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Regulation of transcription by the viral activator VP16

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Erklärung

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Summary

Transcription initiation by RNA polymerase II is finely controlled by a multitude of activators and regulatory factors. The Mediator complex is the central coactivator that enables a response of RNA polymerase II to activators and repressors. During this thesis an inducible VP16 model system was established, which allowed analysis of transcription initiation. Formation of the transcription complex at the example of the general transcription factors TFIIB and TFIIH, PC4, Mediator and Pol II, the recruitment of the histone acetyltransferases CBP and GCN5, polyadenylation complexes CPSF and CstF as well as histone modifications could be followed timely resolved by ChIP analysis. Further, the VP16-specific A-Med complex could be analyzed in vivo and in vitro. The transition from an inactive to an active A-Med complex was accompanied by the loss of MED1 and the Cdk8 kinase module that could be linked to phosphorylation.

Interestingly, A-Med seems to be able to associate with additional transcription cofactors that have histone acetyltransferase (CBP) and histone methyltransferase (Dot1L) activities as well as polyadenylation factors (CPSF and CstF). CBP could be shown to be VP16-dependently recruited in vivo and to interact directly with MED25 through the ACID domain. The Dot1L specific histone modification (metH3K79) could be detected dependent on VP16 transcription in vivo. CPSF-1 and CstF-64 could be detected on the promoter together with its direct interactor PC4. Their appearance on the promoter correlated with the detection of mRNA from the reporter gene. Taken together, A-Med seems to integrate activities used for histone modification, transcription and polyadenylation.

Publications

Current list of publications to which this work contributed:

Mittler, G., Stuhler, T., Santolin, L., Uhlmann, T., Kremmer, E., Lottspeich, F., Berti, L., and Meisterernst, M. (2003)
A novel docking site on Mediator is critical for activation by VP16 in mammalian cells.
EMBO Journal 22(24):6494-504

Uhlmann, T., Boeing, S., and Meisterernst M. (2005).
Complex dynamics of human Mediator.
in preparation

Contents

1 Introduction

1.1 The flow of genetic information

The genome of an organism encodes for the complete set of genes, about 30.000 genes in humans [Bentley et al., 2001]. Only a subset of these genes are transcribed and translated at any given time. Which subset is expressed, depends largely on the developmental state and the environmental needs of a cell. Gene expression is a regulated processes that is tightly controlled by a complex interplay of regulation mechanisms. The DNA encoded gene is transcribed in the nucleus into messenger RNA (mRNA). The mRNA is getting processed and transported to the cytoplasm of the cell where it is translated by the ribosome (Fig.1). Any of these steps allow for regulation of genexpression.

Figure 1: The flow of genetic information.

Transcription is the fundamental process in which the DNA encoded information is transcribed into RNA. The enzymes responsible for this process are the RNA polymerases. The basic transcription reaction is similar in all organisms. In eucaryotes, RNA polymerase II transcribes all the protein coding genes. The function of RNA polymerase II transcription machinery is orchestrated by a myriad of regulatory proteins. Activators bind sequence specifically in the vicinity of genes and recruit additional coactivators and transcription factors. These regulatory proteins influence the activity and processivity of RNA polymerase II and thereby regulate the expression rate of genes. These regulatory proteins can directly influence transcription by stabilizing or modifying the transcription machinery and thus promoting RNA polymerase II from initiation to elongation. Furthermore, these coregulatory proteins may indirectly promote transcription by chromatin decompaction either by modification of the histone tails or by removing complete

histones. Therefore, they generate a chromatin environment that is more competent for transcription.

As soon as transcription starts, the 5' end of the nascent mRNA is modified by the addition of a cap to prevent the newly synthesized mRNA from degradation by RNases. During the transcription elongation, the coding regions (exons) as well as the non coding regions (introns) are transcribed by the RNA polymerase II to form pre-mRNA molecules. Cotranscriptionally, the splicing machinery starts to remove the introns and by this forms a mature mRNA molecule. Upon reaching the termination signal at the end of the gene, RNA polymerase II stops transcribing [Greger and Proudfoot, 1998, Proudfoot, 1989, Aranda and Proudfoot, 2001]. Recognition of the precise termination point is linked to transcription initiation and required for efficient transcription [Birse et al., 1998, Barilla et al., 2001]. In a concerted action of the cleavage and polyadenylation factor (CPSF) and the cleavage stimulatory factor (CstF), the newly synthesized mRNA is cleaved and the polyadenosinetail (polyA) is added. This newly transcribed and processed mRNA is then exported out of the nucleus to the cytoplasm, were translation into a new protein occurs at the ribosome.

