern and degree of DNA methylation changes dramatically during the development of an organism. It has been reported that in mammals, shortly after fertilisation, the entire genome is demethylated, 'resetting' this epigenetic mark (Davis et al., 2000; Mayer et al., 2000; Oswald et al., 2000). When the fertilized egg attaches to the uterine wall, the genome is gradually methylated *de novo*, resulting in new, individual epigenetic marks. After this, *de novo* methylation decreases during differentiation and is, except for a few cases, rare after full differentiation (Ehrlich et al., 1982).

Methylation of DNA is an important contributor to stable gene expression patterns, and together with chromatin modifying enzymes it can contribute to maintain the silent state of chromatin (Urnov and Wolffe, 2001; Wolffe and Matzke, 1999). The methyl group is attached to DNA by DNA methyl transferases (DNMTs), several of which have been identified. Some of the DNMTs have roles in the maintenance of the methylation pattern after cell divisions and some function in *de novo* methylation. DNA methylation has mainly a negative effect on the transcription rate and causes repression for three main reasons: (a) several *transcriptional repressors* can bind to methylated DNA (Hendrich and Bird, 1998; Lewis et al., 1992; Prokhortchouk et al., 2001), (b) proteins that bind to DNA, such as transcription factors, can be excluded (Tate and Bird, 1993; Watt and Molloy, 1988) and (c) DNMTs can be recruited to histone deacetylases (HDACs) and cause *de novo*

methylation in regions already silenced by co-repressors. This can result in a permanently silenced gene (Bachman et al., 2001; Fuks et al., 2001). Even though normally linked to gene silencing, an example of DNA methylation resulting in gene activation is the exclusion of a transcriptional repressor, CTCF, resulting in an active gene (Hark et al., 2000; Ohlsson et al., 2001).

1.2.2 Histone modifications

As mentioned above, the N-terminal histone tails can be modified in several different ways. Here, I will discuss only **acetylation** and **methylation** (see Table.1.1) since these are considered to be the most common modifications.

Histone acetylation is the post-translational histone modification that has been most extensively studied. This modification occurs on lysine residues, particularly on histones H3 and H4. It is a transient, short term regulation that occurs at a low level throughout much of the genome (primarily in euchromatin), resulting from a balance between the activities of histone acetyl transferases (HATs) and histone deacetylases (HDACs) (Vogelauer et al., 2000). In the absence of histone acetylation, the basic histone tails are firmly associated with the acidic DNA backbone. This firm association can prevent transcriptional activators and polymerases getting access to DNA. Histone acetylation can neutralize the charge of the N-terminal histone tails and relieve the association between histones and DNA. The relieved association would in turn ease the access to DNA for transcriptional activators and polymerases (Hansen et al., 1998; Wolffe and Hayes, 1999). A more recent report suggests an alternative way for histone acetylation to alter the conformation of chromatin; by causing an increase of the α-helical content of the histone tails (Wang et al., 2000). A link between DNA methylation and histone acetylation has been proposed. It is shown that DNA methylation can lower the level of histone acetylation (Eden et al., 1998). This connection is probably caused by the ability of the methyl-binding protein MeCP2 to recruit HDACs (Jones et al., 1998; Nan et al., 1998). A protein domain called the bromodomain has been shown to be able to bind to acetylated lysines and it can contribute to epigenetic gene regulation (Haynes et al., 1992) (see section 1.4.1).

Histone methylation occurs on arginine and lysine residues. Histone methylation is associated with both activation and repression of genes; methylation of Lys4 on H3 has been linked to transcriptionally active genes, whereas methylation of Lys9 on H3 has been associated with transcriptional silencing (Jenuwein and Allis, 2001; Zhang and Reinberg, 2001). Several histone methyl transferases (HMTases) have been identified, most of them containing the SET domain (named for its presence in SUV39, E(Z) and TRX, members of the PEV group, the PcG and the trxG of genes in *Drosophila* respectively, see section 1.3.1 and 1.3.2).

The SET domain was first recognized as a conserved pattern in a few chromatin-associated proteins (Tschiersch et al., 1994). Later observation of homology between the SET domain and a plant enzyme led to the characterization of a number of SET domain containing proteins also as enzymes; namely HMTases (Rea et al., 2000). The SET domain is now identified in several hundred proteins (Schultz et al., 2000). Histone methylation is, in contrast to the more transient histone acetylation, a stable and long term modification, and no histone demethylases have yet been identified. Analyses have indicated that there is a link between histone methylation and DNA methylation through heterochromatin protein 1 (HP1). HP1 binds specifically to methylated Lys9 on H3 *in vitro* and recruits a DNA methyl transferase (Jackson et al., 2002; Tamaru and Selker, 2001). HP1 contains a protein domain, the chromodomain (see Table 1.1 and Section 1.4.2) which is able to recognize this histone modification (Nielsen et al., 2002).

1.2.3 The histone code hypothesis

There are several examples that different epigenetic marks are linked to each other, some of which have been mentioned above. The enormous potential of different modification patterns, both on histones and DNA, makes chromatin modification a very complex issue and gives a wide range of possible regulation mechanisms (Turner, 1993). More recently, the different DNA- and histone modifications were proposed to function separately or in combinations as a 'histone code' that may be interpreted in various ways by different proteins (see Section 1.4) (Strahl and Allis, 2000). The idea is that the 'histone code', by being a cause of alteration in the chromatin structure, eventually contributes to epigenetic gene regulation.

1.3 Examples of epigenetic gene regulation

There are a number of interesting and important phenomena linked to epigenetic gene regulation. Below are given a few typical examples of epigenetic regulation and some examples of diseases caused by epigenetic malfunction.

1.3.1 Epigenetic gene regulation in *Drosophila Melanogaster*

The expression state of chromatin in *Drosophila* (*Drosophila melanogaster*) has been found to be altered by the absence of proteins expressed from two groups of genes; the **PcG** (Polycomb group) and the **trxG** (trithorax group) (Eissenberg, 1999; Orlando, 2003; Turner, 2001). The PcG and trxG proteins are involved in the regulation of the homeotic genes, which specify the development of the body segments in most animals. Correct expression patterns are ensured in part by the proteins of the PcG genes. These proteins are involved in chromatin condensation and gene *silencing* of the homeotic genes. The PcG of genes encodes several proteins, such as PC (Polycomb), E(Z) (Enhancer of zeste), PCL (Polycomblike), PSC (Posterior sex combs), ESC (Extra sex combs) and ISWI (Imitation switch). Proteins expressed from the trxG genes counteract the silencing effects of the PcG

proteins to maintain a decondensed form of chromatin, and thus gene *activity*. This group includes proteins like TRX (Trithorax), Brm (Brahma), Zeste and the ASH proteins ASH1 and ASH2 (Absent and small homeotic discs). Homologues of PcG and trxG genes have been identified in a number of other species, including mammals and other vertebrates and it is probable that they exist in all eukaryotic organisms.

1.3.2 Heterochromatic position effect variegation (PEV)

A group of genes related to the trxG and the PcG genes are the **PEV** (Position effect variegation) genes, linked to the epigenetic phenomenon PEV. Examples of proteins in *Drosophila* encoded by these genes is the heterochromatin protein HP1, and the suppressor of variegation proteins, included SUV39 (Brody, 1995; Brown, 1999; Orlando, 2003). PEV was first discovered by H J Muller in 1930. He discovered that the white eye-colour in *Drosophila* varied among the cells of the eye, giving a pattern of red and white. The cause of this variation is explained by a rearrangement of the *white* gene (which is required for a red eye colour) resulting in an inactive gene. The activity of a gene can vary according to its chromosomal position. By different mechanisms, for example chromosomal rearrangement by inversions or translocations, a euchromatic gene can be placed next to a region of heterochromatin, resulting in silencing in regions of originally active genes. The main reason for the silencing is the *cis* spreading of condensed heterochromatin condition past the breakpoint of euchromatin, (Eissenberg, 1999; Turner, 2001; Wakimoto, 1998).

1.3.3 Imprinting and X-inactivation

Genomic imprinting is a phenomenon in which one of a pair of genes is being silenced. The phenomenon is exclusively found in mammals and it involves several mechanisms, including DNA-methylation, histone acetylation and histone methylation. Only a small number of genes are subjected to genomic imprinting (Brown, 1999; Jaenisch, 1997). X-inactivation is a special form of imprinting. In humans, females have two X-chromosomes whereas males have only one. If both of the female X-chromosomes were active, proteins encoded by genes on the X-chromosome would be synthesized at a higher level in females as compared to males. To avoid this, one of the female X-chromosomes is converted to a condensed and transcriptionally silent state of chromatin and is seen in the nucleus as a structure called the Barr body. This condensation occurs through several steps, including DNA methylation, histone modification and the coating of the X-chromosome by *Xist* RNA expressed from the X-chromosome that is to be inactivated. DNA methylation and histone deacetylation is important for maintenance of the inactivated X-chromosome. Only a few short segments containing small clusters of genes remain active, reviewed in (Brown, 1999; Li, 2002).

1.3.4 Epigenetic mechanisms and human disease

There are several examples of malfunction of epigenetic mechanisms leading to disease in human. A few interesting cases are discussed below. Two major reasons for epigenetic malfunction are: (a) mutation in a gene encoding a chromatin-modifying protein and (b) mutation in a gene encoding a protein involved in binding to an epigenetic mark. **ICF syndrome** and **Rett syndrome** are examples of the two cases, respectively. ICF syndrome involves a mutation in a *de novo* DNA methyl-transferase (Xu et al., 1999) and Rett syndrome can be caused by mutations in the gene encoding a methyl-cytosine-binding protein (Amir et al., 1999). The **ATRX** gene is a gene encoding a chromatin-modifying protein in the SNF2 family (proteins involved in chromatin remodelling). A mutation in this gene can cause, among other things, mental retardation (Gibbons et al., 2000). In the area of **cancer**, epigenetics is playing an important role. Alterations in DNA-methylation was found in human cancer cells in 1983 (Feinberg and Vogelstein, 1983) and since then it has been a vast area of research examining epigenetic alterations in human tumours and their role both in the activation of tumour promoter genes and the silencing of tumour suppressor genes (Feinberg et al., 2002). The proteins MLL (1.9.2) and CBP (1.6.1) are both proteins linked to epigenetic regulation, that in cancer cells can be translocated. There are indications that cancer can be promoted also by malfunction in histone acetylation (Mielnicki et al., 1999) and histone methylation (Nguyen et al., 2002).

1.4 Proteins that bind to nucleosomes

As was mentioned in Section 1.2.3, epigenetic marks printed in chromatin can be interpreted by proteins recognizing the marks. Usually these proteins contain one or more of the domains found to interact with nucleosomes. Some of the domains are shown to interact with modified histone tails (the **bromodomain** and the **chromodomain**) and others are proposed to have a similar function (the **SANT domain** and the **PHD finger**). In addition, there are a few proteins binding to nucleosomes by other domains and motifs (**RbAp** and **SIR**).

1.4.1 The bromodomain

The bromodomain was first discovered in the *Drosophila* protein brahma and in the yeast transcriptional activator SWI2/SNF2 (Haynes et al., 1992; Tamkun et al., 1992). It is an evolutionarily conserved domain of ~110 amino acids, found in several chromatin-associated proteins and in many of the known HATs. The structure of a bromodomain is shown in Figure 1.5. The bromodomain adopts a left-handed four-helix bundle with long intervening loops, termed the ZA loop and the BC loop, between the helices A, B, C and Z (Dhalluin et al., 1999). The hydrophobic cleft between the BC and the ZA loop has been proposed to be the recognition site for acetylated histone tails (Dhalluin et al., 1999; Hudson et al., 2000; Jacobson et al., 2000; Owen et al., 2000). Some proteins containing the bromodomain are listed in Table 1.1.

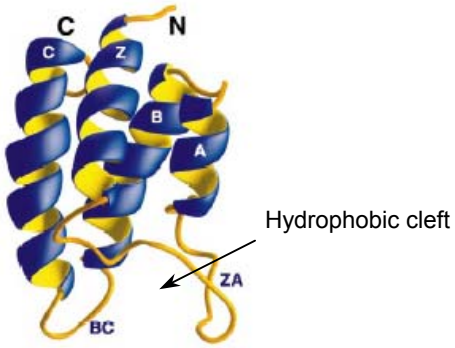


Figure 1.5. The structure of the bromodomain of p300/CBP-associated factor, P/CAF. The bromodomain is a ~110 amino acid module found in chromatin-associated proteins (Dhalluin et al., 1999).

1.4.2 The chromodomain

The **chr**omatin **o**rganisation **mo**difier, the chromodomain, is a conserved ~50 amino acids long motif that is found in a variety of proteins from different species (Paro and Hogness, 1991), reviewed in (Brehm et al., *submitted;* Eissenberg, 2001). In mammals, most proteins containing chromodomains are part of large macromolecular chromatin complexes, or they are proteins involved in chromatin remodelling. Some examples are HP1, Pc and SUV39 (Section 1.3.1 and 1.3.2). The chromodomain (Figure 1.6) has been found to contain three anti parallel β-sheets and a C-terminal α- helix (Ball et al., 1997). Several research groups have suggested a link between the chromodomain and epigenetic mechanisms: The chromodomain of HP1 has been shown to be responsible for binding to a methylated lysine 9 at histone H3 (Bannister et al., 2001; Lachner et al., 2001; Muchardt et al., 2002). There are also reports indicating that some chromodomains can bind to RNA (Akhtar et al., 2000) or to DNA (Bouazoune et al., 2002). Some proteins containing the chromodomain are listed in Table 1.1.

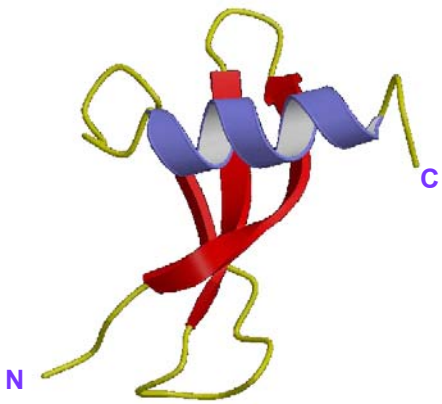


Figure 1.6 The structure of the chromodomain of HP1. The chromodomain contains three anti parallel β-sheets, ending with an α- helix (Nielsen et al., 2002).

1.4.3 The SANT domain

The SANT domain is a small protein domain present in proteins from organisms varying from viruses to vertebrates (Aasland et al., 1996). Its precise function has not yet been convincingly documented, but there are strong indications that it has a function linked to chromatin. It is present in several proteins involved in transcriptional regulation, for example SWI3 (Switching mating type), ADA2 (Adaptor), N-CoR (Nuclear receptor co-repressor) and ISWI, all of which are contained in chromatin remodelling complexes. A fact supporting the chromatin related implication is its relatedness to the DNA binding Myb domain (Aasland et al., 1996) and the SWI/SNF complex (which includes SWI3) that are reported to have DNA binding activity (Quinn et al., 1996). Evidence also indicate that SANT-regions in several co-repressors can bind, and some also activate, HDACs (Guenther et al., 2001; You et al., 2001). Two parts of the SANT domain (SANTa and SANTb) of Ada2 have been studied, where SANT b was shown to interact with the acetylase Gcn5 and SANTa was thought to recognize or interact with chromatin (Sterner et al., 2002). Other experiments have indicated that the SANT domain interacts with the N-terminal tails of the histones (Boyer et al., 2002). No structure is yet available for the SANT domain

1.4.4 RbAp46/48

There are a few examples of proteins binding to nucleosomes without the presence of any of the domains mentioned above. The mammalian nuclear protein **RbAp48** (Retinoblastoma-associated protein 48) is one example. Evidence suggests that RbAp48, and the closely related protein RbAp46, interacts with core histones H2A and H4 (Verreault et al., 1998). RbAp48 from *Xenopus laevis* has been suggested to bind to a segment of the N-terminal tail close to the histone fold domain of histone H4 *in vivo* (Vermaak et al., 1999). The RbAps are present in several protein complexes involved in mechanisms such as histone acetylation and deacetylation, nucleosome disruption and assembly (for example the NuRD complex). RbAp48 contains seven copies of a motif named the **WD repeat** (from the amino acids Trp and Asp) which is predicted to form a β-propeller structure (Sondek et al., 1996; Wall et al., 1995). The amino acids between each WD-repeat are speculated to be solvent exposed and involved in protein-protein contact. These WD-repeats are common also in other proteins, such as the chromatin association factor CAF1 and the nucleosome remodelling factor NuRF (Vermaak et al., 1999).

1.4.5 SIR-proteins

Another group of proteins binding nucleosomes is the Sir-group of proteins (silent information regulators), which contain four proteins; **Sir1p-Sir4p**. They are not structurally homologous, but they are known to form a complex with each other. The Sir2/Sir3/Sir4 complex has been shown to be able to repress transcription, particularly in regions close to telomeres, by modulating chromatin structure. Evidence indicates that Sir3p and Sir4p are involved in the repair of double stranded breaks in DNA (Astrom et al., 1999). Sir3p and

Sir4p have also been reported to interact with the N-terminal part of H3 and H4 (Gasser and Cockell, 2001; Hecht et al., 1995; Nicolas et al., 2001). Recent data identified the protein domain BAH, Bromo-Adjacent Homology (Nicolas and Goodwin, 1996) in Sir3p and it has been suggested that this domain is responsible for interaction with chromatin at the H4 N-terminal tail (Zhang et al., 2002).

1.4.6 The PHD finger

The PHD finger (Plant Homeo Domain) was discovered as a motif Cys_4-His-Cys_3 in the homeodomain protein HAT3.1 from *Arabidopsis thaliana* (Schindler et al., 1993). The motif resembled the metal binding domains RING (Cys_3-His-Cys_4) and LIM (Cys_2-His-Cys_5), but strong evolutionary conservation suggested that this was a novel domain. The conserved motif is indicated in the alignment in Figure 1.7. The PHD finger, which contains 50-80 amino acids, is found in >400 proteins, several of which are nuclear proteins involved in regulation of transcription, including the proteins TRX and PCL. It has therefore been proposed that the PHD finger may play a role in chromatin-mediated transcriptional regulation (Aasland et al., 1995). The crystal structure of the PHD finger from WSTF (Williams Syndrome Transcription Factor, Figure 1.8) show that the conserved cysteins cooridinate two Zn^{2+} ions that fold the PHD finger into a zinc finger structure (Capili et al., 2001; Pascual et al., 2000). Several diseases are linked to mutations in PHD fingers of varying proteins (Capili et al., 2001), suggesting a vital role for this domain. A further discussion on the possible roles for the PHD finger is given in Section 1.5.

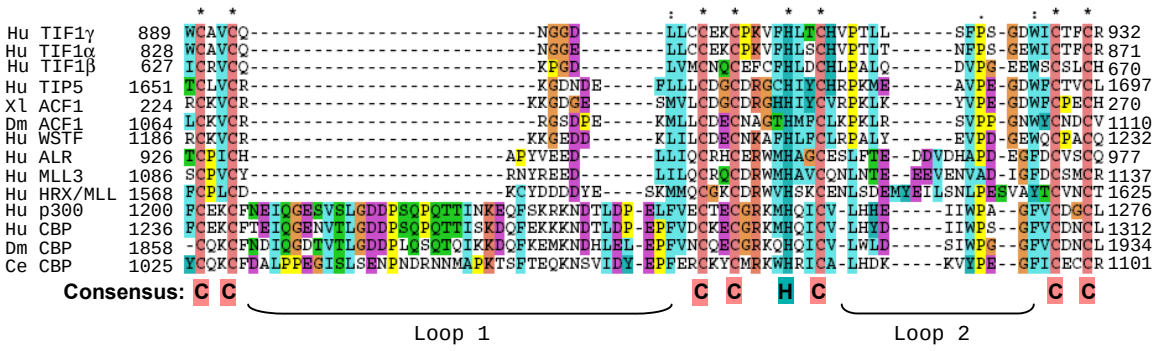


Figure 1.7 Multiple alignment of selected PHD fingers. The figure shows an alignment of PHD finger encoding sequences from the proteins discussed in Section 1.6.1 and 1.9.2 (p300, TIF1γ, TIP5 and MLL) and their close relatives. The pattern Cys_4-His-Cys_3 is indicated as the consensus below the alignment. These amino acids coordinate two zinc atoms. The alignment was made using Clustal X (default parameters), and the sequences with accession numbers are listed in Table 2.5.

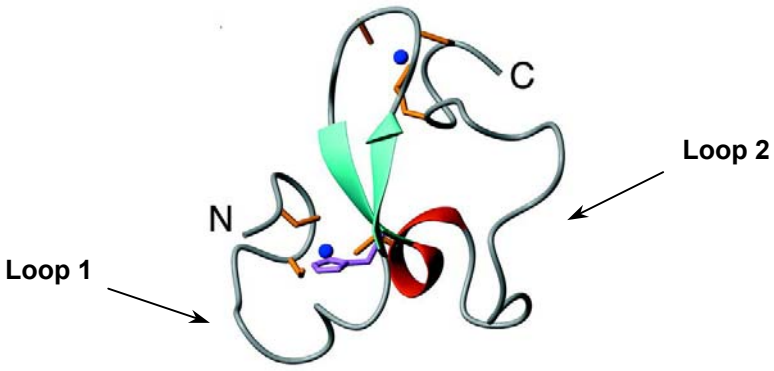


Figure 1.8 Structure of the PHD finger in WSTF. The two blue balls represent zinc ions coordinated by the seven cysteins, indicated in orange, and a histidine, indicated in puple (Pascual et al., 2000).

1.5 Possible functions for the PHD finger

Several functions have been proposed for the PHD finger (Aasland et al., 1995).

(a) As other classes of zinc-containing domains, for example the DNA binding domain of nuclear receptors and GAL4 related proteins, one might speculate that the PHD finger can bind to nucleic acids (Marmorstein et al., 1992).

(b) Other protein domains that are, as the PHD finger, rich in cystein have been suggested to have a protein-protein interacting function. This has been suggested for the LIM domain (Schmeichel and Beckerle, 1994) and also indicated for the RING finger (Burd and Emr, 1998). Several PHD finger proteins exist in complexes, like transcriptional cofactors and epigenetic regulators. The PHD finger might in these cases interact with other protein domains often present in these types of proteins, such as chromodomains, bromodomains or other PHD fingers, situated in juxtaposition or in another protein. An example is the data showing that the PHD finger in the protein KAP1 can interact with the KID domain of Mi2α (Schultz et al., 2001). Also in the protein PCL, the PHD finger is proposed to function as an independent protein interacting domain (O'Connell et al., 2001). Furthermore, the PHD finger in MLL is suggested to interact with the cyclophilin Cyp33 (Fair et al., 2001).

(c) A third possible function is that the PHD finger may recognize a specifically modified histone tail, although there is no data to support this yet.

It has been indicated that the PHD fingers in the closely related proteins p300 and CBP (Section 1.6.1) are not of the same importance in their respective proteins. In p300, the PHD finger is dispensable for histone acetyl transferase (HAT) activity, whereas for CBP it is essential (Bordoli et al., 2001; Kalkhoven et al., 2002). These results propose a new possible function for the PHD finger; (d) as a domain required for HAT activity.

Yet another function was recently proposed for the PHD finger; (e) in the protein ING2, the PHD finger was identified as a nuclear phosphoinositide receptor (Gozani et al., 2003).

Several articles have in the recent past claimed that the PHD fingers of certain protein families have ubiquitin ligase activity (Coscoy and Ganem, 2003; Lu et al., 2002). Bioinformatical analysis has shown that these domains are, in fact, RING fingers (Section 1.4.6) not PHD fingers (Aravind et al., 2003).

1.6 Experimental background

My project has been a part of the research group's main aim of elucidating the function(s) of the PHD finger. As mentioned earlier, the PHD finger is present in several proteins involved in epigenetic gene regulation, including TRX and PCL (Section 1.3.1). The focus in our group has therefore been on the PHD finger as a putative nucleosome interacting domain. The PHD finger studied in the research group originates from the protein p300. Other PHD fingers have been explored but were found difficult to express *in vitro* (Rein Aasland, *pers. comm*.). In p300, the PHD finger is situated next to a bromodomain. Variants of the region containing these two domains (p300BP, p300P and p300B) have been used in nucleosome binding experiments in our group (see Figure 1.9).

1.6.1 Function and structural organisation of p300

The human protein p300, and the very similar protein CBP (CREB binding protein), were initially identified as proteins interacting with the viral oncoprotein E1A and the cellular cAMP-response element CREB (Chrivia et al., 1993; Eckner et al., 1994). The two proteins, often referred to as p300/CBP, originate from genes on two different chromosomes but have nearly identical DNA sequence. Their functions are partly overlapping although some differences have been found (Bordoli et al., 2001; Kawasaki et al., 1998; Kung et al., 2000; Yao et al., 1998). p300/CBP participates in several physiological processes resulting in severe consequences when inactivated, for instance by binding of E1A. One consequence is the loss of controlled cell proliferation, indicating that the proteins may function as tumour suppressors, reviewed in (Chan and La Thangue, 2001). Evidence has shown that the proteins are transcriptional coactivators and that they can activate transcription through several different mechanisms. One route involves the intrinsic HAT domain (Figure 1.9) through which the proteins are found to be capable of acetylating all four core histones (Ogryzko et al., 1996). There are indications that the PHD finger (in CBP) is an integral part of the HAT domain (Kalkhoven et al., 2002), but it is still not clear, however, whether the HAT activity of p300/CBP is directly involved in chromatin remodelling. It has been reported that p300 can interact and form a stable complex with chromatin and that the bromodomain, situated next to the PHD finger, is required but not sufficient for the interaction (Manning et al., 2001). It has for this reason been proposed that the PHD finger might contribute in the binding of p300 to chromatin (Kalkhoven et al., 2002).