1.1.1 Structure of protein-coding genes and their regulatory sequences

Transcription is a highly complex process, that is regulated by the interplay of gene specific transcription factors, general transcription factors and coregulators on the level of DNA. Many regulatory elements are known. One can discriminate between gene-specific elements that act only on one gene and global elements that can act on a group of genes. The gene-specific elements of class II genes contain distal- and proximal promoter elements. Regulatory DNA sequences that are located 5' of the coding region may be part of the promoter (Fig.2). The proximal promoter elements are located close to the actual start site and are called UAS (upstream activating sequence) or URS (upstream repressing sequence) respectively. Typical class II core promoter elements are the TATA box that is located approximately -30 to -25 bp upstream of the start site and the TFIIB recognition element (BRE) located just upstream (uBRE) [Struhl, 1995] or downstream (dBRE) [Wensheng Deng and Stefan G.E. Roberts, 2005] of the TATA box. Further com-

monly found elements are the initiator-region (Inr) that is located around the transcription start site [Furter-Graves and Hall, 1990] and the downstream core-promoter element (DPE) that locates about 30 bp downstream of the initiation site, reviewed in [Smale and Kadonaga, 2003, Lewis et al., 2005]. It is noteworthy that none of these elements are strictly necessary for transcription initiation and that the combination of any of these elements is sufficient to allow basal transcription. The TATA box is the binding site for TBP, the TATA binding protein.

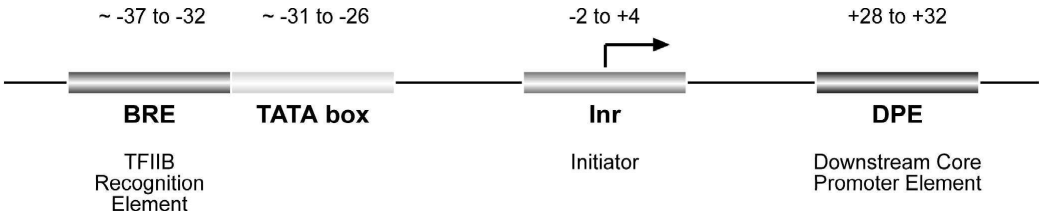

Figure 2: Core promoter elements. This figure depicts some of the elements that can contribute to basal transcription from a core promoter. Each of these elements is only found in a subset of promoters. A specific promoter may contain some, all or none of these elements.

Mutational analysis defined TATA A/T AA as the consensus sequence for the TATA box [Chen and Struhl, 1988, Singer et al., 1990, Wobbe and Struhl, 1990]. This sequence is nearly identical in all eucaryotes and emphasizes the evolutionary conservation of the TBP-TATA interaction. The DPE seems to act together with the Inr element as a binding site for the general transcription factor TFIID (Tab.2). The TBP accessory factors (TAFs) - TAF6 and TAF9 - within the TFIID complex proved to be critical for DPE binding and thus for recognition of TATA-less promoters. Additionally, the DPE element also seems to work through the positive cofactor 4 (PC4) [Lewis et al., 2005].

Distal promoter elements act from the distance and can be located several kilobases (kb) away from the actual transcription start site. As an interesting feature, these distal elements can act independently of their position and orientation. Enhancer stimulate transcription whereas silencer repress transcription. These elements are rather poorly understood and their mode of action is under discussion. Enhancers, like promoters, are bound by sequence specific activators that form a

functional enhancosome and influences the rate of transcription.

Global elements like insulators, MARs (matrix attachment regions) and SARs (scaffold associated regions) are only poorly understood. Insulators often form the border between hetero- and euchromatin and therefore influence the global chromatin structure. MARs and SARs influence the localization of larger DNA stretches by supporting DNA loop formation through attachment to the nuclear matrix.