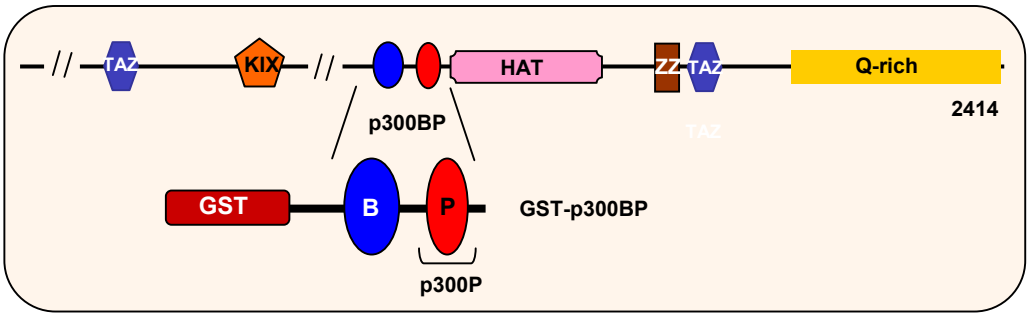


Figure 1.9 Domain organisation of p300. Explanations: TAZ, zinc finger; KIX, CREB binding domain; B, bromodomain (see Section 1.4.1); P, PHD finger (Section 1.4.6 and 1.5); HAT, histone acetyl transferase; ZZ, zinc finger. The region as used as GST-fusion protein (GST-p300BP) and the PHD finger used in the domain swapping experiments (p300P) is indicated below the sequence. The figure is modified from Rein Aasland.

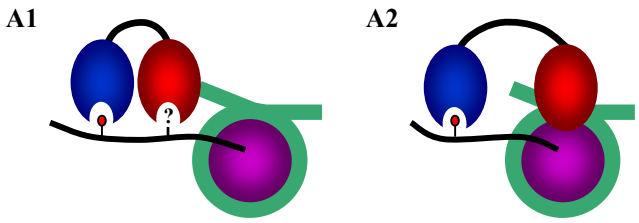
1.6.2 Relation between the bromodomain and the PHD finger in p300

The nucleosome retention assay (see Section 3.8.1) has been used in our group to show that GST-p300BP can bind to acetylated nucleosome *in vitro*. Since several bromodomains are shown to interact with acetylated lysines, (Section 1.4.1) it seems reasonable that the bromodomain in p300 has the same function. It the same assay it was found, however, that both the PHD finger (p300P) and the bromodomain (p300B) is required to detect the nucleosome interaction (Ragvin et al., *in prep*.). No nucleosome interaction has been detected with this assay for neither of the domains separately, possibly because the binding by only the bromodomain is not strong enough. These data show that the bromodomain in GST-p300BP is dependent on the PHD finger, but it is unclear *how* the PHD finger contributes to nucleosome binding.

However, more recently it was found that in the electrophoretic mobility shift assay (EMSA, see Section 3.8.2), both p300P and p300B are on their own able to interact with nucleosomes (Ragvin et al., *in prep*.). In the same assay, it was also shown that a region of the murine protein Requiem, containing two PHD fingers, is able to bind to nucleosomes without any other domains present (Anja Ragvin *pers. comm*.). These findings suggest that not only the bromodomain, but also the PHD finger, might have a nucleosome interacting activity, and that the stringency of the EMSA makes the weak interaction of only one domain detectable.

Despite the findings that both domains interact with nucleosomes, it is still reasonable to assume that *in vivo*, the domains must cooperate to achieve nucleosome binding. Based on the findings listed above, there seem to be two main ways in which p300P can contribute to the binding of GST-p300BP to the nucleosome (Figure 1.10). Either (Figure 1.10, A) the domains function individually and both domains are required simply to get a sufficiently strong interaction or (Figure 1.10, B) an internal interaction between the domains is required for the domains to be able to interact with the nucleosome. For further details, see Figure 1.10, figure text.

A The bromodomain and the PHD finger function independently



B The bromodomain and the PHD finger interact

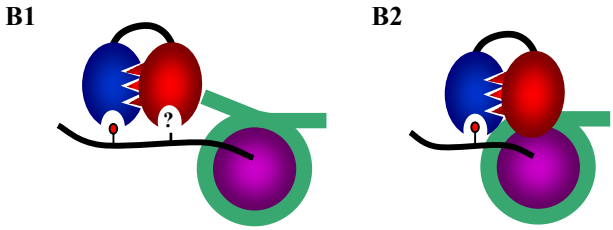


Figure 1.10 Possible models for the interaction of p300BP with the nucleosome. Only one of eight histone tails on the nucleosome is shown, and the red ball refers to an acetylated lysine. The blue domain represents a bromodomain and the red domain represents the PHD finger. **(A)** The bromodomain and the PHD finger function independently. The bromodomain probably binds to an acetylated lysine on one of the histone tails and the PHD finger might serve different roles; A1, it may bind to a specifically modified histone tail or A2, it may bind somewhere on the histone core, to the DNA or at the junction between the core histone and the DNA. **(B)** The bromodomain and the PHD finger must interact with each other to obtain nucleosome interaction. The bromodomain probably binds to an acetylated lysine on one of the histone tails and the PHD finger may have some of the same roles as in A; B1, it may bind to a specifically modified histone tail or B2, it may bind somewhere on the histone core, on the DNA or at junction between the core histone and the DNA. In all cases, the PHD finger may contribute to a stronger and more specific nucleosome binding.

1.7 Questions

The basis for my project was to examine which of the possibilities outlined in Figure 1.10 are most likely to be true. Would it in any of these cases be possible to replace the PHD finger in GST-p300BP with PHD fingers from other proteins and still retain nucleosome binding activity? Two questions are addressed on these experiments: 1) Are the bromodomain and the PHD finger in p300 so dependent on each other that other PHD fingers can not replace the original? 2) Do the size or shape of the inserted PHD fingers affect the bromodomain's ability to bind to nucleosomes? The latter case could make the protein functional with one kind of PHD finger and not with another. Replacement experiments might give some more detailed information about the function of the PHD finger in p300 in particular, and in other PHD finger proteins in general.

1.8 Aim

Two different polypeptides[a], GST-p300BP and GST-TIFPB, were the starting points of the experiments. GST-p300BP, with bromodomain and PHD finger from p300, has been extensively studied in the group and is confirmed to bind to nucleosomes. The other, GST-TIFPB, with PHD finger and bromodomain from TIF1γ (Section 1.9.2), has not been tested previously. Different domain recombinants[b] generated from the two basis vectors pSXG-p300B and pSXG-TIFB (encoding only the respective bromodomains, see Table 2.4) were going to be made by inserting a PHD finger encoding sequence from each of three heterologous proteins (see Section 1.9.2 and Table 2.1). Finally, the nucleosome binding activity of each of the resulting six domain recombinants was going to be tested. The aim was to find out whether other PHD fingers were able to replace the originals in GST-p300BP and GST-TIFPB without loosing the polypeptides' ability to interact with nucleosomes.

1.9 Strategies

1.9.1 Experimental strategy

The plasmids encoding the domain recombinants were to be made by insertion of PHD finger encoding sequences into a linker[c] that had been inserted in the two basis vectors (pSXG-p300B and pSXG-TIFB) in advance. Sequences encoding PHD fingers from p300 (Figure 1.9), TIP5 (Figure 1.11), TIF1γ (Figure 1.12), and the last of the three PHD fingers in MLL (Figure 1.13) were to be used as PHD finger substitutes. The resulting eight recombinant plasmids would encode six domain recombinants and two controls that would have their original PHD finger reinserted (Table 4.1). All polypeptides were to be purified by affinity purification and were then going to be tested for nucleosome binding. Different methods could have been used to detect this interaction, such as BIAcore, sucrose gradients, or protease cleavage. The nucleosome retention assay and the EMSA are good and sensitive methods for such detection, and were also already established in the group. These two methods were therefore chosen to test nucleosome binding in this work. To make an easier comparison to the previous experiments in the group, the same kind of nucleosomes (mononucleosomes, see Table 2.3) were going to be used in these experiments.

1.9.2 PHD finger substitutes

The various PHD finger substitutes in p300 were chosen for different reasons. The ones from TIF1γ and TIP5 were chosen because they are originally situated next to a bromodomain. The idea was that they for this reason might functionally resemble the PHD

[a] Since all plasmids used in this project encode only parts of proteins, e.g. the bromodomain region from p300, all products of the plasmids are referred to as polypeptides instead of proteins.
[b] A domain recombinant refers to a polypeptide that has had one of its domains swapped with the corresponding domain from a foreign protein. In these experiments, the domain is a PHD finger.
[c] The oligonucleotides used in the experiments referred to as linkers, should in fact be called adaptors, since they have cohesive ends and not blunt ends. To be able to refer to these oligonucleotides with the same name before and after insertion into a plasmid, they are called linkers in all cases.

finger of p300. The PHD finger from MLL was chosen because it is *not* originally situated next to a bromodomain, to see if this PHD finger might behave differently than the other three. Some information about the function and structural organisation of these proteins is given below.

TTF-I interacting protein 5 (TIP5)

The gene encoding the human protein TIP5 is a recently cloned gene (Strohner et al., 2001). Together with SNF2h, the mammalian homolog of ISWI (Section 1.3.1), TIP5 forms a complex named NoRC, nucleolar remodelling complex. As the name indicates, NoRC is localized in the nucleolus, where it is involved in the regulation of the transcription of rRNA genes (Strohner et al., 2001). Transcription of these genes is dependent on binding of the transcription terminator factor TTF-1 to the RNA polymerase I transcription site. TTF-1 recruits remodelling complexes to the rDNA promoter, such as NoRC (Längst et al., 1997). TIP5 is the largest unit in the complex (205 kDa) and can interact with TTF-1 (Strohner et al., 2001). By recruitment of HDAC, NoRC works as a repressor, probably through nucleosome sliding. Over-expression of TIP5 is related to an increase in deacetylation, correlating with the report of interaction between TIP5 and HDAC1 (Zhou et al., 2002). In the C-terminal, TIP5 contains a PHD finger and a bromodomain (Figure 1.11). Deletion of this region abolishes the interaction with HDAC1 (Zhou et al., 2002) indicating that this region is involved in the interaction. In addition to recruiting HDAC, NoRC is also found to recruit DNMTs (Santoro et al., 2002). The central part of TIP5 is thought to interact with chromatin and is possibly dependent on histone acetylation for this binding. It has been suggested that the BAZ motif, bromodomain adjacent zinc-finger, is involved in this interaction (Strohner et al., 2001).

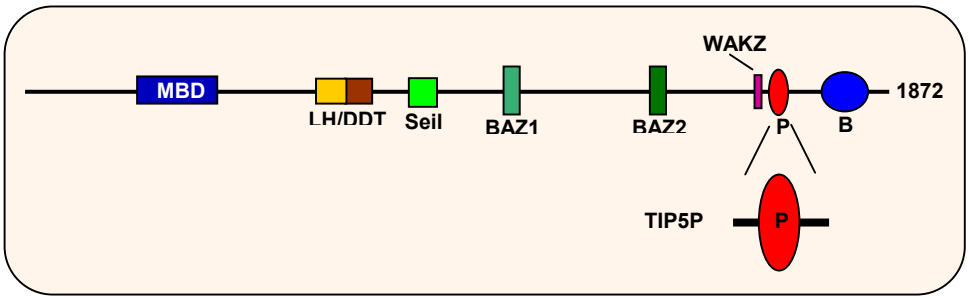


Figure 1.11 Domain organisation in TIP5. Explanations: MBD, methyl-CpG binding domain; LH/DDT, LH rich DNA binding domain often present in chromosome remodelling factors; Seil, conserved region of unknown function, BAZ, Bromodomain adjacent to Zn finger domain; WAKZ, conserved motif also present in Acf1 and WSTF; P, PHD finger (Section 1.4.6 and 1.5); B, bromodomain (Section 1.4.1). The PHD finger used in domain swapping experiments (TIP5P) is indicated below the sequence. The figure is modified from Rein Aasland.

Transcription intermediary factor 1 γ (TIF1γ)

Transcription intermediary factors (TIFs) are proteins that can influence a gene's activity by being mediators between transcription factors and components of the general transcription machinery or proteins involved in chromatin remodelling. The human gene encoding TIF1γ was cloned in 1999. The protein is structurally and functionally related to the other members of the TIF1 family, TIF1α and TIF1 β, but a few functional properties are clearly distinct (Venturini et al., 1999). TIF1α and TIF1β might be involved in heterochromatin-induced gene repression by the interaction with a DNA binding domain, for example the KRAB silencing domain or a protein in the HP1-family (Friedman et al., 1996; Kim et al., 1996; Le Douarin et al., 1996; Moosmann et al., 1997). TIF1α also interacts with several nuclear receptors, which are ligand dependent transcription factors (Le Douarin et al., 1995; vom Baur et al., 1996). Evidence suggests that TIF1α is able to interact with chromatin (Remboutsika et al., 1999), supporting the hypothesis of a link between the TIF1 proteins and chromatin. Furthermore, there are indications that TIF1γ (and the other two TIF1s) can silence genes when recruited to a promoter region of a reporter gene, but no evidence exist indicating that TIF1γ interacts neither with KRAB, the HP1s nor any nuclear receptors. TIF1γ may thus cause repression by a yet unidentified partner (Venturini et al., 1999). The three proteins of the TIF1 family all contain the same protein domains (Figure 1.12). In the central part of the proteins, there is a region of less conservation. In the N-terminal part of this region, though, it is found a conserved sequence named TSS (TIF1 signature sequence). Mutation in this region disrupts repression by TIF1γ and may thus be important for its function (Venturini et al., 1999). A PHD finger and a bromodomain are situated in the C-terminal of the protein.

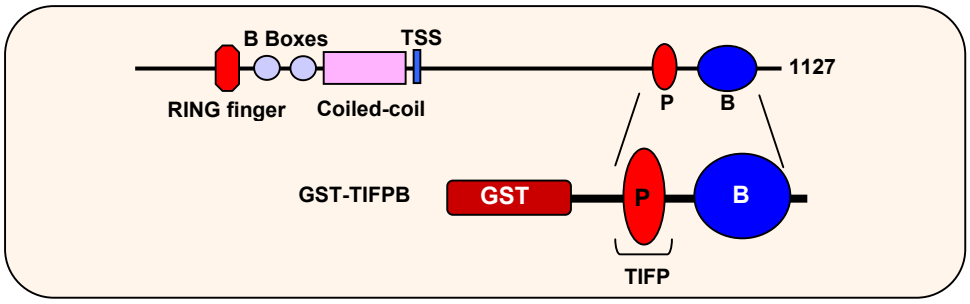


Figure 1.12 Domain organisation of TIF1γ. Explanations: RING finger, protein interacting domain; B Box, cystein+histone rich region binding zinc; Coiled coil, bundles of long α-helices; TSS, TIF1 signature sequence; P, PHD finger (Section 1.4.6 and 1.5); B, bromodomain (Section 1.4.1). The region used as GST-fusion protein (GST-TIFPB) and the PHD finger used for domain swapping experiments (TIFP) are indicated below the sequence. The figure is based on (Peng et al., 2002).

Mixed Lineage Leukemia (MLL)

The 431 kDa human protein MLL (HRX or ALL-1) is one of four mammalian homologues of the *Drosophila* protein TRX (Section 1.3.1). Studies have shown that MLL, as TRX, is needed for the maintenance of the transcription of the HOX genes (Yu et al., 1998). As the

name implies, MLL is linked to different variants of leukaemia. Leukemia in these cases is a result of various translocations of the MLL gene. The 5' part of the gene has been found fused with more than 30 different genes (Gu et al., 1992), expressing fusion proteins interfering with the normal function of MLL. MLL is proteolytically cleaved in two parts that together with at least 27 other proteins form a multiprotein super complex, MLL-MPSC (Nakamura et al., 2002; Yokoyama et al., 2002). The different variants of fusion proteins can either enter the complex, or compete with it for the binding to DNA and thereby disturb its function (Yano et al., 1997).

The normal function of MLL is less understood, but a recent report suggests that the protein might have both (a) activating and (b) repressive roles (Schraets et al., 2003). (a) Genes encoding proteins affecting vital organs and cells, such as muscles, limbs, bones and blood cells were found to be *activated* in the presence of MLL. (b) In the absence of MLL, several genes in malignant cancer cells seem to be over-expressed, indicating that these genes might normally be *repressed* in the presence of MLL. MLL contains, in the central part, three adjacent PHD fingers (Figure 1.13) . In contrast to TIP5 and TIF1γ, the protein does not contain any bromodomains.

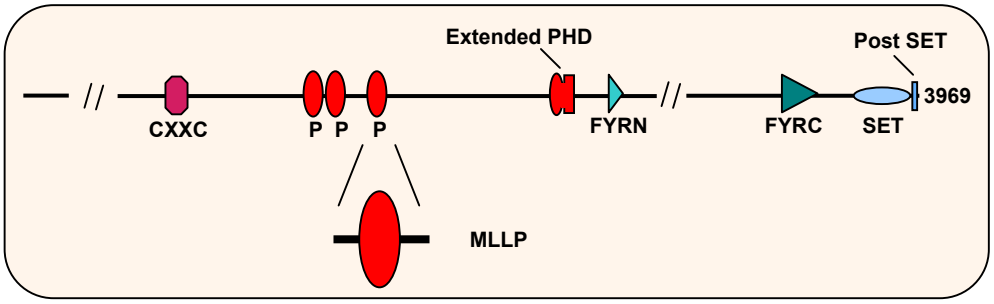


Figure 1.13 Domain organisation of MLL. Explanations: CXXC, Zn finger; P, PHD finger (see Section 1.4.6 and 1.5); B, bromodomain (see Section 1.4.1), Extended PHD finger, special kind of PHD finger; FYR, Phe-Tyr rich region; SET, histone methyl transferase. The PHD finger used in domain swapping experiments is indicated below the sequence. The figure is based on information given in the databases SMART and Pfam.

1.9.3 Possible outcomes

There are several possible outcomes for the experiments outlined in Section 1.9.1:

(a) **All the domain recombinants may bind.** This may raise the question whether GST-p300BP would be able to bind to nucleosomes with all kinds of PHD finger substitutes or maybe even with other kinds of domains, similar in size or structure. A possibility in this case might be that the bromodomain is flexible in its binding; if the PHD finger has a structure that does not fit with the normal binding point of the bromodomain, a possible scenario is that the bromodomain finds 'something else' to bind to, in a position that makes the PHD finger fit better to the nucleosome. This could indicate that also other PHD fingers might have a chromatin binding activity.

(b) **Only some of the domain recombinants bind.** This could indicate that the PHD fingers in the positive domain recombinants have a nucleosome binding activity, and it would make it interesting to see if there are common properties among these specific PHD fingers, or the proteins that they originate from, that could make them more likely to bind than the others.

(c) **None of the domain recombinants bind.** This could be the result if there is a specific interaction between the bromodomain and the PHD finger that prohibits other PHD fingers to replace it, or that the unnatural combination makes the domains not able to cooperate. This result would also appear if the inserted PHD fingers do not have a nucleosome binding activity. One could not exclude, however, that the heterologous domain makes the polypeptide dysfunctional due to improper folding.

MATERIALS

2.1 PHD fingers, bacteria, nucleosomes and plasmids

Table 2.1 PHD fingers used in domain swapping experiments

Origin protein	Refe-rence[a]	Origin plasmid (Table 2.4)	Name of PHD finger	Amino acid sequence [b] encoded by the DNA sequence inserted in acceptor vector	Bases	Amino acids[c]	Mw (kDa)
Hu TIP5	1.9.2	pBSII sk (+) KIAA0314	TIP5P	FQLERSIAWEKSVNKVT**C**LV**C**RKGD NDEFLLL**C**DG**C**DRGC**H**IY**C**HRPKME AVPEGDWF**C**TV**C**KAQQVEGEFTQV	222	74	~8
Hu TIF1γ	1.9.2	pBSII sk (+) KIAA1113	TIFP	FNNKDDDPNEDW**C**AV**C**QNGGDLL**C**C EK**C**PKVF**H**LT**C**HVPTLLSFPSGDWI **C**TF**C**RDIGKPEVEYDV	198	66	~7
Hu MLL	1.9.2	pSXG-MLLP	MLLP	FPGTTPGKGWDAQWSHDFSLCHDCA KLFAKGNF**C**PL**C**DKCYDDDDYESKM MQ**C**GK**C**DRWV**H**SK**C**ESLSGTEDEMY EILSNLPESVAYT**C**VN**C**TERV	288	96	~10
Hu p300	1.6.1	pSXG-p300P	p300P	FIPRDATYYSYQNRYHF**C**EK**C**FNEIQ GESVSLGDDPSQPQTTINKEQFSKRK NDTLDPELFVE**C**TE**C**GRKM**H**QI**C**VLH HEIIWPAGFV**C**DG**C**LKKSARTRKEV	309	103	~12

[a] The reference refers to information about the origin protein, not the plasmid or the PHD finger

[b] The conserved PHD finger pattern is indicated in bold types

Table 2.2 *Escherichia coli* strains

Bacteria	Genotype	Use	Supplier
DH5α	*supE44 supF58 hsdS3(r_B-m_B) dapD8 lacY1 glnV44Δ(gal-uvrB)47 tyrT58 gyrA29 tonA53Δ(thyA57)*	Purification of plasmid	Clontech
BL21 (DE3) pLysS	B F^- *dcm ompT hsdS(r_B-m_B-) gal λ* (DE3)[pLysS Cam^r][a]	Protein expression	Stratagene
XL1-Blue supercompetent	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' *proAB* $lac^qZ\Delta M15$ Tn*10* (Tet^r)]	Mutagenesis	Stratagene

Table 2.3 Nucleosomes

The nucleosomes used in the experiments were purified from human SupT-cells by Anja Ragvin. These cells were used because they are easy to grow in suspension and they have large nuclei. The cells were grown in the presence of Trichostatin A (TSA), which is an inhibitor of deacetylases. This made the isolated nucleosomes hyperacerylated. Fractions containing ~80 % mononucleosomes were selected and used in these experiments. The nucleosomes were radiolabelled in one of two ways; (a) By incorporation of {methyl-^{3}H} thymidine in DNA during cell growth or (b) by 5' phosphorylation of DNA with {γ-^{32}P} ATP after purification.

The procedures are described in (Ragvin, 2001).

Table 2.4 Plasmids

Name	Base-pairs	Explanation	Source
pSGX	4994	pGEX2TK vector from Pharmacia modified by adding a polylinker named ESBSP (*Eco*R I, *Sma* I, *Bam*H I, *Sal* I and *Pst* I) between the *Bam*H I and *Eco*R I sites	Sigrid Erdal, MBI, UiB
pSGX-p300P	5287	pSXG vector with PHD finger encoding sequence from p300 inserted between the *Eco*RI and *Sal* I sites	Sigrid Erdal, MBI, UiB
pSGX-p300B	5458	pSXG vector with Bromodomain encoding sequence from human p300 inserted between the *Eco*RI and *Sal* I sites	Sigrid Erdal, MBI, UiB
pSGX-p300BP	5725	pSXG vector with both PHD- and Bromodomain encoding sequences from p300 inserted between the *Eco*RI and *Sal* I sites	Sigrid Erdal, MBI, UiB
pBSII sk (+) KIAA1113	9385	cDNA encoding TIF1γ inserted in the *Sal* I and *Not* I sites of a p Bluescript II sk+ vector	Takashiro Nagase, Kazusa DNA research Intitute, Japan
pBSII sk (+) KIAA0314	7422	cDNA encoding TIP5 inserted in the *Sal* I and *Not* I sites of a p Bluescript II sk+ vector	
pSGX-TIF1γB	5548	pSXG vector with Bromodomain encoding sequence from TIF1γ added in the *Bam*H I site	Sigrid Erdal, MBI, UiB
pSXG-*m*TIFB	5548	pSGX-TIF1γB with the *Eco*R I site in position 957 removed by site directed mutagenesis	Section 3.1 and 4.1.1
pSXG-PHDc / pSXG-MLLP	5270	pSXG vector containing the last of the three PHD coding regions (PHDc) of MLL inserted between the *Eco*R I site and the *Sal* I site	Sigrid Erdal, MBI, UiB
pSXG-p300B*link*	5482	pSXG-p300B vector containing the linker MS (see Table 2.6) inserted in the *Sal* I site	Section 3.3
pSXG-p300B*link*P	5773	pSXG-p300B*link* containing the PHD finger encoding sequence from p300 inserted in the *Mun* I and *Sal* I sites in the linker	Section 3.4, Table 4.1
pSXG-p300B-TIFP	5662	pSXG-p300B*link* containing the PHD finger encoding sequence from TIF1γ inserted in the *Mun* I and *Sal* I sites in the linker	Section 3.4, Table 4.1
pSXG-p300B-MLLP	5756	pSXG-p300B*link* containing the PHD finger encoding sequence from MLL inserted in the *Mun* I and *Sal* I sites in the linker	Section 3.4, Table 4.1
pSXG-p300B-TIP5P	5686	pSXG-p300B*link* containing the PHD finger encoding sequence from TIP5 inserted in the *Mun* I and *Sal* I sites in the linker	Section 3.4, Table 4.1
pSXG-TIF*link*B	5572	pSXG-*m*TIFB vector containing the linker SE (see Table 2.6) inserted in the *Eco*R I site	Section 3.3
pSXG-TIFP*link*B	5764	pSXG-TIF*link*B containing the PHD finger encoding region from TIF1γ inserted in the *Eco*R I and *Xho* I sites in the linker	Section 3.4, Table 4.1
pSXG-MLLP-TIFB	5858	pSXG-TIF*link*B with the PHD finger encoding region from MLL added in the *Eco*R I and *Xho* I sites in the linker	Section 3.4, Table 4.1
pSXG-p300P-TIFB	5875	pSXG-TIF*link*B containing the PHD finger encoding region from p300 inserted in the *Eco*R I and *Xho* I sites in the linker	Section 3.4, Table 4.1
pSXG-TIP5P-TIFB	5788	pSXG-TIF*link*B containing the PHD finger encoding region from TIP5 inserted in the *Eco*R I and *Xho* I sites in the linker	Section 3.4, Table 4.1

Table 2.5 PHD finger sequences used in alignment in Figure 1.7

Protein[a]	SwissProt Accession number	Protein length (aa)	Position of PHD finger	Explanation
p300 family				
Hu p300	Q09472	2221	1200-1276	E1A- associated protein
Hu CBP	Q92793	2442	1236-1312	CREB binding protein
Dm CBP	Q9W321	3276	1858-1934	CREB binding protein
Ce CBP	P34545	2056	1025-1101	CREB binding protein
TIF1 family				
Hu TIF1α	O15164	1050	828-871	Transcription Intermediary Factor 1 α
Hu TIF1β	Q13263	835	627-670	Transcription Intermediary Factor 1 β
Hu TIF1γ	Q9UPN9	1127	889-932	Transcription Intermediary Factor 1 γ
TIP5 family				
Hu TIP5	Q9UIF9	1872	1651-1697	TTF-interacting protein 5
Hu WSTF	Q9UIG0	1483	1186-1232	Williams Syndrome Transcription Factor
Xl ACF1	Q8UVR5	627	224-270	ATP-utilizing Chromatin assembly and remodelling Factor 1, ISWI ortholog
Dm ACF1	Q9V9T4	1476	1064-1110	ATP-utilizing Chromatin assembly and remodelling Factor 1, ISWI ortholog
MLL family				
Hu HRX/MLL	Q03164	3969	1568-1625	Human trithorax protein
Hu ALR	O14687	4957	926-977	Homolog of ALL1 and trx
Hu MLL3	Q8NEZ4	4911	1086-1137	Mixed Lineage Leukemia 3

[a] The two-letter code in front of each protein name indicates the organism; Hu (*Homo sapiens*), Dm (*Drosophila melanogaster*), Ce (*Caenorhabditis elegans*), Xl (*Xenopus laevis*).

2.2 Molecular weight standards

Table 2.6 Molecular weight standards

Name	Cat. no	Supplier
DNA markers		
1 kb-ladder	15615-024	GibcoRBL
φX174 DNA *Hae* III Marker	G176A	Promega
Protein markers		
BenchMark™ Protein Ladder	10747-012	GibcoRBL
SDS-PAGE Standards, low range	161-0304	Bio-Rad

2.3 Enzymes and buffers, and reagents for PCR and sequencing

Table 2.7 Enzymes

Enzyme	Concentration	Cat No	Supplier
Restriction enzymes			
*Bam*H I	10 U/µl	R6021	Promega
*Eco*R I	12 U/µl	R6011	Promega
Sal I	10 U/µl	R6051	Promega
Spe I	10 U/µl	R6591	Promega
Xho I	10 U/µl	R6161	Promega
Mfe I	10 U/µl	R0589	NEB
Dpn I	10 U/µl	200518	Stratagene
Ava I	10 U/µl	R609A	Promega
Phosphatase			
Calf Intestinal Alcaline Phosphatase (CIAP)	1 U/µl	M1821	Promega
Kinases			
T4 polynucleotide kinase	3 U/µl	M180A	Promega
Ligase			
T4 DNA ligase	3 U/µl	M1801	Promega
Polymerase			
50 x Advantage ® cDNA polymerase mix [a]		S0595	Clontech
Pfu Turbo® polymerase	2.5 U/µl	200518	Stratagene
Others			
Thrombin	1 U/µl	27-0B46-01	Amershan Biosciences

[a] Contains 1.1 µg/µl KlenTaq-1 DNA polymerase and 1.1 µg/µl TaqStart antibody in storage buffer.

Table 2.8 Buffers and reagents for PCR, sequencing and enzymes

Buffer	Cat.no	Supplier
For PCR		
10 x cDNA PCR Reaction Buffer	S0596	Clontech
10 x reaction buffer for site directed mutagenesis	200518	Stratagene
Deoxynucleotide Triphosphates (dNTPs)	U1201, U1211, U1221, U1231	Promega
For sequencing		
BigDye™ Terminator [a]	4314421	Perkin Elmer
For enzymes		
10 x Multicore buffer	R9991	Promega
10 x CIAP buffer	M1833	Promega
10 x T4 DNA ligase buffer	C1263	Promega
10 x NEB Buffer 4	B7004S	NEB
10 x T4 polynucleotide kinase buffer	C131B	Promega
10 x Buffer B, Buffer D, Buffer E, Buffer H		Promega

[a] Terminator Ready Reaction Mixture containing A, C, G and T -Dye terminators labelled with different dichloro-exitation labels, dNTPs (dATP, dCTP, dUTP and dITP, in place of dGTP), AmpliTaq DNA polymerase with thermally stable pyrophosphatase, $MgCl_2$ and TrisHCl buffer, pH 9.0.

2.4 Oligonucleotides

Table 2.9 Oligonucleotides [a] used for linkers, sequencing and PCR

Name	Sequence [b]	Explanation
Used for ligation to form a linker		
SalEco-Upper	5' AATTC CTCGAGGGTCCGACTAGTC 3' <*Eco*R I> *Xho* I	Can anneal with SalEco-Lower to form a linker, SE, that fits in a plasmid opened with *Eco*R I (see Figure 4.6)
SalEco-Lower	5' AATTGACTAGTCGGACCCTCGAGG 3' <*Eco*R I> *Xho* I	Can anneal with SalEco-Upper to form a linker, SE, that fits in a plasmid opened with *Eco*R I (see Figure 4.6).
MunSal-Upper	5' TCGAG CAATTGGGTCCGACTAGTG 3' <*Sal* I> *Mun* I	Can anneal with MunSal-Lower to form a linker, MS, that fits in a plasmid opened with *Sal* I (see Figure 4.5).
MunSal-Lower	5' TCGACACTAGTCGGACCCAATTGC 3' <*Sal* I> *Mun* I	Can anneal with MunSal-Upper to form a linker, MS, that fits in a plasmid opened with *Sal* I (see Figure 4.5).
Used as primers for PCR		
TIP5Eco5	5' GGCGGAATTCCAGCTGGAGAGGTCCATT *Eco*R I GCCT 3'	PCR primer binding at 5' side of the PHD finger encoding sequence in pBSII sk (+) KIAA0314. The PCR product can be digested to get an *Eco*R I sticky end.
TIP5Sal3	5' GCGCGTCGACCTGAGTGAATTCTCCCTCC *Sal* I ACCTG 3'	PCR primer binding at 3' side of the PHD finger encoding sequence in pBSII sk (+) KIAA0314. The PCR product can be digested to get a *Sal* I sticky end.
TIF1GEco5	5' GCGGAATTCAACAATAAAGATGATGACC *Eco*R I CAAAT 3'	PCR primer binding at 5' side of the PHD finger encoding sequence in pBSII sk (+) KIAA1113 PCR. The PCR product can be digested to get an *Eco*R I sticky end.
TIF1GSal3	5' GCGCGTCGACATCATATTCAACTTCTGGC *Sal* I TTTCC 3'	PCR primer binding at 3' side of the PHD finger encoding sequence in pBSII sk (+) KIAA1113. The PCR product can be digested to get a *Sal* I sticky end.
Used as primers for sequencing		
5PGEX	5' GGGCTGGCAAGCCACGTTTGGTG 3'	5' sequencing primer binding pSXG.
3PGEX	5' CCGGGAGCTGCATGTGTCAGAGG 3'	3' sequencing primer binding pSXG.
300 P5	5' GGCCATATGGAATTCATACCTCGTGATG CCACTTA 3'	5' sequencing primer binding to sequence in front of the PHD finger encoding region in pSXG-p300BP.
Used in Site directed Mutagenesis		
Lower EcoMut	5' GAAGCAGGAACAGGCTCCTG GAA**C**TCAATACTTAATTCATGG 3' (*Eco*R I)	5' PCR primer annealing over the *Eco*R I site in position 1071 of pSXG-TIFB, containing a mismatch mutating T 1073 to a C (see Figure 4.2)
Upper EcoMut	5' CCATGAATTAAGTATTGA**G**TTCCAGGAG (*Eco*R I) CCTGTTCCTGCTTC 3'	3' PCR primer annealing over the *Eco*R I site in position 1071 of pSXG-TIFB, containing a mismatch mutating A 1073 to a G (see Figure 4.2)

[a] All oligonucleotides were supplied by MedProbe AS, Oslo

[b] Relevant restriction sites are underlined, compatible sites indicated with arrow brackets and sites with mismatches in parenthesis.

2.5 Chemicals

Table 2.10 Chemicals

Chemical name	Formula	Supplier
Acrylamide, 30% solution (contains 7.8 g/l bisacrylamide 4K)	C_9H_7NO	AppliChem
Acetic acid	$C_2H_2O_2$	KeboLab
Adenodine 5' triphosphate	-	Pharmacia
Agar	-	Merck
Agarose	-	GibcoBRL
Ammoniumperoxodisulphate (APS)	-	Merck
Ampicillin	-	Astra
Bis-acrylamid	$C_7H_{10}O_2N_2$	Kodak
Boric Acid	H_3BO_3	Merch
Bovine Serum Albumin, acetylated (BSA-Ac)	-	Promega
Bovine Serum Albumin (BSA)	-	Promega
β-Mercaptoethanol	$HSCH_2CH_2OH$	Merck
Bromphenol blue	-	Merck
Chicken Egg Albumin (CEA)	-	Sigma
Chloramphenicol	$C_{11}H_{12}Cl_2N_2O_3$	Sigma
Chloroform	$CHCl_3$	Merck
Comassie Brilliant Blue	-	Merck
D(+)-Glucose monohydrate	$C_6H_{12}O_6 \cdot H_2O$	Merck
Dithiothreitol (DTT)	$C_4H_{10}O_2S_2$	Sigma
Ethylene dinitrilo tetraacetic acid (EDTA)	$C_{10}H_{17}N_2Na_2O_8 \cdot H_2O$	Merck
Ethanol 96 %	C_2H_6O	Arcus producter AS
Ethanol 100 %	C_2H_6O	Arcus producter AS
Ethidium Bromide (EtBr)	$C_{21}H_{20}BrN_3$	Merck
Glutathione sepharose TM 4B	-	Amrsham
Glutathione, reduced form	$C_{10}H_{17}N_3O_6S$	Sigma
Glycerol, 87 %	$C_3H_8O_3$	Merck
Glycin	H_2NCH_2COOH	Merck
IPTG	-	Promega
Isoamyl alcohol	$C_5H_{12}O$	Merck
Isopropanole	C_3H_8O	Arcus produkter AS
L-glutamat	$C_5H_9NO_2$	Merck
Magnesium dichloride hexahydrate	$MgCl_2 \cdot 6\ H_2O$	Merck
Magnesium sulphate heptaahydrate	$MgSO_4 \cdot 7\ H_2O$	Merck
N,N,N',N'-tetra methyl ethylene diamine (TEMED)	$C_6H_{16}N_2$	Merck
NZ amine, casein hydrolysat	-	Sigma
Potassium chloride	KCl	Merck
Potassium dihydrogen phosphate	KH_2PO_4	Sigma
Pyronin B	-	Janssen Chimica
Sodium acetate	$NaCH_3COO$	Merck
Sodium hydroxide	NaOH	Merck
Sodium hydrogen phosphate dihydrate	$NaHPO_4 \cdot 2H_2O$	Merck
Sodium chloride	NaCl	Merck
Sodium dodecylsulphate (SDS)	$C_{12}H_{25}O_4SNa$	Sigma
TEMED	$C_6H_{12}N_2$	Merck
Trichostatin A (TSA)	$C_{17}H_{22}N_2O_3$	Wako
Trimethyl chlor silan (TMCS)	$(CH_3)_3SiCl$	Fluca Chemie
Tris-(hydroxymethyl)-aminomethane (Tris)	$NH_2C(CH_2OH)_3$	USB
Triton X-100	-	Sigma
Tryptone / Tryptone Peptone	-	DIFCO
Ultima Gold TM Liquid Scintillation Coctail (LSC)	-	Packard
X-gal	-	Melford Laboratories
Xylene Cyanol FF	-	Internarional Biotechnologies INC
Bacto ™ YeastExtract	-	DIFCO
Zinc acetate dihydrate	$(CH_3COO)_2\ Zn \cdot 2\ H_2O$	Merck

2.6 Kits, consumables and apparatus

Table 2.11 Kits

Kit	Cat. no	Supplier
QuikChange® Site-Directed Mutagenesis Kit	200518	Stratagene
QIAfilter™Plasmid Maxi Kit	12263	Qiagen
Wizard®*Plus* Minipreps	A7510	Promega
D*c*Protein Assay	500-0114	Bio-Rad
QUIAEX®II Gel Extraction Kit	20051	Qiagen

Table 2.12 Consumables [a]

Product	Cat. no	Supplier
Amicon Ultrafree-MC 0.2 µm Filter Unit, nonsterile	UFC30GV00	Millipore
Centricon®Centrifugal Filter Devices	4205	Millipore
Slide-A-Lyzer dialysis cassette, 10000 MWCO	66425	Pierce

[a] Other consumables were standard laboratory equipment

Table 2.13 Apparatus [a]

Type of equipment	Name
Centrifuges	
Nanofuge	Labnet minifuge
Microfuge	Sigma 112 B. Brown Biotech International, 12026 rotor
Megafuge	Megafuge 1.0 R refrigerated, Rotor 3360 Heraeus instruments
Biofuge	Biofuge fresco, refrigerated microfuge, Heraeus instruments
Sorvall centrifuge	RC5C refrigirated
Other equipment	
DNA sequencer	Seq ABI 377 ABI PRISM
French Press	French®Pressure Cell Press, SIM AMINCO, 20 K Cell
PCR machine	Gene Amp PCR System 2400, Perkin Elmer
Scintillation counter	TRI-CARB® 4530, Packard
Phosphoimager	FLA 2000 IP, Fujifilm
Sonicator	Micro Ultrasonic Cell Disrupter, Kontes
DNA/RNA spectrophotometer	GeneQuant II RNA/DNA calcultaor, Pharmacia
Cell spectrophotometer	CO 8000 Cell Densitymeter, WPA biowave
Gel Dryer	Model 583, Biorad
Electroporator	Gene Pulser™, BioRad
Microplate absorbance reader	Labsystem Multiscan MS

[a] Other apparatus were standard laboratory equipment

2.7 Buffers, solutions and cell media

Table 2.14 Cell media

LB medium [a]	**SOB** [a]	**SOC** [b]	**NZY+ broth** [a]
1 % (w/v) Tryptone	2.0 % (w/v) Tryptone	SOB	10 mg/ml NZ amine
0.5 % (w/v) Yeast extract	0.5 % (w/v) Yeast extract	5 mM $MgCl_2$	(casein hydrolysate)
1 % (w/v) NaCl	10 mM NaCl	5 mM $MgSO_4$	5 mg/ml Yeast extract
	2.5 mM KCl	20 mM Glucose	5 mg/ml NaCl
			12.5 mM $MgCl_2$ } [c]
			12.5 mM $MgSO_4$ } [c]
2 x YT [a]	**2 x YT-G** [b]	**GYT** [a]	20 mM Glucose } [c]
1.6 % (w/v) Tryptone	2 x YT	10 ml 87 % (v/v)	
1.0 % (w/v) Yeast Extract	2 % (w/v) Glucose	Glycerol	
0.5 % (w/v) NaCl		0.125 g Yeast extract	
		0.259 g Tryptone	

[a] Sterilized by autoclaving
[b] Prepared immediately before usage
[c] Added immediately before usage

Table 2.15 Agarose gel electrophoresis

5 x TBE	**6 x Loading buffer**	**1 % Agarose**
0.5 M Tris	0.25 % (w/v) Bromphenole blue	0.5 x TBE
0.5 M Boric acid	0.25 % (w/v) Xylene cyanol FF	1 % (v/v) Agarose
10 mM EDTA pH 8.0	30 % (v/v) Glycerol	0.1 % (v/v) Ethidium bromide [a]

[a] Added immediately before use

Table 2.16 SDS-PAGE

10 x Running buffer	**12 % SDS separation gel**	**4 % SDS stacking gel**
0.25 M Tris	0.25 M TrisHCl pH 8.8	0.25 M TrisHCl pH 6.8
2 M Glycine	0.1 % (w/v) SDS	0.1 % (w/v) SDS
1 % (w/v) SDS	12 % (v/v) Acrylamide	4 % (v/v) Acrylamide
	0.2 % (v/v) TEMED	0.2 % (v/v) TEMED
	0.05 % (w/v) APS	0.05 % (w/v) APS
4 x Sample buffer [a]	**Coomassie brilliant blue stain solution**	
125 mM TrisHCl pH 6.8	0.1 % (w/v) Coomassie brilliant blue	
20 % (v/v) Glycerol	40 % (v/v) EtOH	
4 % (w/v) SDS	10 % (v/v) Acetic acid	
Pyronin B		
Bromphenolblue		
5.7 mM β-mercaptoethanol		

[a] Stored at –20 °C before adding β-mercaptoethanol, afterwards at 4 °C.

Table 2.17 Purification of polypeptides

TZNK [a,b]	**TZNK/β/T**	**GST elution buffer** [c]	**PBS, pH 7.4** [a, b]
50 mM Tris-HCl pH 8.5	TZNK	15 mM reduced glutathione	140 mM NaCl
12 mM NaCl	10 mM β-Mercapto-ethanol	50 mM Tris-HCl pH 8.0	30 mM KCl
100 µM ZnAc	0. 1 % Triton X-100	100 µM Zn acetate	10 mM Na_2HPO_4
150 mM KCl			2 mM KH_2PO_4
2.0 mM $MgCl_2$			

[a] Stored at 4 °C. The buffers should not be more than 2-3 months
[b] Sterilized by autoclaving
[c] Stored in aliquots of 2 ml at –20 °C

Table 2.18 Nucleosome binding assays

$TGDZ_{80}$ [a]		**Elution buffer** [b]	**4.5 % PAA gel**
20 mM TrisHCl pH 8.0	Autoclaved	15 mM Reduced glutathione	0.4 x TBnoE [c]
80 mM NaCl		50 mM TrisHCl pH 8.0	4.5 % (v/v) Acrylamide
0.1 % Triton X-100	Added through steril filter		0.17 % (v/v) APS
1 mM DTT			0.07 % TEMED
100 µM ZnAc			
1 mM L-glutamat			

[a] Stored at 4 °C. The buffer should not be older than 2-3 months.
[b] Stored at –20 °C
[c] TBE (Table 2.15) without EDTA

METHODS

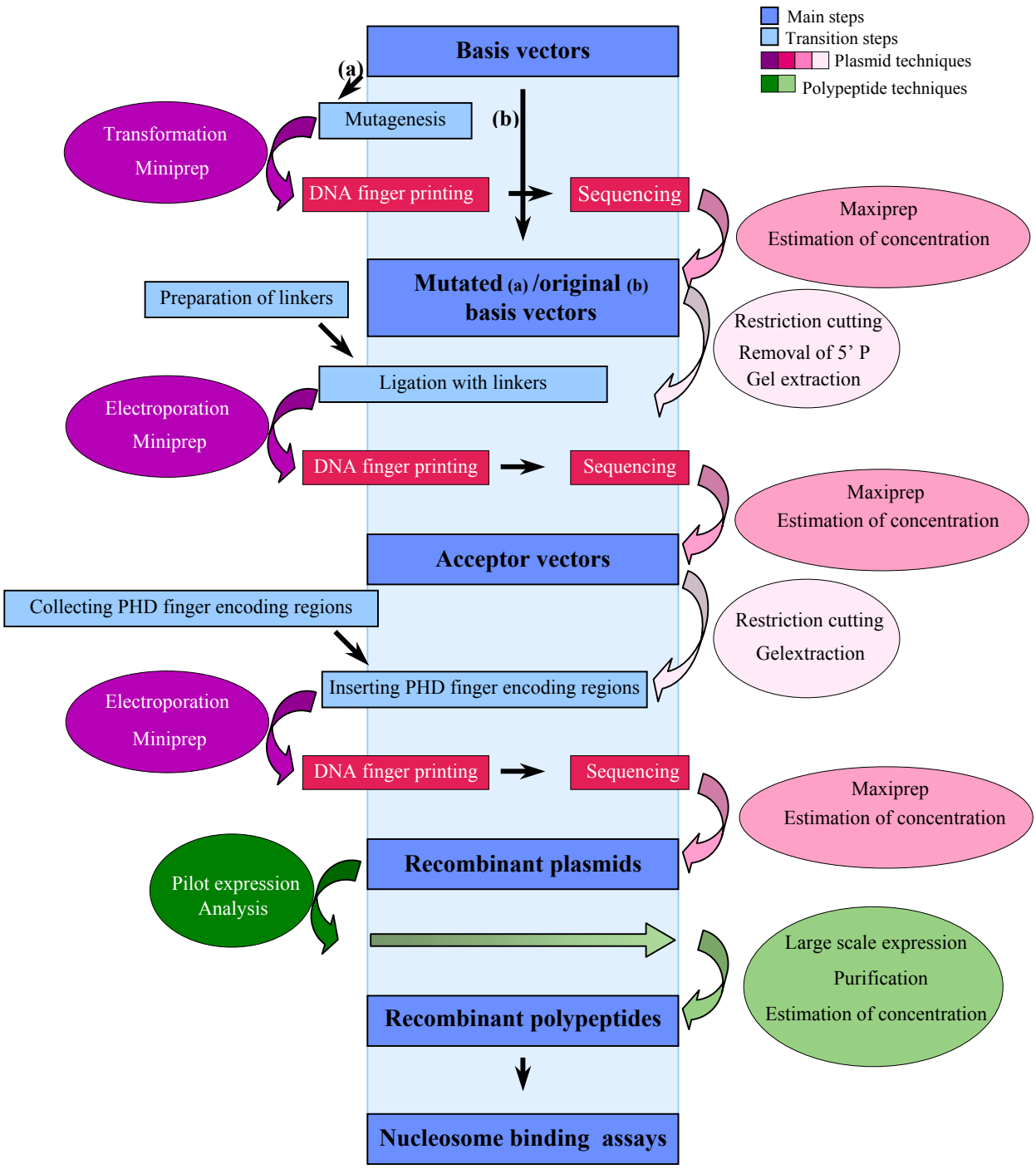


Figure 3.1 Overview of methods and experiments. Main steps are shown in dark blue, transition steps in light blue, and techniques for vector construction and polypeptides in purple and green respectively.

3.1 Site-directed mutagenesis

To remove an *Eco*R I site in pSXG-TIFB that interfered with the vector construction (see Section 4.1.1), a point mutation was introduced in the *Eco*R I recognition sequence using QuikChange® Site-Directed Mutagenesis Kit. Primers with mismatches were designed so that the PCR product would get a silent point mutation (see Figure 4.3). An explanation of the principles of the kit is given in Figure 3.2.

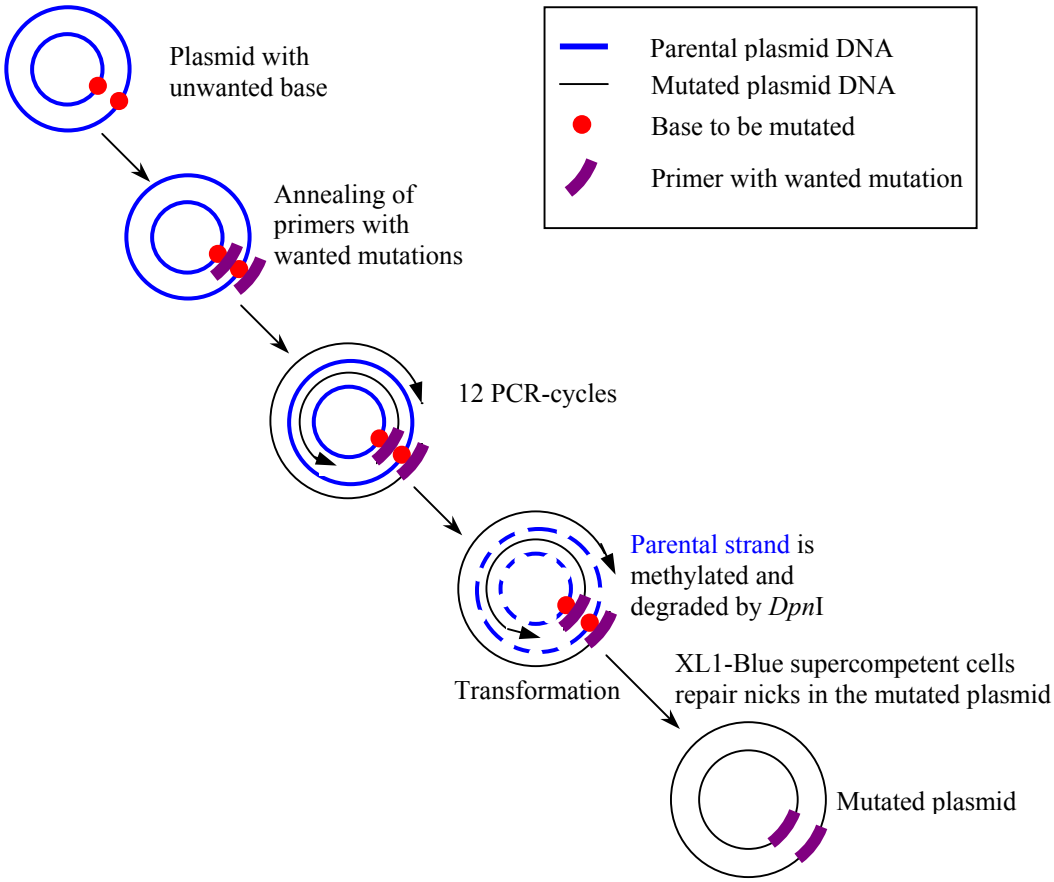


Figure 3.2 Site directed mutagenesis. The figure shows how a site directed mutation is achieved by the QuikChange® Site-Directed Mutagenesis Kit. Primers with wanted mutation anneal to the plasmid and the plasmid DNA is amplified by rounds of PCR. The parental DNA strand is removed from the reaction mixture by digestion with the enzyme *Dpn* I. *Dpn* I is specific for methylated and hemimethylated DNA and will therefore digest only the parental strand, witch is methylated, and not the new strand which has not yet been methylated. The mutated plasmid is transformed into XL1-Blue supercompetent cells for amplification and repairing of nicks from the PCR cycles. The figure is based on a figure in the mutagenesis protocol.