1.2 Eucaryotic RNA Polymerases

Unlike bacteria and archaea that posses only one RNA polymerase, eucaryotes posses three different RNA polymerases that are responsible for the synthesis of the different classes of RNA. According to the elution profile, they were named RNA polymerase I to III [Roeder and Rutter, 1969]. RNA polymerase I (Pol I) is responsible for the synthesis of ribosomal RNA (rRNA). RNA polymerase II (Pol II) transcribes protein coding genes into messenger RNA (mRNA) and RNA polymerase III (Pol III) transcribes transfer RNA (tRNA) [Young, 1991]. Biochemically, the RNA polymerases can be distinguished according to their sensitivity for α-amanitin (Tab.1). RNA polymerase I is not sensitive to α-amanitin whereas RNA polymerase II (stronger) and RNA polymerase III (weaker) exhibit α-amanitin sensitivity [Roeder and Rutter, 1970].

Type	Gene	Transcripts	α-amanitin sensitivity
Pol I	class I	18S-, 5.8S, 28S-rRNA	none
Pol II	class II	mRNA, snRNA	$K_D = 10^{-8}$M
Pol III	class III	tRNA, 5S-rRNA, snRNA	$K_D = 10^{-6}$M

Table 1: RNA-polymerases in the eucaryotic nucleus. Transcripts and sensitivity to α-amanitin are indicated.

In Eucarya, Pol II is highly conserved where it contains 12 subunits (Rpb1 - Rpb12). Several crystal structures of Pol II have been solved which includes the

10 and 12 subunit Pol II, Pol II bound to the general transcription factors TFIIS, TFIIB as well as Pol II with nucleotides [Cramer et al., 2001, Armache et al., 2003] [Kettenberger et al., 2003, Kettenberger et al., 2004, Bushnell et al., 2004] [Armache et al., 2005]. As a feature specific to eucarya, the largest subunit of Pol II - Rpb1 - contains a C-terminal domain (CTD) which consists of 25 to 52 hepta repeats with the consensus sequence YSPTSPS [Corden, 1990], the exact length depends on the organism. The CTD as a whole is essential in yeast, it's deletion is lethal [Nonet and Young, 1989]. The CTD consensus motif is bound by factors that regulate transcription initiation, elongation, termination and mRNA processing [Woychik and Hampsey, 2002, Hahn, 2004, Lima, 2005]. The phosphorylation state of CTD correlates and regulates the transcription cycle [Dahmus, 1996]. Only dephosphorylated CTD is bound by Mediator during initiation, phosphorylation breaks this interaction. Mainly the Cdk/Cyclin pairs of TFIIH (Cdk7/CyclinH) and of Mediator (Cdk8/CyclinC) are responsible for CTD phosphorylation at Ser5 during initiation. During elongation, pTEFb (Cdk9/CyclinT) phosphorylates the CTD preferably at Ser2 [Pinhero et al., 2004]. For transcription reinitiation, the CTD has to be dephosphorylated again. In yeast, Fcp1, Scp1 and Ssu72 are known phosphatases that dephosphorylate the CTD [Lin et al., 2002, Meinhart et al., 2003, Kamenski et al., 2004].

CRSP (cofactor required for Sp1), a human Mediator complex, interacts with Pol II CTD and adopts a specific conformation that differs from free Mediator complex [Naar et al., 2002]. Mediator function needs a CTD interaction, since yeast Mediator can not support transcription of a CTD-less Pol II. Furthermore, only an intact CTD allows the formation of a stable Pol II / Mediator complex [Myers et al., 1998, Asturias et al., 1999].

1.3 Transcription factors

1.3.1 The general transcription factors

The generation of functional class II *in vitro* transcription systems using nuclear extracts and subsequent fractionation of these extracts led to the identification of the general transcription factors (GTFs), TFIIA, TFIIB, TFIID, TFIIE, TFIIF and

TFIIH (Tab.2) [Roeder, 1996, Lee and Young, 2000]. These GTFs are required for start-site recognition, promoter melting and early transcription elongation. They form the minimal set of factors that are required *in vitro* for activated transcription initiation from model promoters [Hampsey, 1998].

Factor		Function
TFIID	TBP	core promoter recognition, binds to the TATA box and bends the DNA
	TAFs	INR and DPE recognition
TFIIA		stabilization of the TBP - DNA interaction
TFIIB		recruitment of RNA polymerase II and TFIIF, determination of the transcriptional start site
TFIIF		recruitment of RNA polymerase to the promoter. Involvement in open complex formation.
TFIIE		recruitment of TFIIH, modulation of the kinase- and helicase activity of TFIIH
TFIIH	XPB	3'-5' helicase activity for promoter melting and open complex formation
	XPD	5'-3' helicase activity for DNA repair (nucleotide excision repair; NER)
	Cdk7	phosphorylation of CTD
	CycH	regulation of Cdk7

Table 2: The human general transcription factors

During transcription initiation, the preinitiation complex (PIC) is formed in a stepwise process that starts with the binding of TFIID to the TATA box. TFIID consists of the TATA-box binding protein (TBP) and TBP-associated factors (TAFs) [Tora, 2002]. TBP is required for TATA recognition and bending of the promoter DNA [Kim et al., 1993a, Kim et al., 1993b]. TBP together with TAFs facilitates basal transcription. The TAFs are required for activated transcription and for recognition of TATA-less promoters. TFIIA enhances and stabilizes the formation of this primary complex [Geiger et al., 1996, Tan et al., 1996]. Subsequent binding of TFIIB, Pol II, TFIIF, TFIIE and finally TFIIH completes PIC

formation [Van Dyke et al., 1988, Buratowski et al., 1989]. The XPB helicase of TFIIH is essential for promoter melting and is ATP dependent. The kinase activity of the Cdk7/CyclinH pair of TFIIH phosphorylates the CTD of Pol II at Ser5 [Hengartner et al., 1998]. This phosphorylation weakens the Pol II / Mediator interaction and releases Pol II in the presence of NTPs into the elongation phase [Serizawa et al., 1993, Kim et al., 1994, Li and Kornberg, 1994].

TBP is a special case, since it is involved in transcription with all three RNA polymerases [Hernandez, 1993]. It is a component of the SL1 complex for class I gens, in TFIID for class II genes and in part of a TFIIIB form at class III genes. Additionally, two more TBP containing complexes are known, the TAC [Mitsiou and Stunnenberg, 2000] complex and B-TFIID [Pereira et al., 2001], that can substitute for TFIID at least *in vitro*. BTAF1 or the yeast homolog Mot1p, were shown to interact with TBP and to regulate TBP function dynamically on promoters of class II genes [Pereira et al., 2003, Geisberg and Struhl, 2004]. Recently, two new groups of TBP paralogs were revealed. One group comprises the TBP-like factor (TLF) and TBP-related factor 1 and 2 (TRF1 and TRF2). These paralogs can bind to DNA sequences different from the canonical TATA box [Dantonel et al., 1999, Rabenstein et al., 1999] and support transcription from TATA-less promoters [Ohbayashi et al., 2003]. The second group of paralogous genes to TBP identified in vertebrates are called TBP2s [Veenstra et al., 2000]. Not much is known about the function of these novel genes.

TFIIB is recruited to the promoter after the binding of TBP and the TAFs. Direct interactions of TFIIB with TBP and the Pol II as well as with DNA have been reported. On the level of DNA TFIIB recognizes and binds to the BRE [Imbalzano et al., 1994]. These interactions help to position the Pol II in the right direction and to select the proper transcriptional start site [Buratowski et al., 1989, Nikolov D. and S., 1995, Bushnell et al., 2004].

TFIIF binds tightly to Pol II, stabilizes the PIC and helps to suppress non-specific DNA binding of Pol II [Conaway and Conaway, 1993]. Additionally, it

plays an important role in elongation, by preventing the pausing of Pol II. Both subunits of TFIIF show some sequence similarities to the σ-factor of the bacterial transcription machinery.

TFIIE interacts directly with Pol II, TFIIF and TFIIH, which it recruits to the PIC [Flores et al., 1989]. Additional functions of TFIIE are the stimulation of the kinase as well as the helicase activity of TFIIH [Lee and Young, 2000]. TATA containing promoters are dependent on TFIIE where a checkpoint function for PIC formation was suggested.