The primers used for mutagenesis were Upper EcoMut and Lower EcoMut (Table 2.9). The mutagenesis was performed as described in the protocol, using the following PCR conditions: 95 ºC/ 30 sec and 12 cycles of {95 ºC/ 30 sec, 55 ºC/ 1 min and 68 ºC/ 12 min}. After the mutagenesis, the mutated plasmid was transformed into XL1-Blue supercompetent cells by heat shock as described in the protocol, and the sells subjected to the steps outlined in Figure 3.1.

3.2 General techniques used during plasmid construction

3.2.1 Transformation of electrocompetent cells

A newly thawed batch of 40 µl electrocompetent *E. coli* DH5α cells was added 1 µl plasmid DNA (60-300 ng) from a miniprep (3.2.2) or a ligation mixture (3.3.3 and 3.4.2). Plasmids that were used for expression of polypeptides were transformed into *E. coli* BL21 (DE3) pLysS cells (cells are listed in Table 2.2). The mixture was incubated on ice for at least one minute and the sample transferred to a chilled cuvette. Electroporation was performed with a resistance of 200 Ohm, 25 mF capasistance and a pulse of 2.5 kV. After electroporation, 1 ml SOC (Table 2.14) was added, reagents were mixed and the whole volume transferred to a 1.5 ml microfuge tube. The cells were then incubated for 45-60 minutes at 37 ºC and vigorous shaking, and 50 µl of the cell suspension was plated on LB-plates supplemented with ampicillin (100 µg/ml) and IPTG (25 µl 400 mM). Bacterial cells containing plasmids that were used for expression of polypeptides were collected by centrifugation in a microfuge at 13000 rpm for 1 minute and all cells plated on 2 x YT-G plates supplemented with ampicillin (100 µg/ml) and chloramphenicol (25 µl 25 µg/ml in 100 % ethanol). The plates were incubated at 37 °C for at least 16 hours.

3.2.2 Isolation of plamids

Miniprep; small-scale isolation of plasmids

Small-scale isolation of plasmids was performed according to the Promega Plasmid Mini Protocol. Briefly, plasmids were separated from genomic DNA, proteins and cell debris by centrifugation, and isolated by affinity purification. One bacterial colony from an LB plate (3.2.1) was inoculated in 5 ml LB-medium (Table 2.14) with 100 µg/ml ampicillin, and the bacteria were grown for 16 hours at 37 ºC and shaking at 250 rpm. Plasmids were isolated from 3 ml cell culture, using a vacuum manifold, according to the Promega protocol. Plasmids were stored at –20 °C.

Maxiprep; large-scale isolation of plasmids

Large-scale isolation of plasmids was performed using the QIAGEN Plasmid Maxi Protocol, which is based on separating precipitated genomic DNA, proteins and cell debris from plasmids through a cartridge, followed by affinity purification of plasmids. One bacterial colony from an LB plate was inoculated in 5 ml LB-medium with 100 µg/ml ampicillin. After inoculation, the cells were grown at 37 ºC with shaking at 250 rpm for 6-8 hours and 100 µl cell was culture transferred to an erlenmeyer in a 1:1000 dilution in LB containing 100 µg/µl ampicillin. The bacterial cells were grown for at least 16 hours at 37 °C with shaking at 250 rpm and plasmids purified from 100 ml cell culture according to the QIAGEN protocol. Plasmids were stored at –20 °C.

3.2.3 Restriction digestion of plasmids

For DNA fingerprinting

New plasmid constructs were analysed by DNA fingerprinting. Plasmids were treated with one or more enzymes giving a pattern different from the parental construct. The reactions were performed in a total volume of 10 µl containing 100-300 ng DNA, 1 µl of each enzyme and 1 x appropriate digestion buffer (Table 2.8). Digestion was performed at 37 °C for 1 hour and the digestion pattern analysed on a 1 % agarose gel {7 x 10 x 0.5}[a] that was run at 70 V for about an hour in 0.5 x TBE.

For further vector construction

When linearizing a plasmid used for vector construction (as in Section 3.3.3), or when cutting out a fragment of DNA (as in Section 3.4.1), restriction digestion was performed in a total volume of 50 µl. The digestion mixtures contained 1-4 µg plasmid, 2-3 µl of each enzyme and 1 x appropriate digestion buffer, and were incubated at 37 °C for 3 hours. The enzymes were inactivated by incubation at 70 °C for 10 min.

3.2.4 Sequencing of plasmids

All new plasmid constructs were, in addition to fingerprinting, verified by DNA sequencing. Sequencing reactions were performed in a total volume of 10 µl containing 200-500 ng plasmid, 2.8 pmol appropriate primer (Table 2.9) and 4 µl Big Dye sequencing cocktail (Table 2.8). The sequencing was based on the chain termination principle and the termination nucleotides contained fluorescent tags detected with a Seq ABI 377 (ABI PRISM) in the sequencing facility. The PCR program used for sequencing was 96 °C/ 5 min and 25 cycles of {96 °C/ 10 sec, 50 °C/ 5 sec and 60 °C/ 4 min}.

3.2.5 Estimating DNA concentrations

By spectrophotometer

Plasmid solutions were diluted 1:100 in ddH_2O and absorbance measured at 260 and 280 nm in a GeneQuant II RNA/DNA calculator spectrophotometer (Table 2.13). DNA concentrations were calculated assuming that 1 A_{260} unit corresponds to 50 µg/ml DNA. The purity of the sample was evaluated assuming that pure DNA has an A_{260}/A_{280} ratio of 1.83.

By agarose-gel electrophoresis

In order to prevent the retention that circular plasmids show on an agarose gel, plasmids were linearized (as in 3.2.3) with a suitable restriction enzyme before analysis on a 1 % agarose gel. A parallel with unlinearized plasmid was included on the gel as a control to verify that the test plasmid had been linearized. The electrophoresis was performed as in

[a] Length x breadth x thickness in cm

3.2.3. A band of the molecular weight standard (MWS) φX174 *Hae* III that was similar in intensity to the plasmid band was used to calculate concentrations:

$$\text{DNA in MWS band [ng]} = \frac{\text{Length of φX174 } \textit{Hae} \text{ III fragment (bp)}}{\text{Length of φX174 (bp)}} \times \text{total MWS [ng]}$$

$$\text{Plasmid concentration [ng/µl]} = \frac{\text{DNA in MWS band [ng]}}{\text{Volume of plasmid sample [µl]}}$$

Final estimations of DNA concentrations were based both on the results from the spectrophotometric measurements and estimation of concentrations by electrophoresis. In my hands, the most reliable, though not totally precise data were obtained by estimating concentrations from the gel, because of considerable variations in the specrophotometric measurements.

3.2.6 Removing 5' phosphate groups

Vectors digested with only one enzyme can easily religate. To avoid religation, free 5' phosphate groups at the ends of these plasmids were removed with Calf Intestine Alcaline Phosphatase (CIAP, Table 2.7) immediately after digestion. Reactions were performed in a total volume of 100 µl containing 1-4 µg linearized plasmid, 1 x CIAP-buffer (Table 2.8) and 0.5 U CIAP. The mixture was incubated for 30 minutes at 37 °C and the enzyme inactivated by incubation at 70 °C for 10 minutes. Plasmids opened with two different enzymes (as in 3.4.2) were not treated with CIAP since these plasmids are unlikely to religate.

3.2.7 Extraction of DNA from agarose gels (gel extraction)

In order to remove buffers and enzymes from a DNA sample after restriction cutting or CIAP treatment, or to separate a DNA fragment digested from its mother plasmid, the DNA was purified by gel extraction. The digestion mixture was run on a 1 % agarose gel as in 3.2.3. The DNA bands were identified by visualization under weak UV-light and excised from the gel. Gel extraction was performed according to the protocol of QUIAEX II Agarose Gel Extraction Kit. Briefly, the gel was solubilized and the DNA bound to silica-gel particles. Superfluous media was removed by centrifugation and the DNA eluted in 10 mM TrisHCl pH 8.5. All DNA extracted from gel was stored at 4 ºC.

3.3 Preparing acceptor vectors

3.3.1 Annealing of primers to form linkers

Two different sets of primers were used, SalEco-Upper / SalEco-Lower and MunSal-Upper / MunSal-Lower (Table 2.9). Each upper primer (100 pmol) was mixed with the corresponding lower primer (100 pmol) and incubated at 85 °C for 5 minutes, at room temperature for 30 minutes and finally on ice for 30 minutes. The final concentration of each linker was 5 µM. The linkers (called SE, SalEco-Upper + SalEco-Lower, and MS, MunSal-Upper + MunSal-Lower) were stored at – 20 °C.

3.3.2 Phosphorylation of 5' ends

Each linker was phosphorylated on the 5' ends to ease ligation into plasmids. Phosphorylation was performed in a total volume of 40 µl containing 100 pmol linker, 1 mM ATP, 1 x T4 polynucleotide kinase buffer (Table 2.8) and 6 U T4 polynucleotide kinase (Table 2.7). The reaction mixture was incubated at 37 ºC for 30 minutes and the phosphorylation reaction stopped by adding 2 µl 0.5 M EDTA. The reaction mixture was subsequently treated with 1 vol. phenol:chloroform:IAA[a]. The reagents were mixed by vortexing and the water phase separated from the phenol phase by centrifugation in a biofuge for 3 minutes at 13000 rpm and 4 °C. The water phase was transferred to a fresh 1.5 ml microfuge tube and the linker DNA precipitated with 0.1 vol. 3 M Na acetate (pH 5.2) and 2 vol. cold 100 % ethanol at –80 ºC for 30 minutes. Precipitated DNA was collected by centrifugation for 15 minutes as above. The DNA was washed with 70 % ethanol and collected by centrifugation for 15 minutes as above. Finally, the linkers were resuspended in 50 µl 10 mM TrisHCl pH 8.5.

3.3.3 Inserting linkers into basis plasmids

To start the construction of acceptor vectors, the basis vectors were opened in the sites in which the linkers were to be added. pSXG-p300B and pSXG-*m*TIFB (Table 2.4, see also footnote page 55) were linearised with *Sal* I and *Eco*R I, respectively (3.2.3), and subjected to further steps as outlined in Figure 3.1. Ligation of linkers into acceptor vectors was performed as follows: Mixtures with DNA from either *Sal* I cut pSXG-p300B or *Eco*R I cut pSXG-*m*TIFB were made in a total volume of 10 µl containing about 50 ng vector DNA, 3 U T4 DNA ligase, 1 x T4 DNA ligase buffer and appropriate linkers in varying amounts of 0-25 pmol. Mixtures with no linker served as negative controls. Ligation was performed room temperature for ~16 hours and subjected to subsequent steps as indicated in Figure 3.1. The resulting acceptor vectors were named pSXG-p300B*link* and pSXG-TIF*link*B.

[a] The reagents are mixed in ratios of 25:24:1 respectively

3.4 Construction of vectors encoding domain recombinants

3.4.1 Isolation of PHD finger encoding sequences

The PHD finger encoding sequences from pSXG-MLLP and pSXG-p300P (Table 2.4) were both isolated from the plasmids by restriction digestion (3.2.3) with *Eco*R I and *Sal* I and purified by gel extraction (3.2.7). To collect PHD encoding sequences from pBSII sk (+) KIAA 0314, TIP5P region, and pBSII sk (+) KIAA 1113, TIF1γP region (Table 2.4), the easiest approach was to use PCR. The primers TIP5Eco5, TIP5Sal3, TIF1γEco5 and TIF1γSal3 (Table 2.9) were designed flanking the PHD encoding sequences and with 5' ends that after PCR could be digested to get *Sal* I and *Eco*R I ends that fit into the sites of the acceptor plasmids. PCR mixtures were prepared in a total of 50 µl containing 0.5 µg plasmid, 25 nmol of each of the appropriate primers, 20 nmol of each deoxynucleotide (dATP, dCTP, dGTP, dTTP), 0.5 µl 50 x Andvantage cDNA polymerase mix and 1 x cDNA PCR Reaction Buffer (Table 2.7 and 2.8). PCR was performed using this program: 94 ºC/ 2 min, 25 cycles of {94 ºC/ 30 sec, 55 ºC/ 50 sec and 70 ºC/ 1 min} and a hold at 70 ºC for 10 min. The PHD finger encoding sequences were isolated by gel extraction (3.2.7) and given *Sal* I and *Eco*R I compatible ends by restriction digestion (3.2.3).

3.4.2 Inserting PHD finger encoding sequences into acceptor vectors

Acceptor vectors were linearised by restriction digestion (3.2.3), pSXG-TIF*link*B with *Eco*R I and *Xho* I and pSXG-p300B*link* with *Mfe* I (an isoschizomer of *Mun* I,) and *Sal* I (see Figure 4.5 and 4.6). *Mfe* I and *Sal* I do not have any suitable digesion buffers in common. pSXG-p300B*link* was therefore treated with one enzyme at a time, and each digestion purified by gel extraction (3.2.7). Ligation of vectors with PHD finger encoding sequences was performed in a total volume of 10 µl. The ligation mixture contained 50 ng acceptor vector, 10 ng PHD finger encoding DNA, 3 U T4 DNA ligase and 1 x T4 ligase buffer (Table 2.7 and 2.8). Ligation was performed at room temperature for ~16 hours. An overview of the new vector construction is given in Figure 4.1 and the name of the resulting constructs listed in Table 4.1

3.5 General techniques used with polypeptides

3.5.1 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Aliquots of 6 µl from the soluble and the insoluble fractions from pilot expression (3.6), 10 µl polypeptide sample from large scale expressions (3.7) or 10 µl fractions from nucleosome retention assays (3.8.1) were added 1 x Sample buffer (Table 2.16) and the polypeptides denaturated by heating at 95 °C for 5 minutes. The aliquots were analyzed on 12 % SDS-polyacrylamide gels by the method of Laemmli (Laemmli, 1970). As molecular weight standard, 10 µl BenchMark™ Protein Ladder or SDS-PAGE standards, Low range (Table 2.6) was applied. The gel {6 x 8 x 0.05}[a] was run at 200 V for about 50 minutes and polypeptides visualized by staining with Coomassie brilliant blue stain solution by warming to boiling point. Gels were destained in water by warming to boiling point.

3.5.2 Estimating polypeptide concentration

Concentration of polypeptides was estimated using DC Protein Assay from Bio-Rad (Table 2.11). The principle of the assay is based on the elaborated method of Lowry; the BCA (bicinchoninic acid) method (Hill and Straka, 1988). In this method, the peptide and Cu^{2+} ions react to form a coloured product that is increased by the presence of BCA and can be detected by spectrophotometer. The β-mercaptoethanol present in the polypeptide solutions can interfere with this reaction and the kit is designed to tolerate this reagent by preincubation of the proteins with iodoacetamide in large excess.

Dilutions of polypeptides in TZNK/β/T (from 1:10 to 1:1) of unknown concentration and BSA with known concentration (from 200 to 1400 µg/ml) were added to a microplate, and the reaction performed as explained in the protocol, with the volumes adjusted to a microplate (to 1/2000 compared to the protocol which gave; 5 µl iodoacetamide, 25 µl reagent A' and 200 µl reagent B). A Multiscan MS (Table 2.13) was used to measure the absorbance in the microplate and the software 'Ascent Software for iEMSReader' was used to interpret the results, create a standard curve from the BSA dilutions and from this calculate concentrations of polypeptides in the samples. In some cases, concentrations were also estimated by analysis by SDS-PAGE, by comparing bands containing polypeptides with bands containing known dilutions (as above) of BSA.

3.5.3 Concentrating dilute polypeptides

In cases where concentrations of polypeptides were too low for use in nucleosome binding experiments (below 14 µM, see Table 4.3), the polypeptides were concentrated using 'Centricon Centrifugal Filter Devices' (Table 2.12). The devices were used as described in the user guide. All centrifugations were performed in a megafuge at 4 °C. The device was equilibrated with 500 µl TZNK/β/T (Table 2.17) by centrifugation for 8 minutes at 2400 rpm. The polypeptide sample was added to the column and an

[a] Length x breadth x thickness in cm

appropriate amount of buffer removed by centrifugation at 2400 rpm to achieve the desired concentration. Centrifugation for 45 minutes reduced the volume with ¾. Finally, the polypeptides were collected by centrifugation for 2 minutes at 1700 rpm and the new concentration estimated as in 3.5.2.

3.5.4 Preparing 50 % glutathione sepharose 4B in TZNK or PBS

The glutathione sepharose 4B beads (Table 2.10) for purification of polypeptides were delivered as a 75 % solution in ethanol, but were to be used in TZNK (Table 2.17) for polypeptide purification and in PBS (Table 2.17) for nucleosome retention assays. The ethanol was therefore removed by centrifugation and the glutathione sepharose 4B resuspended in TZNK or PBS. A volume of 1.33 ml glutathione sepharose 4B was added to a 15 ml polypropylene tube and separated from the ethanol by centrifugation in a megafuge for 5 minutes at 1700 rpm and 4 °C. The beads were washed twice in 10 ml cold TZNK or PBS and finally resuspended in 1 ml TZNK or PBS. Glutathione sepharose 4B in TZNK or PBS was stored up to a month at 4 °C.

3.5.5 Releasing fusion polypeptide from glutathione sepharose 4B

The GST fusion polypeptide bound to glutathione sepharose 4B (about 500 µl, Section 3.7) was added 1 ml GST elution buffer (Table 2.17) containing reduced glutathione (Table 2.10), and the resuspension transferred to a 2 ml microfuge tube. Elution of polypeptides from the beads was performed for 30 minutes at 75 rpm shaking, and the polypeptides separated from the beads by centrifugation in a biofuge for 1 minute at 13000 rpm and 4 °C. A second elution was performed in 500 µl GST elution buffer. To remove the GST elution buffer (containing the reduced glutathione), the polypeptides were dialysed against TZNK/β/T (Table 2.17). Dialysis was performed in Slide-A-Lyser dialysis cassettes (Table 2.12) at 4 °C for minimum 4 hours with slow stiring and the polypeptides transferred to fresh 2 ml microfuge tubes. All polypeptides were stored at 4 °C. The procedure is overviewed in Figure 3.3.

3.5.6 Cleavage of polypeptides from fusion partner with thrombin

Thrombin was delivered as a solid of 500 U, and was added 1 x PBS to a concentration of 1 U/µl and stored at -80 °C. To release the polypeptide from GST, the fusion complex bound to glutathione sepharose (about 500 µl, Section 3.7) was resuspended in 1 ml TZNK/β/T and the polypeptide cleaved with 50 U thrombin in PBS. Digestion was performed for two hours with shaking at 75 rpm and room temperature. Beads bound to GST were removed by centrifugation in a biofuge for 1 minute at 13000 and 4 °C. Analysis by SDS-PAGE was included to test that the cleavage was complete. The free polypeptides were stored at 4°C. The procedure is overviewed in Figure 3.3.

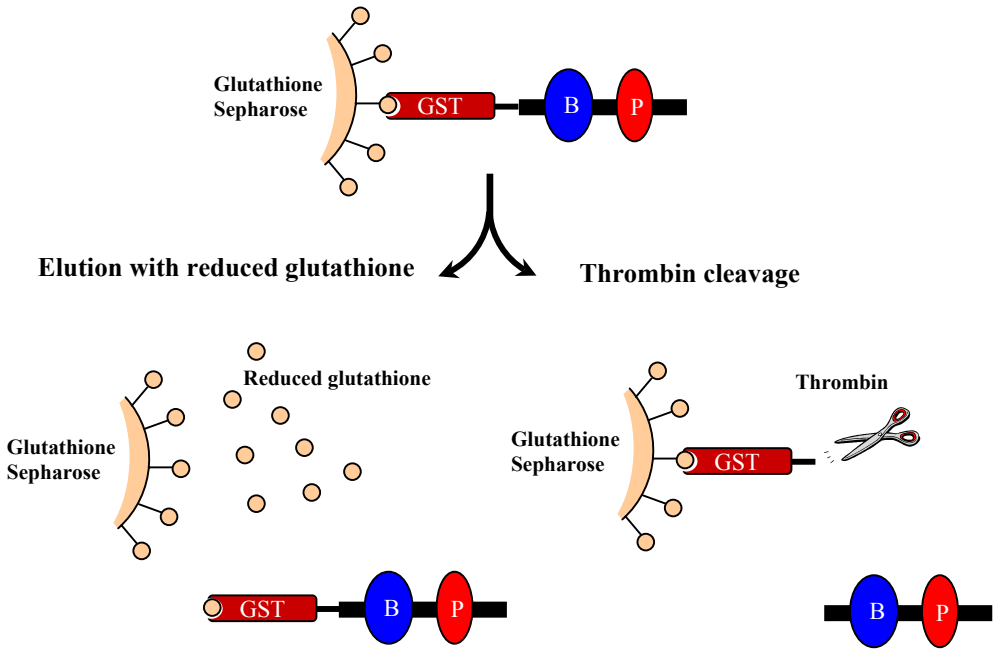


Figure 3.3 Two ways of recovering polypeptides from glutathione sepharose. Polypeptides fused to GST were either eluted from sepharose with reduced glutathione (for nucleosome retention assay, Section 3.8.1) or cleaved from the fusion partner by thrombin treatment (for EMSA, Section 3.8.2).

3.5.7 Scintillation counting

Aliquots of 50 µl of fractions from the nucleosome retention assay (IN-N, FT-N, W1-W5, E1-E3 and R), including a sample only with 50 µl $TGDZ_{80}$ (negative control), were added to a 5 ml polyethylene scintillation tube, mixed with 4.5 ml UltimaGold LSC (Table 2.7) and analysed for radioactivity by counting disintegrations of ^{3}H-atoms in a scintillation counter. The energy range measured in the counting channel was 0–9 keV and in the correction channel 2-19 keV. The radioactivity in the input material was set to 100 %. Relative radioactivity in each fraction from the nucleosome retention assay was calculated as percent of input.

3.6 Pilot expression

To determine the expression conditions giving the largest amount of soluble polypeptide, pilot expression was performed for all the domain recombinants. For each temperature to be tested, about 50 colonies from a 2 x YT-plate (Section 3.2.1) was inoculated in 5 ml 2 x YT-G containing 100 µg/ml ampicillin, 25 µg/ml chloramphenicol and 2 µM Zn acetate. Cells were grown at 37 °C with shaking at 250 rpm. At the OD_{600}s 0.2, 0.4 and 0.7, two aliquots of 1.5 ml were taken from the culture for expression of proteins, one treated with 0,4 mM IPTG (for induction of the gene encoding the GST fusion polypeptide) and one negative control. Parallels of polypeptides were expressed for two hours at 26 °C or 37 °C. Polypeptides tested at 18 °C were expressed for ~16 hours. Bacterial cells in 1 ml sample were then collected in a 1.5 ml microfuge tube by centrifugation in a biofuge for 3 minutes at 13000 rpm and 4 °C. Finally, the cells were resuspended in 200 µl TZNK/β/T.

The cells were lyzed by sonication for 30 seconds at 30 - 40 intensity using a 2 mm tip, while kept in ice. Insoluble material was removed by centrifugation in a biofuge for 10 minutes at 13000 rpm and 4 °C. The supernatant (soluble polypeptide fraction) was transferred to a fresh tube and the pellet (insoluble polypeptide fraction) was resuspended in 200 µl TZNK/β/T. All aliquots were analyzed by SDS-PAGE (3.6.1).

3.7 Large scale expression and -purification of polypeptides

All colonies from a 2 x YT-plate (3.2.1) (>1000 colonies) were inoculated in 1000 ml 2 x YT-G (Table 2.14) containing 100 µg/ml ampicillin and 25 µg/ml chloramphenicol. The bacterial cells were incubated at 37 °C until the optimal OD_{600} of 0.7 (see Table 4.2) was reached. Expression of GST fusion polypeptides was induced by adding IPTG to 0.4 mM and Zn acetate to 2 µM. The polypeptides were then expressed at their optimal temperature of 26 °C (see Table 4.2) (37 °C for GST-p300BP, GST-TIFPB and GST) for two hours. The bacterial culture was divided on four GSA tubes and the cells harvested in a Sorvall centrifuge (Table 2.13) for 5 minutes at 8000 rpm and 4 °C, using a GSA rotor. The surface of each pellet was carefully washed twice with 5 ml cold 1 x PBS and the pellet resuspended in 3.75 ml cold 15 % glycerol in PBS. The cell suspension was divided on three 50 ml tubes and stored at –20° C over night.

The cell suspension was defrosted on ice and cells collected by centrifugation in a megafuge for 10 minutes at 5400 rpm and 4 °C. Each pellet was washed once by resuspention in 10 ml cold 1 x PBS and the cells collected by centrifugation as above. The pellets were then resuspended in 10 ml cold TZNK/β/T, collected in two 50 ml tubes and the volume adjusted to 25 ml with TZNK/β/T. The cells were lysed in the French Press (Table 2.13) by passing twice at 650 psi (pounds per inch), continuously kept on ice. To prevent precipitation, the lysate was incubated with a final concentration of 1 % Triton X-100 (Table 2.10) for 30 minutes at 75 rpm shaking and room temperature. The lysate was then transferred to a fresh tube and cell debris and insoluble polypeptides were removed by centrifugation in a Sorvall centrifuge for 3 minutes at 10000 rpm and 4 °C, using an SS-34 rotor.

The supernatant containing the polypeptides was transferred to a 50 ml tube and added 1 ml 50 % glutathione sepharose 4B in TZNK (3.5.4). The binding of the GST-tag to glutathione sepharose was allowed for 30 minutes at 50 rpm shaking and room temperature. The beads with polypeptides were collected by centrifugation in a megafuge for 6 minutes at 3500 rpm and 4 °C. The beads were washed twice with 20 ml TZNK/β/T with centrifugations as above, resulting in about 500 µl GST-fusion polypeptide bound to glutathione sehparose. At this stage, the polypeptides were either eluted from sepharose by free glutathione and kept bound to GST (3.5.5), or they were treated with thrombin to be released from GST (3.5.6), as shown in Figure 3.3. Size and quality of purified polypeptides were analysed by SDS-PAGE.