TFIIH contains four enzymatic activities, two ATP dependent DNA helicases [Schaeffer et al., 1993], a DNA dependent ATPase [Roy et al., 1994] and a CTD kinase [Lu et al., 1992]. It is an essential factor in transcription as well as in nucleotide excision repair (NER) [Svejstrup et al., 1995]. The ATP dependent 3' - 5' helicase activity of XPB is required for promoter melting [Wang et al., 1992]. The kinase domain containing the Cdk7/CyclinH pair is involved in the transition from transcription initiation to elongation [Dvir et al., 1997].

1.3.2 Activators and coactivators

DNA bound transcription activators transmit many different signals to the transcription machinery. These gene specific activators can recruit additional coactivators by forming direct protein-protein interactions. Typically, a transcription activator contains a DNA binding domain as well as one or more activation domains. The DNA binding domain allows sequence specific binding to promoter regions. Several different DNA binding domains have been characterized. A common DNA binding motif is the Zinc finger, where a Zn^{2+}atom is coordinated by two cysteines and two histidines (e.g. in SP1). Other DNA binding domains contain a helix-loop-helix (HLH) motif (e.g. in USF) or a leucine-zipper (e.g. in CREB, Myc). The leucine-zipper is also a dimerization motif which is formed by a series of leucines at every seventh position of an α-helix.

The activation domains comprise normally 30 to 100 amino acids. These domains are often highly flexible in solution and form secondary structures only upon interaction with their target proteins that can be described by an induced fit mechanism. Activation domains are categorized according to the nature of their amino acid sequence. Commonly found activation domains are glutamine rich (SP1, Oct1, CREB), proline rich (SMAD4, AP-2), serine-/threonine rich (Sox-2, Sox-4) and acidic (VP16, NFκB, E1A, GAL4, p53).

1.3.3 The viral activator VP16

A well studied activator that is commonly used as a model system for acidic activators is the Herpes simplex virion particle protein 16 (VP16). The protein contains a N-terminal DNA binding domain (amino acids 49 - 412) and a C-terminal activation domain (amino acids 411 - 490) [Triezenberg et al., 1988, Cousens et al., 1989]. The crystal structure of the DNA binding domain has been solved. It shows no preferred binding to specific DNA sequences alone but recognizes target sequences of Herpes simplex virus (HSV) immediate early genes in conjunction with Oct-1 and HCF-1[Liu et al., 1999, Babb et al., 2001]. The small activation domain can be divided into two even smaller independent functional subdomains termed VP16:H1 (aa 411 - 456) and VP16:H2 (aa 457 - 490) (Fig.3) [Walker et al., 1993].

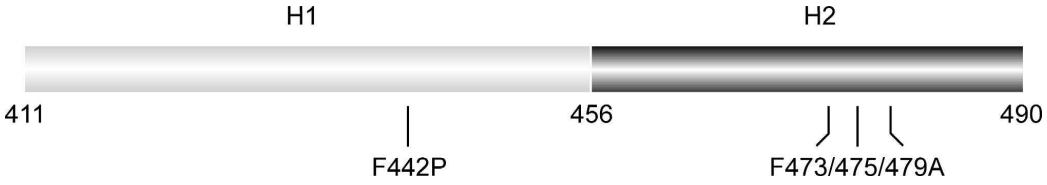

Figure 3: Schematic overview of the VP16 activation domain. Both subdomains, H1 and H2, can function separately from each other. The functionally relevant point mutations targeting the phenylalanines are indicated.

Under physiological conditions, the activation domain is not structured but forms a random coil instead [Donaldson and Capone, 1992]. At low pH or under hy-

drophobic conditions, however, helical structures could be observed. Additionally, interaction of VP16 with TAF9 or PC4 converts the unstructured activation domain into an α-helix [Shen et al., 1996, Uesugi et al., 1997, Jonker et al., 2005]. Mutational analysis revealed point-mutants in VP16:H1 and VP16:H2 that compromise transcription in the context of transient reporter assays as well as in viral infection. Several phenylalanine residues in both subdomains proved to be critical for activation of transcription (VP16:H1mt, Phe442Pro; VP16:H2mt, Phe473/475/479Ala) [Cress and Triezenberg, 1991, Sullivan et al., 1998]. The strong transcription activation potential of VP16 is probably due to the fact that it interacts with many components of the transcription machinery as well as with chromatin modifying complexes. Interactions with the VP16 activation domain were shown for the general transcription factors TFIIA [Kobayashi et al., 1995], TFIID [Goodrich et al., 1993], TFIIF [Zhu et al., 1994], TFIIH [Xiao et al., 1994], the cofactor PC4 [Kretzschmar et al., 1994a], the histone acetyl-transferase CBP/-p300 [Kraus et al., 1999, Ikeda et al., 2002] and the yeast histone acetyltransferase complexes SAGA and NuA4 [Utley et al., 1998] and the Swi/Snif histone remodeling complex [Neely et al., 1999]. Furthermore, VP16 interacts with human Mediator through the MED17 [Ito et al., 1999] and MED25 [Mittler et al., 2003] subunits.