3.8 Nucleosome binding assays

3.8.1 Nucleosome retention assay

In the nucleosome retention assay, polypeptides are bound to glutathione sepharose 4B via the GST tag and ^{3}H labelled nucleosomes applied. The binding reaction is performed in Amicon filter units, 20 µm filter (Table 2.12) placed in 1.5 ml microfuge tubes. By elution of polypeptides from glutathione sepharose, the retained nucleosomes can be detected by determining the radioactivity in the eluted material. The experiment is performed at room temperature, but all buffers added are cold (4 °C) except for the Elution buffer (Table 2.18), which has room temperature. An overview of the assay is given in Figure 3.4.

The filter in the Amicon filter unit was moistened with 50 µl TZNK/β/T pH 8.5 before adding 150 µl 50 % slurry of Glutathione Sepharose beads in 1 x PBS (3.5.4). Redundant fluid was removed by centrifugation. All centrifugations in the assay were performed in a nanofuge at 6000 rpm for about 20 seconds. When adding solutions and during incubations, the tubes were rolled several times to ensure that the beads of glutathione sepharose were properly covered with solution. The beads were washed once with 50 µl TZNK/β/T pH 8.5 before adding 200 µl 12-20 µM polypeptide (see Table 4.3), polypeptide input (IN-P), to attach to the beads. The binding was allowed for 1 hour. Non-binding polypeptides, polypeptide flow through (FT-P), was removed by centrifugation and kept for analysis. The beads with bound polypeptides were washed with 200 µl $TGDZ_{80}$ pH 8.0. To coat the beads not bound to polypeptides, 150 µl BSA-Ac (Table 2.10), diluted to 10 ng/µl in $TGDZ_{80}$, was added and allowed to bind for 30 minutes. The flow through was discarded. Nucleosomes, 200 µl 15-20 nM, labelled with ^{3}H (Table 2.3), nucleosome input (IN-N), were then added to the polypeptides on the beads and the reaction incubated for 1 hour. Non-bound nucleosomes, nucleosome flow through (FT-N), were kept for analysis. Bound material was washed four times with 200 µl $TGDZ_{80}$ (Table 2.18) and once with 200 µl 50 mM Tris-HCl pH 8.0, with incubation for 10 minutes at each washing. All wash fractions (W1-W5) were kept for analysis. The polypeptides bound (or not bound) to nucleosomes were then eluted with 200 µl Elution buffer (Table 2.18). Three elutions were performed, each with 20 minutes incubation. During elution, the mixture was gently vortexed every 5 minutes. Each fraction was kept for analysis (E1-E3). Finally, the beads left on the filter were resuspended in 200 µl Elution buffer and kept for analysis (R). The fractions were analysed by scintillation counting (3.5.7) and SDS-PAGE (3.5.1) and kept at –20 °C.

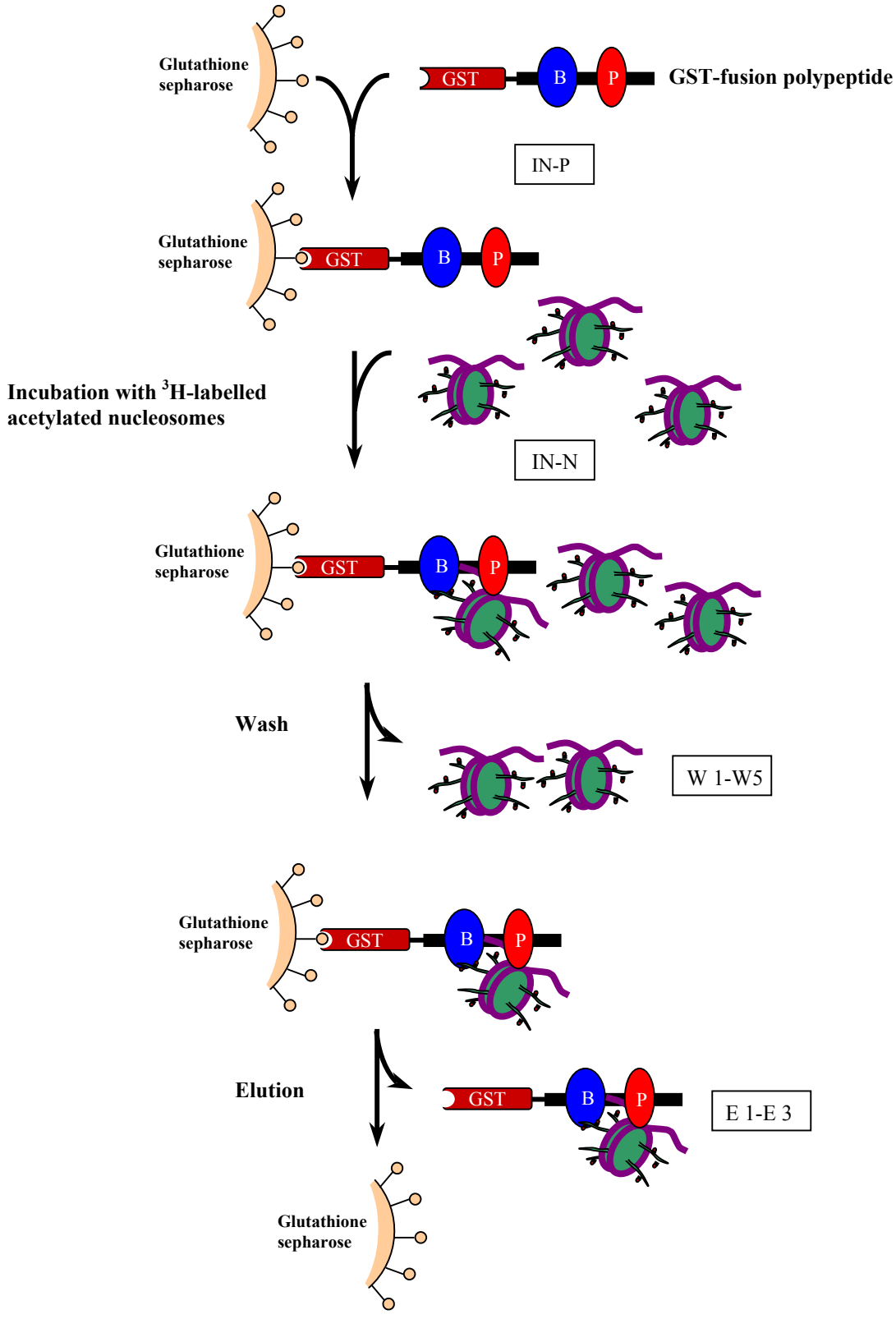


Figure 3.4 Overview of the main steps of the nucleosome retention assay.

3.8.2 Electrophoretic Mobility Shift Assay (EMSA)

EMSA is a method for detecting the interaction between a protein and DNA, or as in this case, nucleosomes. The protein is incubated with ^{32}P-labelled nucleosomes (Table 2.3). The protein-nucleosome complexes are separated from the free nucleosomes by migration through a native polyacrylamide (PAA) gel, with the complexes migrating more slowly.

The glass plates used for electrophoresis were siliconated with 4 % TMCS (Table 2.10) in chloroform and polymerisation of the native 4.5 % PAA gel (Table 2.18) was performed o/n. The gel was prerun at 100 V for at least 1 hour in 0.4 x TBnoE (TBE, Table 2.15 without EDTA). A mastermix was made sufficient for the number of reactions containing all reagents except polypeptide. For each binding reaction, the mixture contained 2 µl $TGDZ_{80}$, ~2.5 fmol ^{32}P- labelled nucleosomes (in my case 3 µl) and 0.3 µl 20 ng/µl CEA (Table 2.10). The mastermix (5 µl for each binding reaction) was added the polypeptides GST-TIFPB and TIFPBt (Table 4.3) in amounts of 100, 300, 500, 700 and 1000 pmol. A control reaction was included containing only mastermix. Reaction volumes with a total volume less than 20 µl were added $TGDZ_{80}$ to increase the volume to 20 µl. The reaction volumes were thus between 20 µl and 80 µl.

Binding was performed at room temperature for 20 minutes on a platform rocker at 20 rev/min. Reaction mixtures were added 5 % glycerol and the entire reaction loaded on the native 4.5 % PAA gel. As positive and negative controls, 1000 pmol p300BPt and GST (not in Table 4.3, purified by Anja Ragvin) were used, respectively. To track the electrophoresis, 6 µl 6 x loading buffer (Table 2.15) was added in two empty wells. The gel was run at 60 V for 1 hour and then at 100 V for about 5 hours, it was dried and subsequently exposed on a 20 x 40 cm BAS Imaging Plate for 24 hours. The BAS Imaging plate was scanned using a FLA-2000 IP / Fluorescent Reader (Table 2.13) as described in the manual of MacBAS (Bio Imaging Analysing System) V2.x (Fujifilm). The scanned image was read and analysed using the software Image Reader V1.8J and Image Gauge V3.41 respectively. The softwares are described in the MacBAS V2.x operation manual.

RESULTS

The aim of the project has been to test whether heterologous PHD fingers are able to replace the one in p300BP without loosing the nucleosome binding activity. Different domain recombinants were made containing PHD fingers from three heterologous proteins, using gene technology (Figure 4.1). The domain recombinants were expressed as GST-fusion polypeptides and tested for nucleosome binding by the nucleosome retention assay (Section 3.8.1). Domain recombinants were also made by swapping the PHD finger in TIFPB. The results are divided in three parts. The first part includes the results from the vector construction and the expression of polypeptides from these. The second part includes the testing of nucleosome binding of polypeptides expressed from the control constructs and the last part shows the results from nucleosome binding of the domain recombinants.

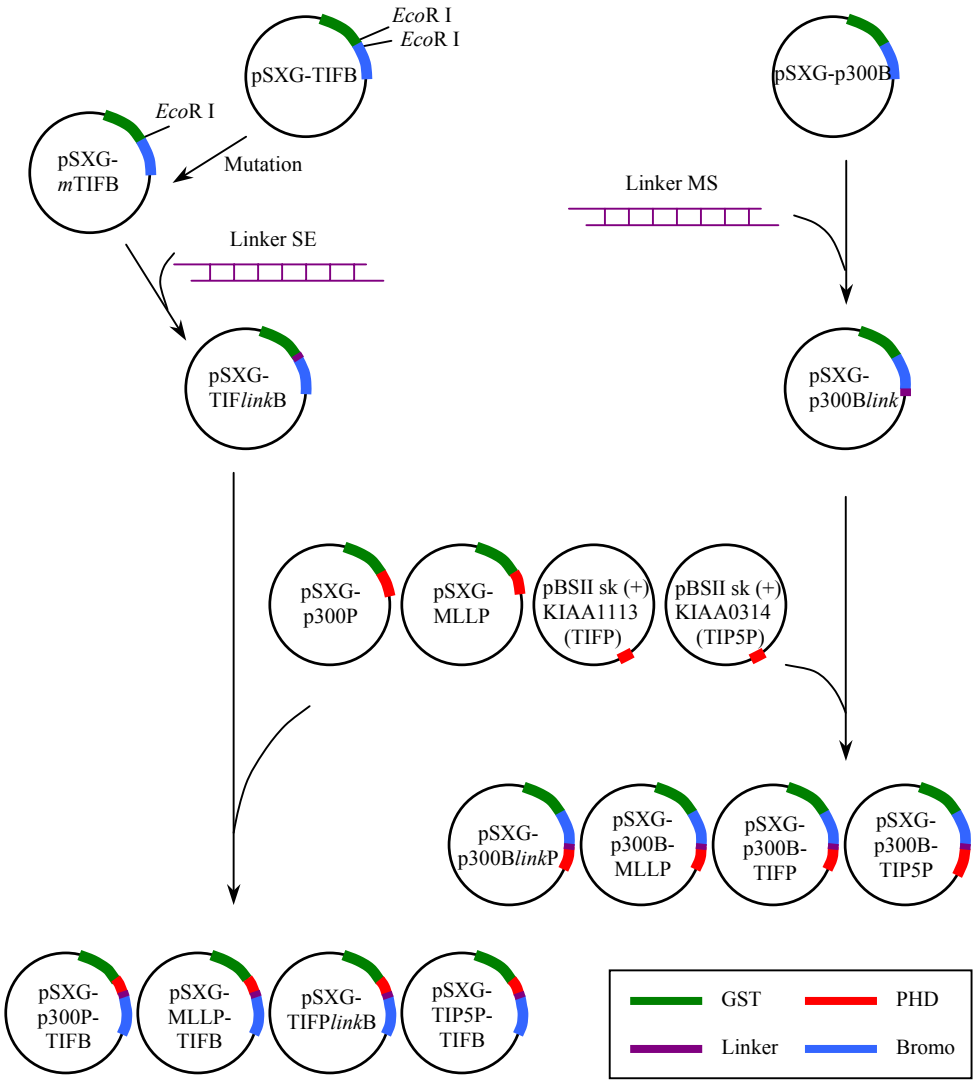


Figure 4.1 Construction of vectors encoding domain recombinants. The figure shows the procedure used to swap the PHD finger encoding regions.

PART I, VECTOR CONSTRUCTION AND POLYPEPTIDE EXPRESSION

4.1 Preparing acceptor vectors[a]

To make the domain swapping[b] easier, the basis vectors pSXG-p300B (the vector coding for the bromodomain region of p300) and pSXG-TIFB (the vector coding for the bromodomain region of TIF1γ) were added linkers with appropriate restriction sites for insertion of PHD finger encoding regions. This generated the acceptor vectors pSXG-p300B*link* and pSXG-TIF*link*B.

4.1.1 Introduction of a silent mutation in pSXG-TIFB

The linker in pSXG-TIFB was to be added in the *Eco*R I site in position 957-962. An additional *Eco*R I site existed in position 1071-1076 (Figure 4.2). This site therefore had to be mutated before starting the vector construction with pSXG-TIFB. The mutation was introduced using site directed mutagenesis (Section 3.1).

Choice of strategy

Instead of using mutagenesis, two alternative strategies were considered. (a) A new linker could have been designed, a linker that could be inserted into another site than *Eco*R I. The linker would have to contain new restriction sites for insertion of PHD finger encoding sequences, which in their turn would have to have ends corresponding to the new sites. Most primers (for linker and for PCR reactions) were already available, and this alternative would demand a range of different new primers. (b) The vector construction could have been done by insertion of sequences in a different succession, starting with an empty pSXG. The linker would have to be inserted first, followed by the PHD finger encoding sequences and finally the sequence encoding the bromodomain (containing the *Eco*R I site). This was not an ideal solution because the additional *Eco*R I site would still be present within the bromodomain encoding sequence (this also accounts for alternative a), and the enzyme *Eco*R I could not be used at later stages if further vector construction should be desirable. The alternative (c) of mutating the *Eco*R I site within the bromodomain was therefore chosen.

[a] Acceptor vector refers to a plasmid (pSXG-p300B*link* or pSXG-TIF*link*B) that is to accept a sequence encoding a PHD finger.
[b] Domain swapping refers to the method of swapping a domain from one protein with the corresponding domain from a foreign protein at the DNA level using plasmids, in these experiments the swapped domain is a PHD finger.

Site directed mutagenesis

To make the mutation, primers, Upper EcoMut and Lower EcoMut, were designed containing a base mismatching a base in the *Eco*R I site of the plasmid sequence. Lower EcoMut contained a cytosine mismatching adenine number 1073 in the plasmid and Upper EcoMut contained a guanine mismatching the thymine 1073 (Figure 4.2). A mutagenesis kit was used to introduce a site directed, silent point mutation without destroying the reading frame for the bromodomain.

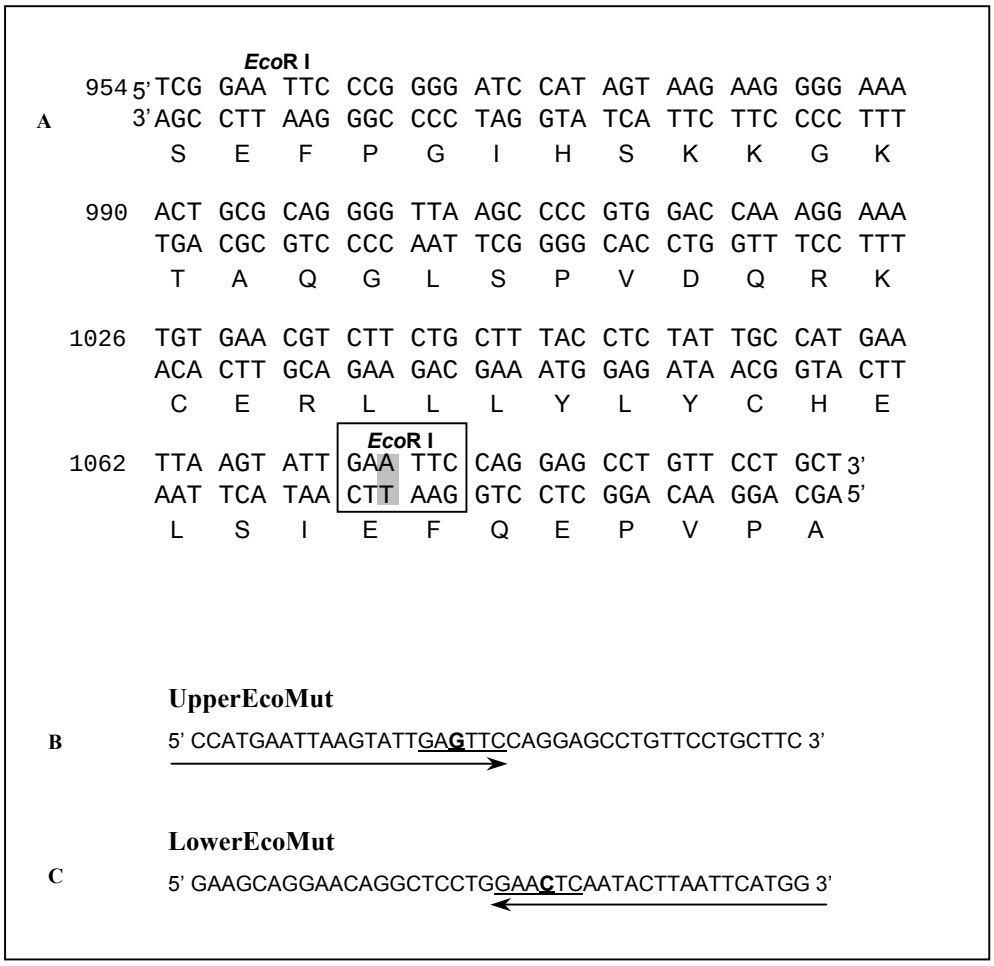
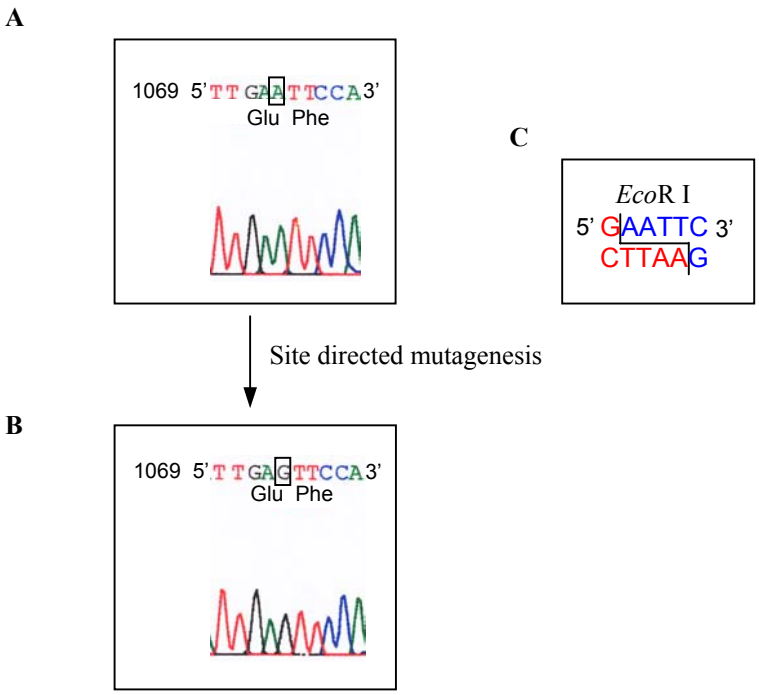


Figure 4.2 Plasmid sequence of pSXG-TIFB, and primers for site directed mutagenesis. (A) The sequence of pSXG-TIFB with the two *Eco*R I sites. The *Eco*R I site to be mutated is marked with a square and the bases to be mutated are shaded in grey. **(B)** The primer Upper EcoMut (anneals to lower strand of the plasmid) contained a mismatch of **guanine** against adenine of the original sequence. **(C)** The primer Lower EcoMut (anneals with upper strand of the plasmid) contained a mismatch of **cytosine** against thymine of the original sequence. The arrows indicate the direction in which the primers anneal.

The mutation was confirmed by DNA sequencing and sequence alignment of the mutated plasmid with the original plasmid. As can be seen in Figure 4.3, the mutagenesis procedure had been successful and the desired mutation of adenine 1073 to guanine was obtained. The mutated plasmid was called pSXG-*m*TIFB[a] and was used in subsequent cloning steps.

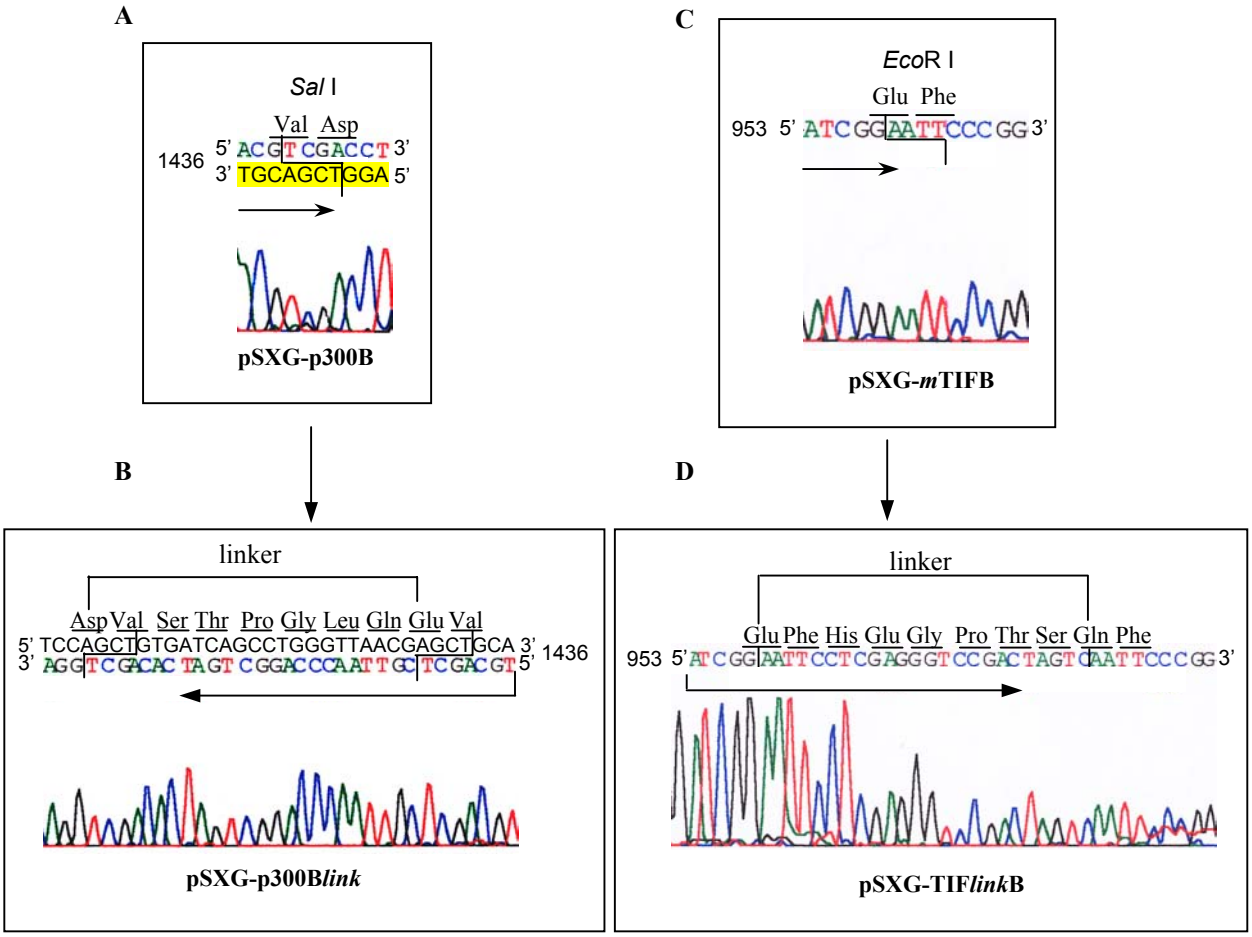


Figur 4.3 Verification of the mutation of A to G in pSXG-TIFB by DNA sequencing. **(A)** Part of the sequence of pSXG-TIFB before mutagenesis. The adenine to be mutated is indicated. **(B)** Sequencing results of pSXG-TIFB after site directed mutagenesis. A silent mutation was made in the sequence. The sequence still encodes a glutamine after mutation from adenine to guanine. **(C)** The recognition site of *Eco*R I for comparison with the mutated and the non-mutated sequences.

4.1.2. Construction of acceptor vectors, pSXG-p300B*link* and pSXG-TIF*link*B

The vector pSXG-p300B was opened in the *Sal* I site, position 1437, and pSXG-*m*TIFB was opened in the *Eco*R I site, position 957. The linkers MS and SE were made by ligation of two primers, as described in Section 3.3.1. To make acceptor vectors, the linker MS was ligated into the *Sal* I site of pSXG-p300B and SE was ligated into the *Eco*R I site of pSXG-*m*TIFB. The resulting acceptor vectors, pSXG-TIF*link*B and pSXG-p300B*link*, now contained new unique *Xho* I and *Mun* I restriction sites respectively to be used in further vector construction. The acceptor vectors were eventually sequenced (for the whole process, see Figure 3.1), to verify that only one linker had been inserted and that it had been inserted in the correct orientation (the linkers had palindromic ends). The results from the sequencing are given in Figure 4.4. pSXG-p300B*link* and pSXG-TIF*link*B were used as acceptor vectors in subsequent cloning steps for domain swapping.