1.4 Mediator complexes

A general transcription cofactor that transmits regulatory information to the basal transcription machinery is the large multi subunit Mediator complex that contains 22-28 subunits [Myers and Kornberg, 2000, Bjorklund and Gustafsson, 2004] [Blazek et al., 2005, Kornberg, 2005] that binds tightly to activators as well as Pol II and controls transcription from class II genes. By binding to Pol II, it is responsible for basal transcription [Mittler et al., 2001, Baek et al., 2002] as well as activated transcription [Flanagan et al., 1991]. Mediator is also part of a scaffold complex that is necessary for transcription reinitiation [Yudkovsky et al., 2000]. The Mediator contains a Cdk8/Cyclin C Kinase module that phosphorylates the CTD of Pol II at serine 5 [Hengartner et al., 1998, Borggrefe et al., 2002]. In addition Cdk8 phosphorylates Cyclin H which stimulates the CTD phosphorylation

activity of the TFIIH associated kinase Cdk7. Therefore, Mediator does not only serve as an assembly platform for the transcriptional machinery but also stimulates the transition from initiation to elongation of transcription.

Mediator complexes have first been purified from yeast *S. cerevisiae*. Biochemical screens revealed factors that bind tightly to activators [Kelleher et al., 1990, Berger SL., 1990] this activity was further characterized and tested in a purified transcription system [Flanagan et al., 1991]. SRB genes were shown to associate with the CTD of the largest subunit of Pol II [Thompson et al., 1993] and with yet unknown proteins to form the large Mediator complex [Kim et al., 1994]. Despite the low sequence conservation among different species, Mediator seems to be conserved throughout evolution [Boube et al., 2002, Bourbon et al., 2004]. However, the low conservation, the enormous size of 1-2 MDa and the complexity made it difficult to isolate human homologs of Mediator. Using the activation domain of the Thyroid-hormone receptor, R.G. Roeders laboratory identified the TRAP complex [Fondell et al., 1996]. Using different purification schemes several labs were able to purify large protein complexes that showed minor differences in their subunit compositions (ARC/CRSP, DRIP, PC2, SMCC and TRAP). All these complexes turned out to represent human Mediator complexes [Naar et al., 1999, Ito et al., 1999, Naar et al., 2001, Sato et al., 2004, Blazek et al., 2005].

1.4.1 Human Mediator complexes

Biochemical screens aiming at the identification of cofactors that bind the ligand dependent activation function (AF2) of the thyroid- and the vitamin D receptor led to the discovery of the TRAP (thyroid hormone receptor associated proteins) [Fondell et al., 1996] and DRIP (vitamin D receptor interacting proteins) [Rachez et al., 1999] complexes. A Cdk8-associated complex, SMCC (Srb/Mediator containing complex) [Gu et al., 1999] proved to be identical to the TRAP complex [Ito et al., 1999]. From the earlier mentioned coactivator fraction [Meisterernst et al., 1991] PC2 [Kretzschmar et al., 1994b], and the CRSP complex (cofactor required for Sp1) [Ryu and Tjian, 1999] were isolated. In contrast to TRAP/SMCC and DRIP, PC2 and CRSP lack the kinase module (comprised of MED12, MED13, Cdk8 and CyclinC). Furthermore, affinity purification us-