[a] All further constructs with bromodomain from TIF1γ were from this stage based on pSXG-*m*TIFB. For this reason, the *m* indicating the mutation is left out when mentioning constructs originating from pSXG-*m*TIFB, for example pSXG-TIFB*link*P and pSXG-p300P-TIFB.



Figur 4.4 Confirmation of acceptor vectors by DNA sequencing. (A) Part of the sequence from pSXG-p300B before inserting linker. The linker was inserted in the *Sal* I site. To ease the comparison with B, complementary sequence is outlined in yellow and amino acids are indicated. **(B)** Sequencing results of pSXG-p300B*link*. Note that the sequence must be read from right to left and compared with the complementary sequence outlined in yellow in A. The amino acids Val and Asp have been separated by the linker. **(C)** Part of the sequence of pSXG-*m*TIFB inserting linker. The linker was inserted in the *Eco*R I site. Amino acids are indicated to ease the comparison with D. **(D)** Sequencing results of pSXG-TIF*link*B. The two amino acids Glu and Phe have been separated by the linker.

4.2 Construction of vectors for expression of domain recombinants

PHD finger encoding sequences

The presence of the entire amino acid sequence might be important for whether the PHD finger will function or not. A strategy has been used in the group to make sure that the whole sequence is present: The outer limits of the conserved pattern (see Section 1.4.6) are first detected. Next, the regions flanking the domain that seem flexible (regions of low conservation between different species and containing few hydrophobic residues and many polar residues; Glu, Ser, Thr and also Pro) are determined. In these flexible regions, the boundaries are placed. The whole PHD finger is therefore assumed to be present in the recombinant polypeptides.

Domain swapping

The construction of recombinant plasmids based on pSXG-TIFB was performed in parallel with the construction of plasmids based on pSXG-p300B, therefore domain recombinants of pSXG-TIF*link*B were made even though the wild type pSXG-TIFPB had not been tested for nucleosome binding. An overview of the vector construction is given in Figure 4.5 and 4.6. To make domain recombinants, the two acceptor vectors pSXG-p300B*link* and pSXG-TIF*link*B were opened with restriction enzymes within the linker, the first vector with *Mun* I (position 1441) and *Sal* I (position 1459) and the latter with *Eco*R I (position 957) and *Xho* I (position 963). Two PHD finger sequences were removed from a donor plasmid with enzymes (see Section 3.4.1): The PHD finger encoding sequence in pSXG-MLLP was collected with *Eco*R I (position 957) and *Sal* I (position 1249) and the sequence in pSXG-p300P collected with the same enzymes (position 957 and 1266 respectively). The two last PHD fingers were collected by PCR (see Section 3.4.1); the TIP5P encoding region (position 3696-3918 in pBSII sk (+) KIAA 0314) and the TIF1γP encoding region (position 4499-4697 in pBSII sk (+) KIAA 1113. The sequences were ligated into the acceptor plasmids (Section 3.4.2). The new plasmids and their expressed polypeptides were given names according to where their domains are taken from (Table 4.1.).

Table 4.1 Overview of domain recombinants [a]

PHD / Bromo	p300P	TIFP	MLLP	TIP5P
p300B	-p300B*link*P	-p300B-TIFP	-p300B-MLLP	-p300B-TIP5P
TIFB	-p300P-TIFB	-TIFP*link*B	-MLLP- TIFB	-TIP5P- TIFB

[a] Domain recombinants were given names indicating where their domains are taken from. The names in red indicate a PHD finger and the names in blue indicate a bromodomain. The names are used for both plasmids and polypeptides, with the prefix pSXG- or GST- indicating if it is a plasmid or a GST-fusion polypeptide respectively. For example, pSXG-p300B*link*P refers to a plasmid and GST-p300B*link*P refers to its expressed polypeptide.

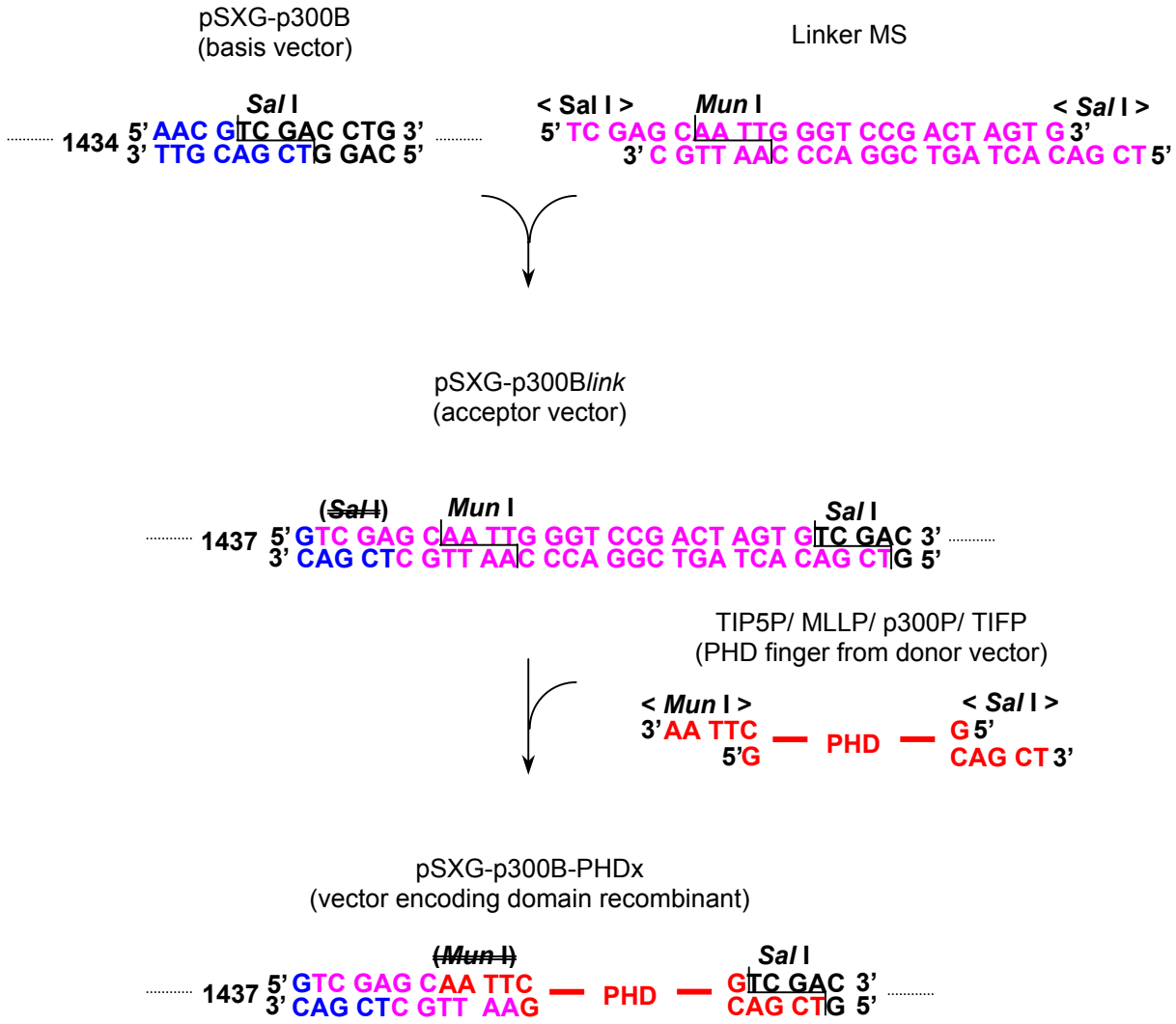


Figure 4.5 Construction of vectors encoding domain recombinants from basis vector pSXG-p300B. The linker MS is inserted in the *Sal* I site of pSXG-p300B, resulting in an acceptor vector. A PHD finger encoding sequence from a donor vector is inserted into the *Mun* I and *Sal* I sites of the acceptor vector resulting in vector encoding a domain recombinant. Blue colour represents the sequence encoding the bromodomain; red the sequence encoding the PHD finger; purple represents sequence of the linker; black represents the area outside the domains. Note that when inserting p300P, part of the between the bromodomain and the PHD finger occurs twice; p300P contains part of this sequence, which is also already present in pSXG-p300B. This results in pSXG-p300B*link*P containing 60 bases more than pSXG-p300BP in addition to the six bases from the linker. The corresponding polypeptide thus contains 33 additional amino acids. Enzyme names shown in arrow brackets indicate compatible sites. Enzyme names in parenthesis, and doubly over-striked, indicate junctions where the restriction sites were not regenerated.

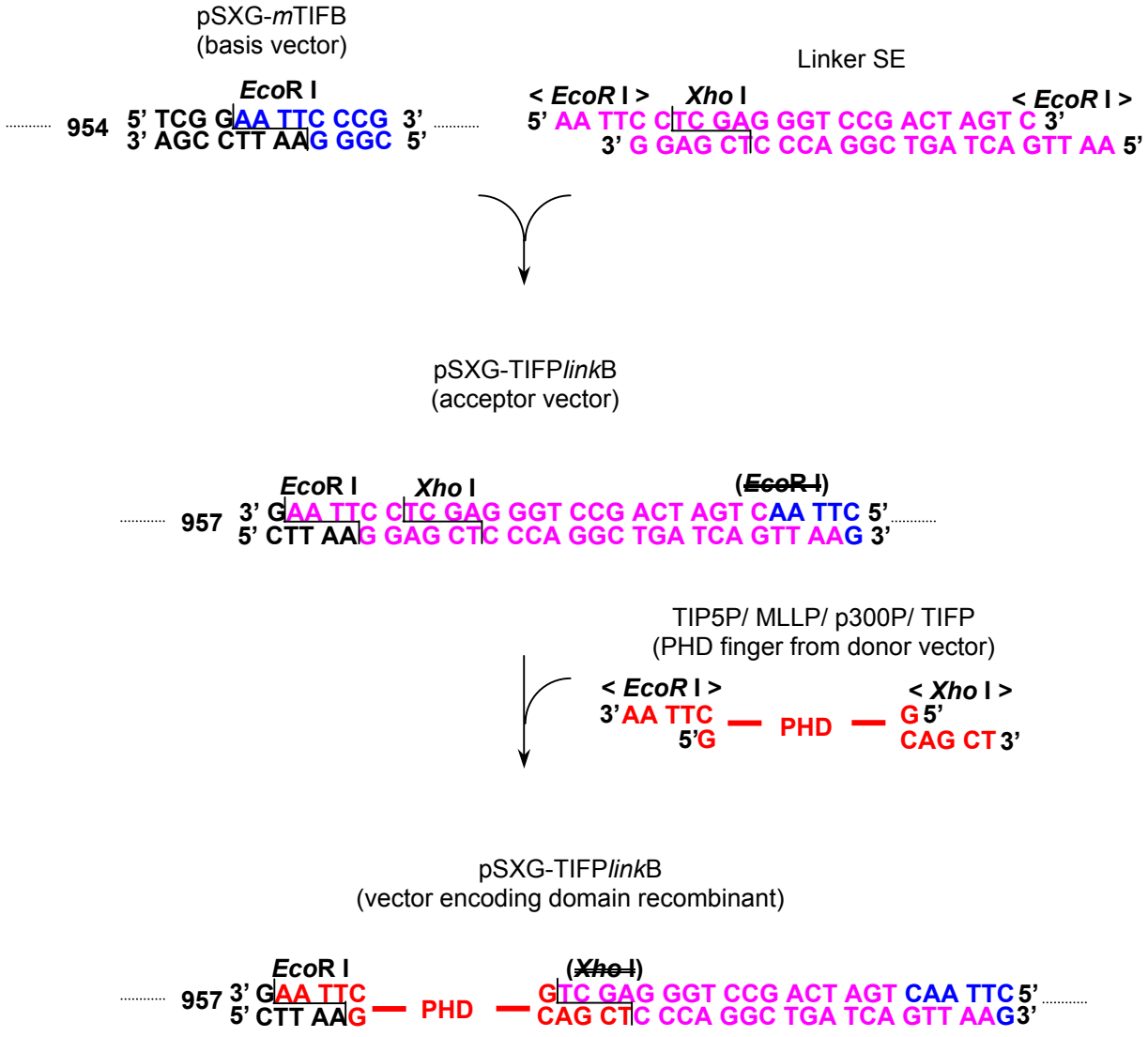
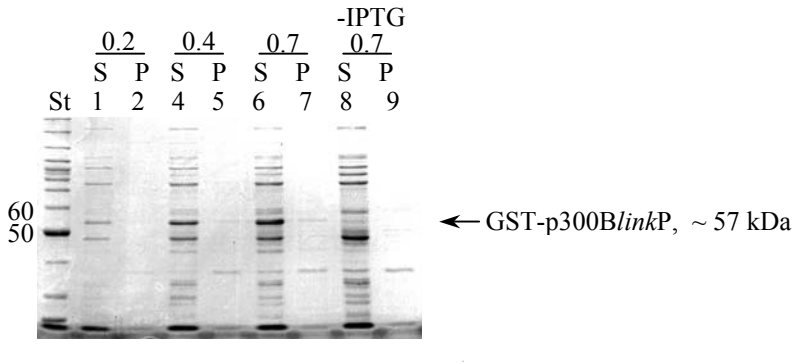


Figure 4.6 Construction of vectors encoding domain recombinants from basis vector pSXG-*m*TIFB. The linker SE is inserted in the *Eco*R I site of pSXG-*m*TIFB, resulting in the acceptor vector pSXG-TIF*link*B. A PHD finger encoding sequence from a donor vector is inserted into the *Eco*R I and *Xho* I sites of the acceptor vector, resulting in a plasmid encoding a domain recombinant. Blue colour represents the sequence encoding the bromodomain; red, the sequence encoding the PHD finger; purple represents the sequence of the linker; black represents the area outside the domains. Sites and junctions are indicated as in Figure 4.5.

4.3 Pilot expression

After the acceptor plasmids had been added the PHD finger encoding sequences, the polypeptides were expressed in small-scale bacterial cultures (pilot expression, Section 3.6) to find the best conditions for producing soluble polypepetides. Different culture densities and incubation temperatures during protein expression were tested since these are known to be critical parameters for protein yield and solubility. The polypeptides were analysed by SDS-PAGE and the size and intensity of the bands from the soluble fractions estimated and compared to find the band of highest intensity. The insoluble fractions indicated how much of the polypeptide that had precipitated. An example of a gel, to show the general amounts expressed, is given in Figure 4.7. An overview of results from pilot expression of all polypeptides is given in Table 4.2 A and 4.2 B.



Figur 4.7 Pilot expression of GST-p300B*link*P at 26 °C. Aliquots of 10 µl from soluble and insoluble fractions in pilot expression were analysed by SDS-PAGE and the gel stained in Coomassie brilliant blue stain solution. The gene encoding the polypeptide was induced for expression at three different cell densities (OD_{600}): 0.2, 0.4 and 0.7. Abbreviations: St, BenchMark™ Protein ladder; S, Supernatants, soluble fractions; P, pellets, insoluble fractions; Lane 8 and 9 are negative controls (polypeptides expressed without the inducer IPTG). The arrow indicates the position of GST-p300B*link*P, which is 57 kDa, and is, as expected, *not* expressed in lanes without IPTG. Relative amounts were estimated by a plus as follows: Lane 1, +; lane 4, ++; lane 6, +++. These signs, representing approximately the same amounts, are used in table 4.2 A and B, to indicate amounts in fractions from pilot expression of the other polypeptides.

Table 4.2 A Pilot expression of domain recombinants based on pSXG-p300B*link* [a]

°C	OD_{600}	MLLP	p300P	TIFP	TIP5P
	0.2			-	-
37 °C	**0.4**		+	-	-
	0.7	++	++	-	-
	0.2		+	+	
26 °C	**0.4**	++	++	++	+
	0.7	++	+++	+++	++
	0.2		-	-	-
18 °C	**0.4**	++	-	-	-
	0.7	+	-	-	-

Table 4.2 B Pilot expression of domain recombinants based on pSGX-TIF*link*B [a]

°C	OD_{600}	MLLP	p300P	TIFP	TIP5P
	0.2				+
26 °C	**0.4**		++	+	+
	0.7	+++	++	+++	++

[a] Note that the prefix GST-p300B- in Table 4.2 A and GST-TIFB- in Table 4.2 B is left out in the tables, only the name of the inserted PHD fingers are indicated. The relative amount of polypeptide is indicated by a plus. The amounts represented by the pluses are shown on the gel in Figure 4.7. The empty spaces in the table mean that no polypeptide appeared on the gel. The squares marked with – mean that expression was not performed under these conditions.

All domain recombinants were expressed as soluble polypeptides in reasonable amounts. For each domain recombinant, the conditions resulting in the largest amount of soluble polypeptide were chosen for large-scale expression. For all domain recombinants, the conditions chosen were culture densities of OD_{600} 0.7 and expression temperatures of 26 °C. Optimal conditions for GST, GST-p300BP and GST-TIFPB had been determined previously in the group, and they were expressed at 37 °C and OD_{600} 0.7.

4.4 Large-scale expression

The polypeptides were expressed in large-scale bacterial cultures (large-scale expression, Section 3.7) at their optimal conditions, purified and analysed by SDS-PAGE to verify that a polypeptide of the correct size was produced (Figure 4.8). Concentrations were calculated by DcProtein Assay from BioRad and the results are shown in Table 4.3 together with the respective sizes of the polypeptides. GST-TIFPB and GST-TIFP*link*B were purified twice and fractions from both preparations are shown on the gel. In Table 4.3, one concentration value is given for each preparation, and the respective usage is indicated behind.

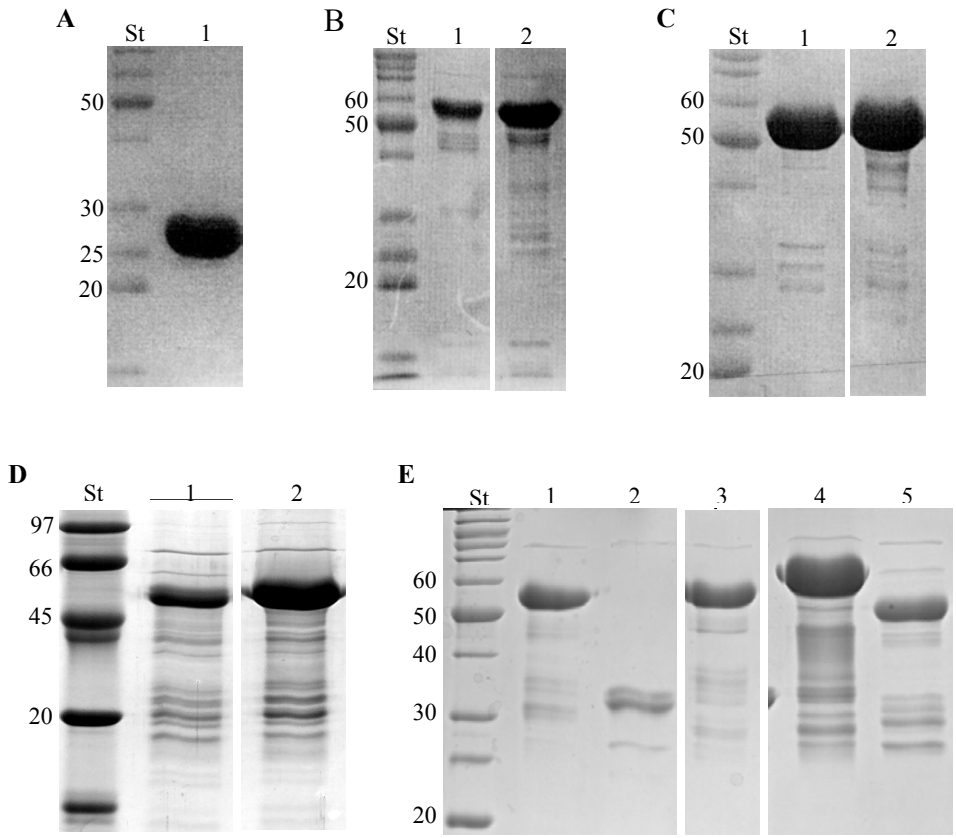


Figure 4.8 Large-scale expression of polypeptides. All polypeptides were purified from *E. coli* lysates (Section 3.7). Aliquots of 10 µl from eluate 1 and 2 (eluate 2 not shown) were analysed by SDS-PAGE (Section 3.5.1). The bands were visualised in Coomassie brilliant blue stain solution. The sizes of the bands (in kDa) of the BenchMark^{TM}Protein Ladder (St) are indicated to the left and is used on all gels except for D, where the SDS-PAGE Standards, low range (Bio-Rad) is used (Table 2.6). **(A)** GST, **(B)** lane 1: GST-TIFPB, lane 2: GST-TIFP*link*B, **(C)** lane 1: GST-p300BP, lane 2: GST-p300B*link*P, **(D)** lane 1: GST-p300B-TIP5P, lane 2: GST-p300B-MLLP, **(E)** lane 1: GST-TIFPB, lane 2: TIFPBt[a] (thrombin treated GST-TIFPB from lane 1), lane 3: GST-TIFP*link*B, lane 4: GST-p300P-TIFB, lane 5: GST-p300B-TIFP. Note that GST-TIFPB and GST-TIFP*link*B was purified twice, once in B and once in E. The sizes and the estimated concentrations of the polypeptides are given in Table 4.3.

[a] The t represents the thrombin cleavage (Section 3.5.6) that has cleaved the polypeptide from GST.

Table 4.3 Concentrations [a] of polypeptides

Polypeptide	Concentration	Size	Reference Figure 4.8
GST	40 µM→ 17 µM	~ 28 kDa	A
GST-TIFPB[b]	7 µM	~ 57 kDa	B, lane 1
GST-TIFP*link*B[b]	20 µM	~ 57 kDa	B, lane 2
GST-p300BP	32 µM→ 24 µM	~ 57 kDa	C, lane 1
GST-p300B*link*P	25 µM→ 20 µM	~ 59 kDa	C, lane 2
GST-p300B-TIP5P	5.5 µM→13 µM	~ 56 kDa	D, lane 1
GST-p300B-MLLP	15 µM	~ 55 kDa	D, lane 2
GST-TIFPB[c]	5.5 µM→12 µM	~ 57 kDa	E, lane 1
TIFPBt[d]	6.5 µM→17 µM	~ 30 kDa	E, lane 2
GST-TIFP*link*B[e]	6.5 µM	~ 57 kDa	E, lane 3
GST-p300P-TIFB	15 µM	~ 62 kDa	E, lane 4
GST-p300B-TIFP	14 µM	~ 55 kDa	E, lane 5

[a] Ideal concentrations for nucleosome binding assays are between 15 and 20 µM (see Section 3.8). Fractions with concentrations higher than 20 µM were diluted in TZNK/β/T to prevent precipitation, and most fractions with concentrations lower than 15 µM were concentrated using Centricon® Centrifugal Filter Devices (Section 3.5.3). The arrow represents dilution or concentration

[b] Batch used in nucleosome retention assays (Figure 4.10)

[c] Batch used in electrophoretic mobility shift assay (Section 4.11)

[d] Thrombin treated GST-TIFPB in the line above (of concentration 5.5 µM, Figure 4.5 E, lane 1).

[e] Batch not used

Figure 4.8 and Table 4.3 show that all the polypeptides could be purified, but in quite varying amounts. GST gave best yield (40 µM). GST-p300BP and the control GST-p300B*link*P were also slightly higher (32 µM and 25 µM respectively) than the other polypeptides (from 5.5 µM to 15 µM), indicating that the polypeptides with the low concentrations are more unstable, and might have precipitated during the purification process. All the polypeptides thus gave a concentration, or were adjusted to concentrations that could be used for nucleosome retention assays (ideally between 15 and 20 µM).

PART II, EVALUATING POSITIVE CONTROLS

To be able to evaluate the ability of the domain recombinants to bind nucleosomes, it was necessary to determine whether the controls, GST-p300B*link*P and GST-TIFP*link*B that had their own PHD finger reinserted, bound to nucleosomes as well as their respective wild types. Nucleosome binding of the wild type GST-TIFPB had not been tested before, so this was tested at the same time as the recombinant, GST-TIFP*link*B. Nucleosome retention assays (Section 3.8.1) were therefore performed with GST-p300B*link*P, GST-TIFPB and GST-TIFP*link*B.

4.5 Nucleosome retention assay with GST-p300B*link*P

The polypeptide expressed from pSXG-p300B*link*P contained 33 additional amino acid residues between the bromodomain and the PHD finger (see figure text, Figure 4.5) compared to the wild type. To test whether this, or the vector construction procedure, could have interfered with the polypeptide's nucleosome binding activity, a nucleosome retention assay was performed with GST-p300B*link*P, two different preparations of GST-p300BP as positive controls and with GST as negative control (Figure 4.9).

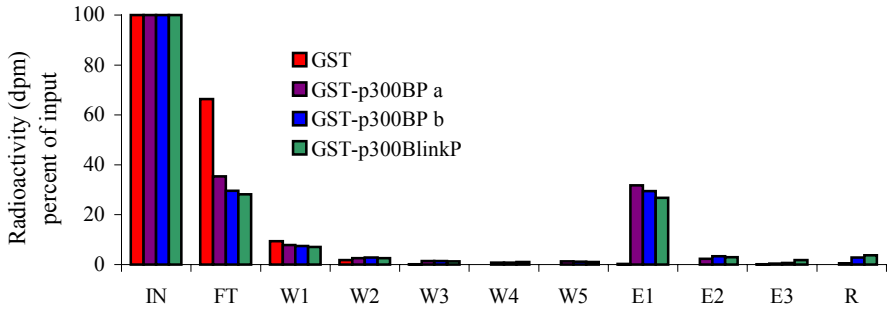


Figure 4.9 GST-p300B*link*P binds to nucleosomes as well as GST-p300BP. The figure shows the results of the nucleosome retention assay (Section 3.8.1) testing the binding of GST-p300B*link*P to ^{3}H-labelled nucleosomes (Table 2.3). The amount of nucleosomes (percent of input) retained by four polypeptides (Table 4.3) is represented with different colours, as indicated on the figure: GST, negative control; two different preparations of GST-p300BP, positive controls; and GST-p300B*link*P. IN, input; FT, Flow Through (the nucleosomes not retained on the nucleosomes); W1-W5, Wash 1 – Wash 5; E1-E3, Eluate 1 – Eluate 3; R, material retained on the glutathione sepharose beads after elution. From all fractions, material was taken for scintillation counting (Section 3.5.7) and the radioactivity in the input material (862 dpm/µl) was set to be 100 %. The radioactivity of all fractions, in dpm and percent of input, is given in Appendix.

The data show that GST-p300B*link*P binds to nucleosomes (27 %) as well as the wild type: The two different preparations of GST-p300BP both bind to nucleosomes (32 % and 29 %). GST does not reveal any nucleosome retention (0 %). For exact values, see Appendix 1. These results demonstrate that neither the extra amino acids inserted between the bromodomain and the PHD finger nor the procedure of vector construction have altered the ability of the protein to interact with nucleosomes. Further experiments testing nucleosome binding of the domain recombinants were therefore performed (Section 4.8).

4.6 Nucleosome retention assay with GST-TIFPB and GST-TIFP*link*B

The polypeptide expressed from pSXG-TIFP*link*B had six additional amino acid residues between the PHD finger and the bromodomain compared to the wild type (see Figure 4.6). A nucleosome retention assay was performed with the wild type, GST-TIFPB, and GST-TIFP*link*B (Figure 4.10). GST-p300BP and GST were used as positive and negative controls respectively.

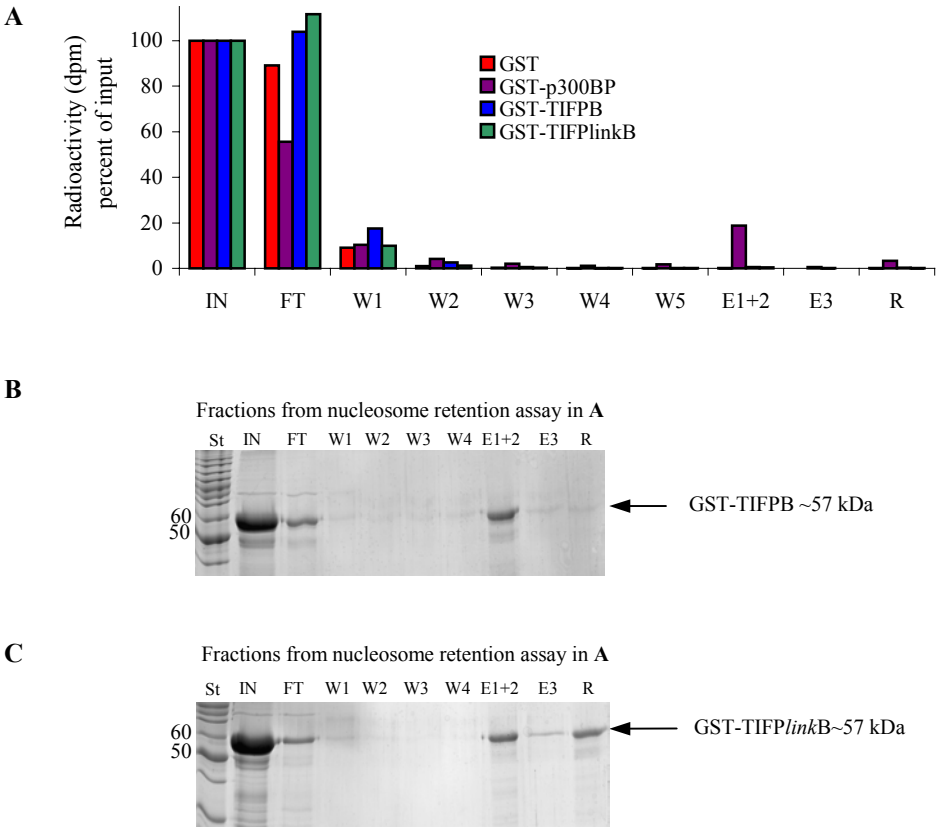


Figure 4.10 No nucleosome binding was detected, neither for GST-TIFPB nor GST-TIFP*link*B. **(A)** Nucleosome retention assay (Section 3.8.1) testing the binding of GST-TIFP*link*B and GST-TIFPB (Table 4.3) to ^{3}H-labelled nucleosomes (Table 2.3). The diagram shows the amount of radioactivity (percent of input) in each fraction of the four polypeptides tested. The polypeptides are represented with different colours, as indicated on the figure: GST, negative control; GST-p300BP, positive control; GST-TIFPB (this polypeptide had a concentration of 7 µM (see Table 4.3), and in stead of concentrating the sample, a double volume, 400 µl, was applied); and GST-TIFP*link*B. Abbreviations, IN, FT etc, are as in Figure 4.9. The radioactivity of the input, 610 dpm/µl was set to be 100 %. **(B/C)** Aliquots of 10 µl of each fraction from nucleosome retention assay with GST-TIFPB and GST-TIFP*link*B analysed by SDS-PAGE (Section 3.5.1). The gels were stained in Coomassie brilliant blue stain solution. The sizes of the bands (in kDa) of BenchMark^{TM}Protein Ladder (St) are indicated to the left. Other abbreviations are as in Figure 4.9, except that IN and FT refers to the polypeptide fractions, IN-P and FT-P respectively (see Section 3.8.1). The radioactivity of all fractions, in dpm and percent of input, is given in Appendix.

Neither GST-TIFPB nor GST-TIFP*link*B showed any ability to bind to nucleosomes. Compared to the input, 0.6 % and 0.4 % retained nucleosomes were obtained respectively, which was similar to the negative control, GST (0.2 %). In the eluate of the positive control, GST-p300BP, 19 % retained nucleosomes was detected compared to the input. The gels

shown in Figure 4.4 B and C confirm that the polypeptides appear in the eluate, E1+2, and not in the washing steps, W1-W4. This means that the polypeptides were bound in the column when the nucleosomes were applied, before the washing steps. Since neither GST-TIFPB nor GST-TIFP*link*B showed any nucleosome binding in the nucleosome retention assay, an alternative method, electrophoretic mobility shift assay (EMSA) was tried.

4.7 Electrophoretic mobility shift assay with GST-TIFPB and TIFPBt

To test whether the EMSA (Section 3.8.2) could be used to detect binding of GST-TIFPB to nucleosomes, GST-TIFPB and TIFPBt were tested in this assay (Figure 4.11). TIFPBt has had its GST tag removed by thrombin cleavage (Section 3.5.6). TIFPBt was tested to see if GST could have any influence on the polypeptide's ability to interact with nucleosomes. Increasing concentrations of polypeptides were incubated with ^{32}P-labelled nucleosomes. The samples were analysed on a 4.5 % native PAA gel followed by visualizing by a radio phosphoimager.

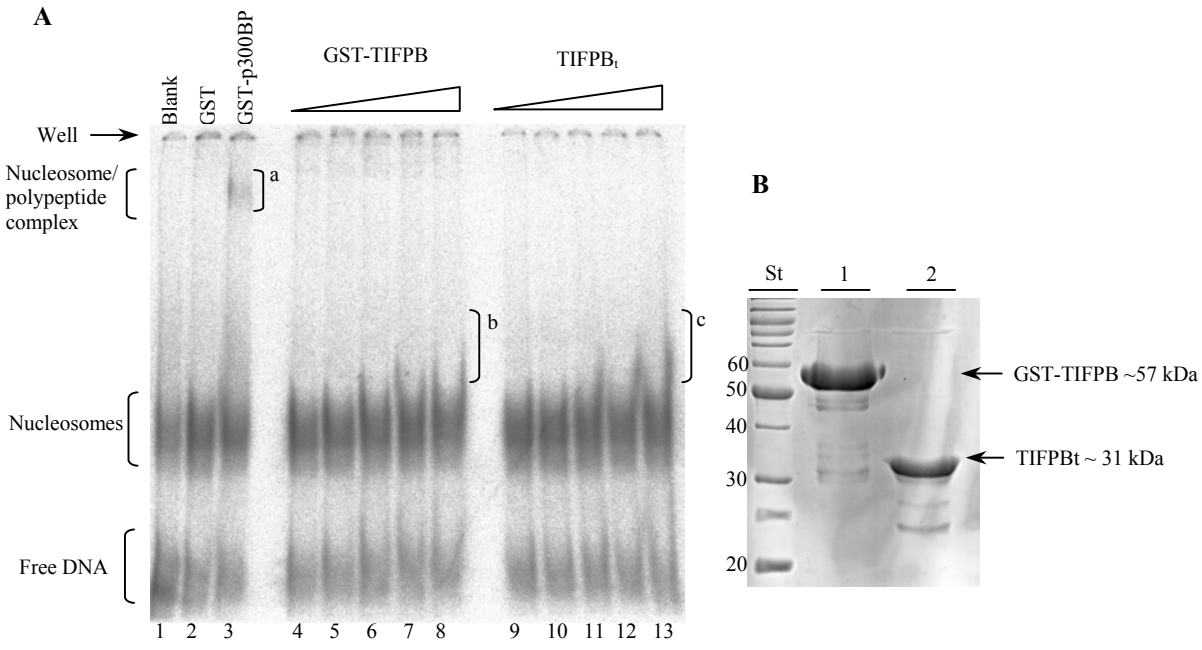


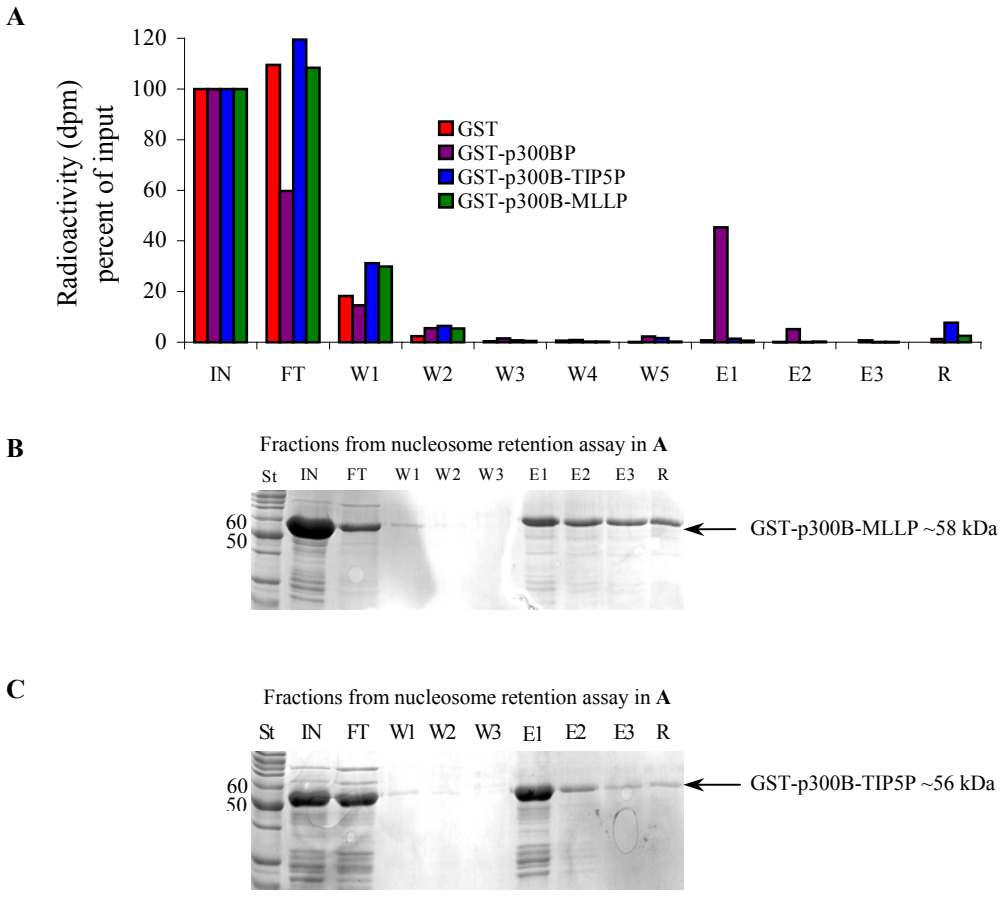
Figure 4.11 No nucleosome binding was detected by EMSA, neither with GST-TIFPB nor TIFPB. An EMSA (Section 3.8.2) was performed testing the binding of GST-TIFPB and TIFPBt (Table 4.2) to ^{32}P-labelled nucleosomes (Table 2.3). **(A)** Each binding reaction contained approximately 2.5 fmol nucleosomes and: lane 1, no polypeptide; lane 2, 1000 pmol GST, negative control; lane 3, 1000 pmol GST-p300BP, positive control; lanes 4-8 GST-TIFPB in amounts of 100, 300, 500, 700 and 1000 pmol respectively; lane 9-13, TIFPBt in amounts of 100, 300, 500, 700 and 1000 pmol respectively. Free nucleosomes and free DNA is visible as a band in the middle and on the bottom of the gel respectively. a, Nucleosome – GST-p300BP complex; b/c, with increased concentration of polypeptide, the bands containing nucleosome is seen further up in the gel, most probably caused by unspecific binding (Anja Ragvin *pers. comm*.). **(B)** SDS-PAGE gel showing the polypeptides used in the EMSA (after concentration by Centricon®Centrifugal Filter Devices) stained in Coomassie brilliant blue stain solution. The sizes of the bands (in kDa) of BenchMark^{TM}Protein Ladder (St) are indicated to the left; Lane 1, 120 pmol GST-TIFPB; lane 2, 170 pmol TIFPB.

There is no visible complex formed between GST-TIFPB or TIFPB and nucleosomes (lanes 4-13). With GST-p300BP a complex is visible as expected (lane 3) and GST shows no binding (lane 2). The negative results for GST-TIFPB and TIFPB indicated that most likely the other domain recombinants with an identical bromodomain would not bind to nucleosomes. These recombinants were therefore not tested for binding to nucleosomes. An exception is GST-p300P-TIFB (with PHD finger from p300); which was tested (Figure 4.12 D-F) since this PHD finger causes binding in p300BP.

PART III, EVALUATING DOMAIN RECOMBINANTS

4.8 Nucleosome retention assay with domain recombinants

Since GST-p300B*link*P showed efficient binding in the nucleosome retention assay, the panel of domain recombinants with the bromodomain from p300 and PHD fingers from heterologous proteins was evaluated (Figure 4.12). Even if the nucleosome retention assay with GST-TIFP*link*B was negative, one of the domain recombinants with TIFB, GST-p300P-TIFB, was evaluated to see if the PHD finger of p300 could cause nucleosome binding in the context of another bromodomain. GST-p300BP and GST were used as positive and negative controls as in previous experiments.



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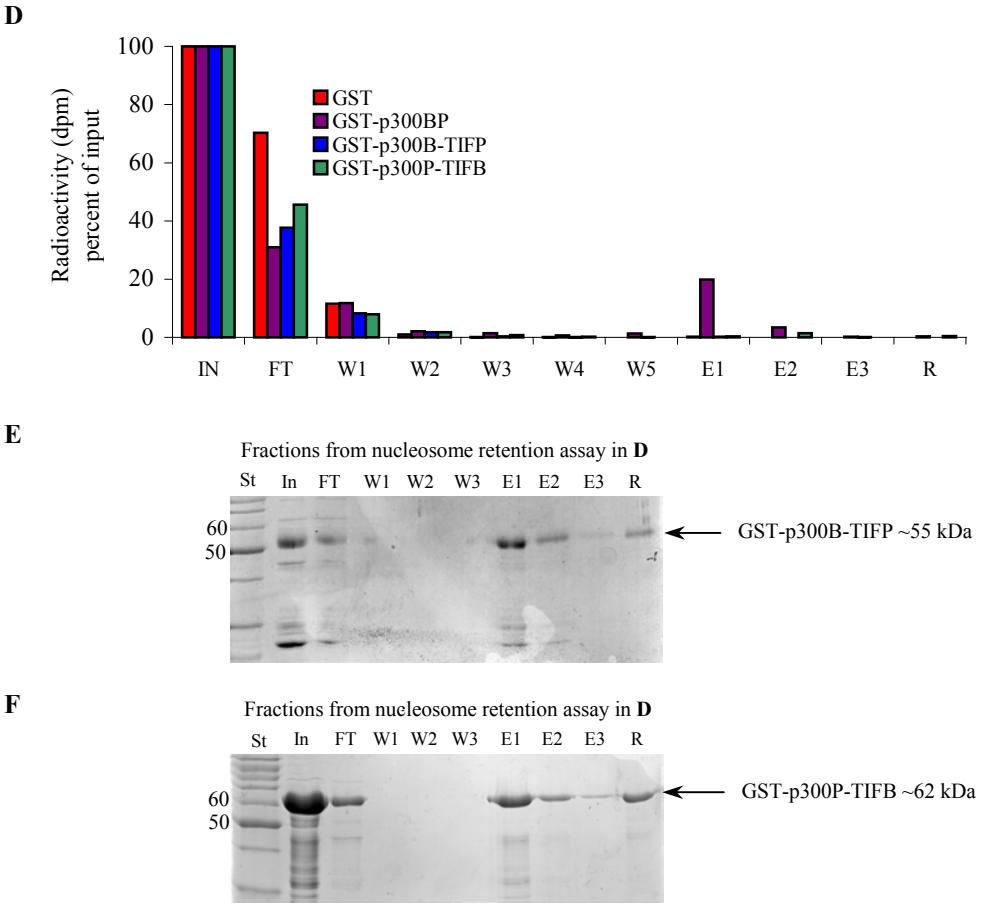

Figure 4.12 No nucleosome interaction is detected for the domain recombinants. **(A)** Nucleosome retention assay (Section 3.8.1) testing GST-p300B-TIP5P and GST-p300B-MLLP (Table 4.2) for binding to ^{3}H-labelled nucleosomes (Table 2.3). The diagram shows the amount of radioactivity in all fractions of the four polypeptides tested, represented with different colours, as indicated in the figure: GST, negative control; GST-p300BP, positive control; GST-p300B-TIP5P and GST-p300B-MLLP. The radioactivity in the input material, 528 dpm/µl was set to be 100 %. **(B)/(C)** Aliquots of 10 µl of each fraction from nucleosome retention assays with GST-p300B-MLLP (B) and GST-p300B-TIP5P (C) analysed by SDS-PAGE (Section 3.5.1) The gels were stained in Coomassie brilliant blue stain solution. The sizes of the bands (in kDa) of BenchMark^{TM}Protein Ladder (St) are indicated to the left. Other abbreviations are as in Figure 4.9, except that IN and FT refers to the polypeptide fractions, IN-P and FT-P respectively (see Section 3.8.1). **(D)** Nucleosome retention assay testing GST-p300B-TIFP and GST-p300P-TIFB for binding to ^{3}H-labelled nucleosomes. The diagram shows the amount of radioactivity in all fractions of the four polypeptides tested, represented with different colours, as indicated in the figure: GST, negative control; GST-p300BP, positive control; GST-p300B-TIFP and GST-p300P-TIFB. The radioactivity in the input material, 836 dpm/µl was set to 100 %. **(E)/(F)** Aliquots of 10 µl of each fraction from nucleosome retention assays with GST-p300B-TIFP (E) and GST-p300P-TIFB (F), analysed by SDS-PAGE (Section 3.5.1). The gels were stained in Coomassie brilliant blue stain solution. The sizes of the bands (in kDa) of BenchMark^{TM}Protein Ladder (St) are indicated to the left, other abbreviations, IN, FT etc, are as described above. The radioactivity of all fractions, in dpm and percent of input, is given in Appendix.

The results in Figure 4.12 show that the positive control in the assays, GST-p300BP, binds nucleosomes as expected (45 % and 20 % of the input nucleosomes were retained in A and D respectively), and GST is negative (0.9 % and 0.2 % of the input nucleosomes were retained in A and D respectively). For the domain recombinants, however, no significant nucleosome retention is detected (from 0.2 % to 1.3 % of input). The gels pictured in Figure 4.12 B, C, E and F confirm that the polypeptides appear in the eluates, E1-3, and not in the

washing steps, W1-W4. This shows that the polypeptides were bound to glutathione sepharose when the nucleosomes were applied. These results indicate that the PHD fingers of TIP5, MLL and TIF1γ cannot replace the PHD finger of p300 without loosing the nucleosome binding activity, and that p300P is not sufficient for GST-p300P-TIFB to bind to nucleosomes.

DISCUSSION

The PHD finger is a protein domain occurring in a number of proteins involved in epigenetic gene regulation. The bromodomain is another domain often present in such proteins. Several bromodomains occur next to a PHD finger, and it has been found by our research group that the bromodomain-PHD finger region of the protein p300 can bind to acetylated nucleosomes (Ragvin et al., *in prep*.). In experiments with this region of p300 in the nucleosome retention assay (Section 3.8.1) both domains are required to detect an interaction with nucleosomes (Ragvin et al., *in prep*.). No nucleosome interaction has been detected in this assay for each domain alone. In the EMSA however, nucleosome interaction has been detected for both domains separately. The aim of this project was to use nucleosome retention assay to study domain recombinants of GST-p300BP, in order to determine whether PHD fingers from heterologous proteins are able to substitute for the original.

5.1 Nucleosome binding assays

Six recombinant plasmids encoding domain recombinants were made by swapping the sequences encoding the PHD fingers in pSXG-p300BP and pSXG-TIFPB with other PHD finger encoding sequences. While GST-p300BP had been thoroughly tested for nucleosome binding in our laboratory previously, GST-TIFPB had not been tested. In my experiments, no nucleosome interaction was detected for GST-TIFPB. With one exception (see below), the domain recombinants of GST-TIFPB were therefore not further tested. The results of the nucleosome binding experiments are discussed in the following.

5.1.1 Nucleosome interaction by the domain recombinants

Three of the vectors encoding domain recombinants were made based on pSXG-p300B. The control polypeptide GST-p300B*link*P showed nucleosome retention similar to the wild type polypeptide (Figure 4.9), but when the PHD finger was swapped with a heterologous PHD finger, the nucleosome binding activity was lost (Figure 4.10 and 4.12). One domain recombinant of GST-TIFPB was also tested; the domain recombinant with the PHD finger from p300 and the bromodomain from TIF (GST-p300P-TIFB, Figure 4.12). No nucleosome interaction, however, was detected with this polypeptide.

Interaction between the bromodomain and the PHD finger in p300

The negative results of nucleosome binding assays with the domain recombinants of GST-p300BP may suggest that there exists an interaction between the bromodomain and the PHD finger in p300. A negative result may thus implicate that the interaction between the domains is lost, giving a dysfunctional polypeptide.

The negative result in nucleosome binding also with GST-p300P-TIFB may further indicate that an interaction is needed between the two domains in p300. Both the bromodomain and the PHD finger in GST-p300P-TIFB may be expected to cause nucleosome binding; the PHD finger of p300 causes binding when situated next to its

original bromodomain and the bromodomain is expected to interact with an acetylated lysine. However, no interaction is detected when altering the domain combination. The negative result with GST-p300P-TIFB may, however, also be explained by the function of the bromodomain: Even though other bromodomains are known to bind to acetylated lysines, the one in TIF1γ might have another function. This possibility must be considered since no interaction was detected in the wild type GST-TIFPB (see Section 5.1.2). The putative interaction between the bromodomain and the PHD finger is further discussed in Section 5.3.2.

Influence of the linkers inserted in the vectors on the expressed polypeptides' function

Experiments have been done in our research group extending the region between the bromodomain and the PHD finger in p300 (Tufteland, 2002). A region of as much as 77 amino acids was inserted between the domains, but the insertion did not interfere with the polypeptide's nucleosome binding activity. The inserted linker in pSXG-p300B*link* therefore most likely has nothing to do with the loss of nucleosome binding of the domain recombinants. Furthermore, the positive control GST-p300B*link*P (which has 33 additional amino acids compared to GST-p300BP, see figure text in Figure 4.5) binds to nucleosomes essentially as well as the wild type, supporting that the linker does not affect the nucleosome binding activity.

The experiment with the extended region between the bromodomain and the PHD finger might be in conflict with the hypothesis of an interaction between the bromodomain and the PHD finger. Those results may indicate that the domains function independently. However, it is not unlikely that despite the extended region between the domains, they might still interact, only with a larger loop between them.

Disruption of the putative interaction between the bromodomain and the PHD finger

By performing domain swapping, the putative interaction between the bromodomain and the PHD finger may be disrupted because a heterologous PHD finger might not be able to interact with the bromodomain (see Figure 5.1).

The structure of the heterologous PHD fingers present in the domain recombinants is a factor that may affect the PHD finger's cooperation with the bromodomain. Even though all PHD fingers contain the conserved pattern of Cys_4-His-Cys_3, there are non-conserved amino acids between these. It is therefore likely that each PHD finger adopts a 3D-structure slightly different from other PHD fingers. They may even be so divergent that the PHD fingers used in my experiments, when fused to the bromodomain of p300, do not physically fit to the bromodomain.

The size of the PHD finger might also be important in this context. The PHD fingers inserted are all smaller than the one in p300, which contains the stretch of amino acids situated after the first two cysteins of the conserved motif (see Figure 1.7 and Table 2.1).

Alternative explanations for the domain recombinants being dysfunctional

The possibility cannot be excluded that the PHD fingers from TIF1γ, TIP5 and MLL do not have any nucleosome binding function in their natural contexts (see Section 1.9.2) and therefore do not bind *in vitro*. Alternatively, the PHD fingers do have nucleosome binding activity, but do not recognise their target on the nucleosome (such as a modification or a particular structure in the nucleosome) because the target is not present in the nucleosomes used in my experiments. Alternatively, the target is present but situated in a disadvantageous position compared to the binding site of the bromodomain. Perhaps nucleosomes purified from another type of cells than SupT-cells, grown under other conditions, would give nucleosomes containing the relevant epitopes. However, nucleosome interaction has been detected for GST-p300BP also with recombinant nucleosomes, containing acetylation as the only modification (Ragvin et al., *in prep*.). The PHD finger of p300 therefore does not seem to be dependent on other modifications.

An aberrant folding of the polypeptides might also explain the loss of nucleosome interaction. Since, however, the positive control, GST-p300B*link*P, was functional (in which the plasmid has been through the same process of vector construction) one can assume that they have proper folding. The folding is further discussed in Section 5.2.

5.1.2 Nucleosome interaction by GST-TIFPB

The polypeptide GST-TIFPB and the recombinant GST-TIFP*link*B did not show any nucleosome binding in the nucleosome retention assay. GST-TIFPB and TIFPB were also tested in EMSA, but no nucleosome interaction was detected. It is hard to make any assumptions based on these results because there is no positive control as with p300BP. An explanation may quite simply be that the PHD finger in TIF1γ does not interact with nucleosomes *in vivo*. It is also possible that there is a nucleosome interaction *in vivo* and that some of the factors mentioned in Section 5.2 (the nature of the nucleosomes, the experimental conditions or the folding of the polypeptides), made it difficult to detect the interaction *in vitro*.

5.1.3 The degree of binding in the nucleosome retention assay

In my nucleosome retention assays, the binding percentages of GST-p300BP vary significantly, from 19 % to 45 %. Previous experiments in our research group have shown binding percentages from 6 % and up to 39 % (Tufteland, 2002). It is not likely that this variation is caused by variable ability of GST-p300BP to bind to nucleosomes. Other experimental factors might, however, cause variation in the binding percentages: Variable concentrations and qualities of polypeptides and nucleosomes, variable results from the scintillation counter, varying pipetting accuracy and practical performance of the experiment. For these reasons it is hard to compare the degrees of nucleosome binding, especially between polypeptides that have not been tested in the same experiments. The experiments in this thesis were designed, however, to test qualitatively a panel of domain

recombinants for nucleosome binding. Therefore, no conclusions have been drawn on the degree of binding. Note also that each experiment has been performed only once. Since the controls have acted as expected, it is reasonable to assume that the experiments are representative.

5.1.4 Determination of radioactivity in nucleosome retention fractions

In the nucleosome retention assays, the binding of polypeptides to nucleosomes was determined by measuring radioactivity (^{3}H) in each fraction (IN-N, FT-N, W1-W5, E1-E3 and R) by scintillation counting. The input value was set to 100 % and the relative amount of radioactivity in the fractions was calculated based on this value (Section 3.8.1). When adding up the relative radioactivity in all fractions, one would expect to get a value close to 100.

In previous experiments in our research group, a recovery of 61 % - 127 % has been observed (Tufteland, 2002). In my experiments, the recovery varied from 48 % with GST-p300B-TIFP (Figure 4.12 D, Table A.4) to as much as 168 % with GST-p300B-TIP5P (Figure 4.12 A, Table A.3), although normally lying in the area 60-70 % (see Appendix). In two incidences, the FT value was even higher that the IN value (Figure 4.10 and 4.12 A).

The most obvious explanation for not recovering all the material is that the fractions taken for counting after nucleosome retention were not precise. When pipetting from 11 different tubes, some material is lost in each pipette tip and the sum will be lower than the input. The reason for getting a recovery of higher than 100 % can also be explained by pipetting; the pipette might have been adjusted imprecisely when taking fractions for input compared to the rest of the fractions. Such inaccuracy may also result in a FT fraction that has a higher value than the IN.

^{3}H is a low energy β-emitting nucleide and the radioactivity is therefore easily quenched. Corrections are made in the scintillation counter that converts cpm (counts per minute) to dpm (disintegrations per minute). The correction is done using a standard curve, assuming that the quenching is the same in all fractions. The quenching most likely varies, however, and in a fraction with high degree of quenching, not all disintegrations will be detected. The result will be a lowered cpm value. Another factor influencing with the counting is the sensitivity to static electricity, which could raise the observed cpm value. Quenching and static electricity are both factors that could have influenced the results of each counting.

The dpm values of the input fractions also varied. The input values in Figure 4.9 and Figure 4.12 D were approximately 43000 dpm and 42000 dpm respectively (see Appendix). However, in the cases where the flow through value was higher than the input, the dpm values in the input fractions were approximately 30000 dpm and 26000 dpm (Figure 4.10 and Figure 4.12 A, respectively). Although the concentrations of the nucleosomes are slightly variable, this comparison suggests that the input value in the two latter cases might have been higher than detected. Quenching or static electricity might also account for the

particularly high binding percentage of the GST-p300BP (45 %) and the relatively high percentage in the R fraction of GST-p300B-TIP5P (7.6 %) in the same experiment (Figure 4.12 A).

Despite the variable values of recovery, input material and binding percentages, the pattern of binding/not binding was easily detectable in all experiments. All experiments are therefore assumed to be reliable although the values may be somewhat imprecise.

5.2 Folding and stability of the polypeptides

The domain recombinants were obtained in quite low concentrations. Normally the concentrations were ~15 μM, but the lowest as low as ~5 μM. These concentrations are rather low compared to 25-40 μM in the wild types GST and GST-p300BP, as well as the control GST-p300B*link*P (Table 4.3). The polypeptides GST-TIFPB and GST-TIFP*link*B (which did not either show nucleosome binding) were also obtained at relatively low concentrations, with the exception of the latter polypeptide giving a concentration of 20 μM in one of two preparations, see Table 4.3).

Such low concentrations of polypeptides could be a result of either a decreased stability by introducing a heterologous domain or the expression conditions not being optimal. Most of the instable or erroneously folded complexes would be expected to precipitate during the purification process. A tendency of the recombinant polypeptides to precipitate from the buffer not long after purification suggests that some incorrectly folded polypeptides might have been present in the solutions. However, precipitation also occurred with GST, GST-p300BP and GST-p300B*link*P, which were functional and therefore were considered to have a proper folding. The reason for the precipitation in all solutions might therefore be that a proportion of the polypeptides formed complexes with each other or that they did not have the optimal conditions for storage.

The polypeptides used in the nucleosome binding assays were always freshly purified, being less than a couple of days old for the recombinants, and up to a month old for GST and GST-p300BP. It is therefore reasonable to believe that the polypeptides used in my experiments had proper folding.

To prevent precipitation, other conditions for storage could have been tried, but storage at 4 °C in TZNK/β/T had been satisfying with GST-p300BP previously in the research group, giving low level of precipitation. These conditions were therefore also used in my experiments. Storage at -20 °C had been tested (Tufteland, 2002) but small amounts of precipitation was also seen at this temperature

5.3 The role of the PHD finger in p300 and other proteins

5.3.1 The role of the PHD finger in p300BP

What do the experiments presented here say about the role of the PHD finger in p300? We already knew in our research group that the PHD finger had to be present to detect nucleosome binding by GST-p300BP in the nucleosome retention assay (Ragvin et al., *in prep*.). The fact that the PHD finger has been shown by EMSA to be able to interact with nucleosomes without the bromodomain suggests that the PHD finger in p300 alone has a nucleosome binding activity (Ragvin et al., *in prep*.). In my experiments, both the PHD finger and the bromodomain in the polypeptide had to originate from p300 to detect an interaction with nucleosomes in the nucleosome retention assay.

In the two nucleosome binding assays (Section 3.8), the nucleosomes and the polypeptides are treated differently. In the nucleosome retention assay, it seems like an interaction of only one domain is too weak to persist in all the centrifugations during the washing steps, explaining why the domains are dependent on each other in this assay. In the EMSA, however, the nucleosomes and the polypeptides are simply mixed and subsequently analysed by electrophoresis, no centrifugations are required. A weak interaction is therefore more easily detected in this assay.

Assuming that the domain recombinants in my experiments are folded correctly and that the inserted PHD fingers also have the ability to interact with nucleosomes, one should expect the domain recombinants to be able to interact with nucleosomes. However, they do not, possibly (as mentioned in Section 5.1.1) due to the binding between the bromodomain and the PHD finger being lost. A nucleosome binding by both the PHD finger and the bromodomain in p300 therefore seems to be difficult if the domains are not, at the same time, able to interact with each other.

Some of the models discussed in Figure 1.9, now seem less plausible. The findings mentioned above narrows down the most likely function of the PHD finger to two models; the models where the bromodomain and the PHD finger interact, and the PHD finger binds to either the histone tail (alternative A1) or somewhere in the histone core (alternative A2). When the results of my experiments are taken into account, some new models may be considered (Figure 5.1). A PHD finger from a heterologous protein may not be able to interact with the bromodomain in p300. The heterologous domain disrupts the cooperation between the bromodomain and the PHD finger, and thereby the binding by at least one of the domains to the nucleosome. The interaction between the domains gives p300BP binding-specificity and stronger affinity to the nucleosome. The cooperation between the domains is discussed below.

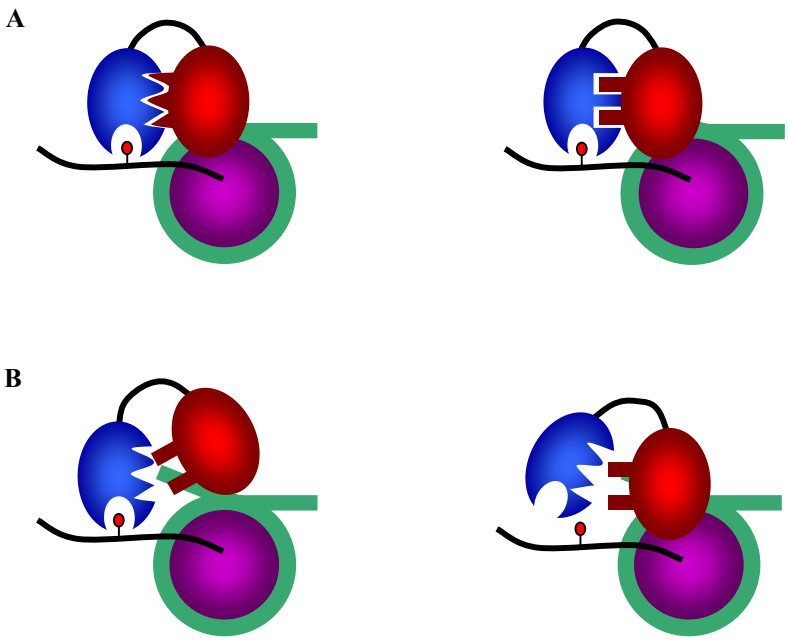

Figure 5.1. A heterologous PHD finger in p300 makes the cooperation with the bromodomain difficult. The PHD finger is illustrated in red and the bromodomain in blue. Only one of the eight histone tails are shown and the red ball refers to an acetylated lysine. **(A)** A protein with its two original domains present functions normally, both domains bind to their respective targets. **(B)** When a domain, in this case the PHD finger, from a heterologous protein replaces the original, the cooperation becomes difficult and only one domain is able to bind to its target.

5.3.2 Cooperation between the bromodomain and the PHD finger in p300

The PHD finger has, as mentioned in the introduction, two flexible loops of less conservation (see Figure 1.7 and 1.8). This region has in fact been suggested to be involved in the binding of the PHD fingers to other protein domains, and because of its low conservation, may serve to give each PHD finger an individual binding property (Capili et al., 2001; Pascual et al., 2000). If the flexible loop reflects a diversity and specificity of PHD fingers, this may give an obvious explanation for the results of my domain swapping experiments and would support the hypothesis presented in Figure 5.1; each PHD finger has a region that fits only its natural ligand, irrespective of whether it is a bromodomain or another domain.

The presence of two such flexible areas might in fact serve as an interaction point for the bromodomain in one loop and the nucleosome in the other. The ability to interact with two different ligands may reflect a function common for all PHD fingers.

An interaction between the domains is further supported by a very recent examination of the flexible loops. It is found that the PHD finger in Mi2β can tolerate extensive substitutions and expansions in this area without affecting the zinc finger fold (Kwan et al., 2003). Because of this stability, it was possible to insert a CtBP2 (a corepressor) binding site in this region. The result was a PHD finger that could specifically

interact with CtBP2, confirming that an interaction with a corepressor is possible in this region.

An article was published in 2000, showing the crystal structure of the two adjacent bromodomains of the human $TAF_{II}250$ protein (Jacobson et al., 2000). In this protein, there are two points of interaction between the bromodomains. The first site is formed by electrostatic interactions between Glu residues on the one domain and Lys residues on the other. The second interaction point is formed between Tyr, Lys, Ile and Thr in one domain and Pro, Asn, Lys and Tyr in the other domain. When studying the sequence of the PHD finger from p300, some of these amino acids (Ile, Thr, Asn and Lys) are very frequent particularly in the flexible area between the second and the third Cys (Figure 1.9, loop 1). Perhaps some of these amino acids are involved in a similar interaction?

In the same article it was suggested that the substrate specificity of the double bromodomain would be expected to be tightly coupled to the relative orientation of the domains. The two binding pockets for the bromodomains span 28 Å, which require about seven amino acids (Jacobson et al., 2000). This is consistent with the distance between the acetylations seen on H4 in vivo (K5, K8, K12, K16). Possibly this is the case also with the bromodomain and the PHD finger; that the distance between the nucleosome binding sites of the domains reflects the distance between their epitopes on the nucleosome. In the same article, it was also suggested that the nucleosome binding could be enhanced by the cooperative binding by the first and the second pocket. These suggestions are consistent with the hypothesis presented about the PHD finger in p300.

As mentioned in the introduction, a number of transcriptional mediators/coactivators that function at the chromatin level has been found to contain PHD fingers (Aasland et al., 1995). About ~30 PHD finger proteins also contain the bromodomain (Capili et al., 2001). It is possible that some, or even all of these PHD fingers, also function in the same way as the p300 PHD finger, being involved in both protein- and chromatin interaction.

5.3.3 The role of the PHD finger in general

Although most PHD fingers seem to contain two loops of low conservation, the putative function of the PHD finger as a nucleosome-binding domain may not be common for all PHD fingers. It is possible that (a) the function of a PHD finger in one protein differ from the function of a PHD finger in another protein, or that (b) the PHD finger in one protein can play several different roles. An alternative interpretation of my experiments may, as mentioned in 5.1.1, support the idea that the function varies among different proteins; that the function of the PHD finger in p300 might be different from the function in TIF1γ, TIP5 and MLL.

In the Introduction, several suggested functions were outlined for the PHD finger (see Section 1.5). Some of these functions are supported by my experiments. Several groups have proposed that the PHD finger exhibit a protein-protein interaction (O'Connell et al.,

2001; Schultz et al., 2001). My experiments may indicate that this is the case also with the PHD finger in p300.

Another possible function mentioned was that the PHD finger was a domain required for HAT activity (Bordoli et al., 2001; Kalkhoven et al., 2002). It was indicated that in CBP the PHD finger is required for HAT activity. In p300/CBP the HAT domain is situated close to the B-P region (see Figure 1.9) and it has in fact been proposed that the PHD finger is an integral part of this domain (Kalkhoven et al., 2002). If the B-P region of p300 is responsible for the binding of p300 to chromatin, the domains may in fact assist in recruiting HAT activity to chromatin. Even though it is found that the PHD finger in p300 is not essential for HAT activity (Bordoli et al., 2001), it is obvious that a binding by the PHD finger to chromatin can ease histone acetylation by the HAT domain. Through the adjacent HAT domain, the PHD finger may actually contribute in histone acetylation, and thus serve a very important role in chromatin remodelling.

5.4 Concluding remarks

The aim of this project was to examine whether heterologous PHD fingers are able to replace the ones in GST-p300BP and GST-TIFPB without loosing the polypeptides' ability to interact with nucleosomes. To test this, nucleosome binding experiments with domain recombinants that had had its original PHD finger swapped with one from another protein was performed. Some models for possible functions of the PHD finger in p300 were considered in Figure 1.10 and the results of my experiments led me to suggest a new model (Figure 5.1) for the role of the PHD finger in p300. The model may also be relevant for other proteins containing a bromodomain and a PHD finger.

The results indicate that the bromodomain in p300 is dependent on its original PHD finger present because of an essential interaction between the domains. Possibly this may reflect an individual pattern in the flexible loop of the PHD finger. An interaction between the domains could in turn give the protein an increased affinity and specificity in the nucleosome interaction. The results thus indicate that the PHD finger in p300 has a nucleosome binding *and* a protein binding activity. A final suggestion is that there may be a link between the PHD finger in p300 and the adjacent HAT domain, possibly indicating an involvement in chromatin remodelling.

5.5 Future perspectives

5.5.1 Interaction between the bromodomain and the PHD finger in p300

In future experiments it would be interesting to explore the hypothesis that there is an interaction between the bromodomain and the PHD finger in p300. An effort was made in this study (results not shown) to detect this interaction. GST-p300P was attached to glutathione sepharose and p300B (GST-p300B cleaved with thrombin to release GST) was added. The complex was eluted and the eluate examined by SDS-PAGE. A similar experiment was performed with p300P added to GST-p300B attached to glutathione

sepharose. However, the experiments were inconclusive because when p300B and p300P were added to the GST-fusion polypeptides it looked like the thrombin left in the solutions cleaved also the polypeptides attached to the beads. The eluate (when adding p300Bt to GST-p300P) would therefore contain a mixture of (a) p300P cleaved from the GST tag (b) putative p300B that had interacted with the GST fusion polypeptide, and (c) GST. The polypeptides GST, p300B and p300P are of very similar sizes and cannot be distinguished on an ordinary 12 % SDS-PAGE-gel. The polypeptides should rather have been distinguished on another kind of gel (for instance a gradient gel) or by using antibodies. Alternatively, the experiment could have been repeated, and the thrombin removed from the polypeptide solution before the electrophoresis.

A second way to examine if an interaction exists between the domains could be to make domain recombinants of GST-p300BP with another PHD finger, a PHD finger that is shown by EMSA to interact with nucleosomes. If also this domain recombinant is negative in a nucleosome retention assay, the loss of binding in this case most probably is caused by a loss of interaction between the domains.

Point mutations could have been made in the flexible region of the PHD finger in p300BP, and the polypeptide tested for nucleosome binding. It would be difficult, using nucleosome retention assay, to decide whether a loss of nucleosome interaction is caused by a loss of the PHD finger's ability to bind to the bromodomain *or* to the nucleosome. By using EMSA, however, a loss of nucleosome interaction would be much easier to interpret.

An interaction between the domains can also be examined using biacore (by keeping one domain immobilized and testing whether the other domain is able to attach to it) or by protease cleavage (to see if an area of one of the domains is protected from protease). Solving the crystal structure of the bromodomain-PHD finger region of p300 could of course provide direct evidence that the two domains interact.

An examination of the folding of the domain recombinants could have been performed to verify that they had folded properly. However, no particularly good procedures and equipment for this existed in the laboratory. (An effort had been done previously in the group with other recombinants (Tufteland, 2002), by treatment with trypsin and examination of the digesting product by SDS-PAGE. A conclusion in this experiment was hard to draw; a digestion pattern for GST-p300BP was not detected because most of the bands on the gel originated from GST.)

If the site of interaction between the domains lies within the flexible region, it is possible that inserting this region into one of the PHD fingers could in fact make this PHD finger functional together with the p300 bromodomain.

5.5.2 The nucleosome binding site of the p300 PHD finger

To test if the PHD finger interacts with a histone tail, experiments can be performed with recombinant or trypsin treated nucleosomes that have all the histone tails removed. Interaction with such histones have been tested previously with the ATPase ISWI (Clapier

et al., 2001). Alternatively, recombinant nucleosomes can be made with one or more tails missing, or they can be made containing specific modifications, as methylation or phosphorylation. By using EMSA and test the binding of the PHD finger to such nucleosomes, information can be obtained about a putative interaction with a histone tail.

An alternative would be to try to detect an interaction between the PHD finger and free histone octameres. (An interaction with free DNA has been tested and found negative, Ragvin et al., *in prep*.). A problem with an experiment with a free histone octamer is that this would be a somewhat artificial situation since histone octameres do not exist *in vivo.* In addition, histone octameres are positively charged and might attract more than its natural ligands. An alternative way to elucidate the binding site, though probably a difficult task, may be by trying to hide the prospective position on the nucleosome by covering the site with another protein or a chemical to see if the interaction is lost.

5.5.3 Function of other PHD fingers

The PHD fingers of MLL, TIP5 and TIF1γ can be tested by EMSA when not in construct with a bromodomain. By elucidating the functions of these PHD fingers, further information from the experiments presented in this thesis could be obtained.

Several diseases are linked to one or more mutations in a PHD finger, usually in one or more of the eight zinc-coordinating residues resulting in a wide variety of diseases (Capili et al., 2001), as mental retardation, cancer and immunodeficiency. Studying these diseases more carefully could provide more information about the function of the PHD finger.

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APPENDIX

Table A.1 Radioactivity in fractions from nucleosome retention assay in Figure 4.9

Mean dpm values of two counts (of 3 minutes), minus blanc (13.6 dpm)

	GST	GST - p300BP a	GST- p300BP b	GST - p300B*link*P
IN	43087.5	43087.5	43087.5	43087.5
FT	28555.2	15256.3	12731.5	12112.2
W1	4053.1	3373.4	3205.2	3024.0
W2	755.9	1127.1	1192.5	1131.5
W3	62.1	583.4	597.0	577.3
W4	9.7	352.6	326.7	427.8
W5	-8.9	555.5	495.3	423.6
E1	97.1	13677.1	12655.3	11509.0
E2	-13.2	1017.5	1435.3	1283.9
E3	65.6	151.8	265.9	758.0
R	-2.5	232.1	1207.3	1628.5

Values in percent of input

	GST	GST - p300BP a	GST - p300BP b	GST - p300B*link*P
IN	100	100	100	100
FT	66.3	35.4	29.5	28.1
W1	9.4	7.8	7.4	7.0
W2	1.7	2.6	2.8	2.6
W3	0.1	1.3	1.4	1.3
W4	0.0	0.8	0.8	1.0
W5	0.0	1.3	1.1	1.0
E1	0.2	31.7	29.4	26.7
E2	0.0	2.4	3.3	3.0
E3	0.1	0.3	0.6	1.8
R	0.0	0.5	2.8	3.8
Sum (FT to R):	77.8	84.1	79.1	76.3

Table A.2 Radioactivity in fractions from nucleosome retention assay in Figure 4.10

Mean dpm values of two counts (of 3 minutes) minus blanc (10.8)

	GST	GST-p300BP	GST-TIFPB	GST-TIFP*link*B
IN	30542.0	30542.0	30542.0	30542.0
FT	27241.9	16997.2	31729.7	34079.2
W1	2778.4	3162.7	5365.6	3043.7
W2	288.7	1297.4	822.3	376.1
W3	77.1	657.4	180.5	100.1
W4	53.1	329.1	50.5	42.1
W5	27.5	564.3	43.5	60.1
E1+2	50.7	5730.0	190.7	130.4
E3	16.5	165.6	27.0	19.0
R	26.9	1039.9	74.5	37.8

Values in percent of input

	GST	GST-p300BP	GST-TIFPB	GST-TIFP*link*B
IN	100	100	100	100
FT	89.2	55.6	103.9	111.6
W1	9.1	10.4	17.6	10.0
W2	1.0	4.2	2.7	1.2
W3	0.2	2.1	0.6	0.3
W4	0.2	1.1	0.2	0.1
W5	0.1	1.8	0.1	0.2
E1+2	0.2	18.8	0.6	0.4
E3	0.0	0.5	0.1	0.1
R	0.1	3.4	0.2	0.1
Sum (FT to R):	100.1	97.9	126.1	124.0

Table A.3 Radioactivity in fractions from nucleosome retention assay in Figure 4.12 A

Main dpm values of two counts (of 3 minutes) minus blanc (8.3)

	GST	GST - p300BP	GST - p300B-TIP5P	GST - p300B-MLLP
IN	26376.4	26376.4	26376.4	26376.4
FT	28894.5	15728.4	31533.6	28606.5
W1	4804.3	3851.8	8218.3	7865.7
W2	638.7	1439.4	1702.2	1435.6
W3	86.1	376.1	207.3	109.4
W4	162.6	211.0	45.1	65.0
W5	34.4	601.9	438.3	58.7
E1	204.6	11958.7	345.9	169.8
E2	36.9	1349.5	36.9	46.9
E3	-5.3	189.4	10.6	19.5
R	3.6	317.1	2014.2	654.2

Values in percent of input

	GST	GST - p300BP	GST - p300B-TIP5P	GST - p300B-MLLP
IN	100	100	100	100
FT	109.5	59.6	119.5	108.4
W1	18.2	14.6	31.1	29.8
W1	2.4	5.5	6.4	5.4
W3	0.3	1.4	0.8	0.4
W4	0.6	0.8	0.2	0.2
W5	0.1	2.3	1.7	0.2
E1	0.8	45.3	1.3	0.6
E2	0.1	5.1	0.1	0.2
E3	0.0	0.7	0.0	0.1
R	0.0	1.2	7.6	2.5
Sum (FT to R):	132.1	136.5	168.7	147.93

Table A.4 Radioactivity in fractions from nucleosome retention assay in Figure 4.12 D

Mean values of two counts (of 3 minutes) minus blanc (7.3)

	GST	GST-p300BP	GST-p300B-TIFP	GST-p300P-TIFB
IN	41807.3	41807.3	41807.3	41807.3
FT	29425.5	12945.8	15762.7	19080.2
W1	4833.6	4913.4	3448.0	3324.3
W2	398.5	879.8	755.8	741.5
W3	69.0	585.7	134.3	301.4
W4	39.2	298.1	65.8	82.1
W5	11.9	554.4	67.2	4.7
E1	95.2	8307.1	103.6	155.1
E2	16.5	1450.5	12.5	581.5
E3	-3.8	110.6	57.9	2.7
R	3.2	136.0	6.2	204.1

Values in percent of input

	GST	GST-p300BP	GST-p300B-TIFP	GST-p300P-TIFB
IN	100	100	100	100
FT	70.4	31.0	37.7	45.6
W1	11.6	11.8	8.2	8.0
W2	1.0	2.1	1.8	1.8
W3	0.2	1.4	0.3	0.7
W4	0.1	0.7	0.2	0.2
W5	0.0	1.3	0.2	0.0
E1	0.2	19.9	0.2	0.4
E2	0.0	3.5	0.0	1.4
E3	0.0	0.3	0.1	0.0
R	0.0	0.3	0.0	0.5
Sum (FT to R):	83.5	72.2	48.8	58.5