new Name	*S. cerevisiae* (old name)	*H. sapiens* (old name)
MED1	Med1	TRAP220
MED2	Med2	-
MED3	Pgd1/Hrs1/Med3	-
MED4	Med4	TRAP36/DRIP36
MED5	Nut1	-
MED6	Med6	hMed6/Drip33
MED7	Med7	hMed7/Drip34
MED8	Med8	Arc32
MED9	Cse2/Med9	Med25
MED10	Nut2/Med10	hNut2/hMed10
MED11	Med11	HSPC296
MED12	Srb8	TRAP230/DRIP240
MED13	Srb9	TRAP240/DRIP250
MED14	Rgr1	TRAP170/DRIP150/CRSP150
MED15	Gal11	ARC105/PCQAP
MED16	Sin4	TRAP95/DRIP92
MED17	Srb4	TRAP80/DRIP77/CRSP77
MED18	Srb5	p28b
MED19	Rox3	LCMR1
MED20	Srb2	hTRFP/p28a
MED21	Srb7	hSrb7/p21
MED22	Srb6	Med24/Surf5
MED23	-	βTRAP150/DRIP130/CRSP130/hSur2
MED24	-	TRAP100/DRIP100/CRSP100
MED25	-	ARC92/ACID1
MED26	-	ARC70/CRSP70
MED27	-	TRAP37/CRSP347
MED28	-	Fksg20
MED29	-	Hintersex
MED30	-	TRAP25
MED31	Soh1	hSoh1
Cdk8	Srb10/Ssn3/Ume5	hSrb10/CDK8
Cyclin C	Srb11/Ssn8/Ume3	hSrb11/CycC

Table 3: Mediator subunit nomenclature according to [Bourbon et al., 2004].

ing the activation domains of SREBP (sterol receptor enhancer binding protein) and VP16 identified ARC [Naar et al., 1999] a complex that proofed to be highly related to the DRIP complex. In conclusion, two prominent forms of human Mediator exists, the large forms containing the kinase module (TRAP/SMCC and ARC/DRIP) and the smaller forms that lack the kinase module (PC2 and CRSP).

1.4.2 Organization and structural properties of Mediator

The mammalian Mediator complex contains 28 to 30 subunits [Sato et al., 2004, Malik and Roeder, 2005a] (Fig.4). Presumably, the murine and human complex are also organized into subcomplexes and modules similar to the yeast complex [Dotson et al., 2000]. In yeast, genetic [Lee et al., 2000], biochemical [Myers and Kornberg, 2000] and structural studies [Dotson et al., 2000] revealed the modular organization of Mediator. Even though comparable data for the metazoan complex is lacking, an analogous organization is most likely.

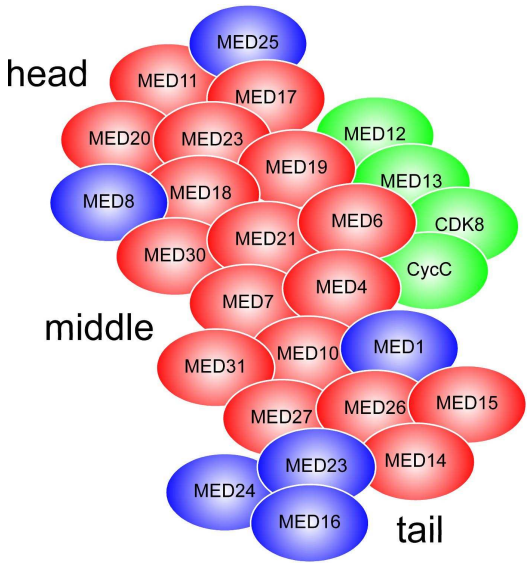

Figure 4: Subunit structure of the Mediator complex. Subunits present in most Mediator preparation are shown in red. Subunits only loosely attached to this core Mediator are depicted in blue. The Kinase module that is restricted in the large Mediator forms is shown in green. (Adapted from [Malik and Roeder, 2005a])

The "small" Mediator complexes, B-Med, PC2 and CRSP have a set of 18 to 20 subunits in common that might correspond to a central core of tightly associated proteins [Sato et al., 2004, Malik and Roeder, 2005a]. Other proteins seem to be less strongly associated and might contribute to important mechanistic aspects of Mediator function. These proteins are, MED1, MED23, MED24, MED16, MED25, MED13, MED12, Cdk8 and CyclinC. The subunits MED23, MED24 and MED16 most likely constitute a submodule, because Mediator complexes isolated from mouse cell deficient of either MED23 [Stevens et al., 2002] or MED24 [Ito et al., 2002a] lack each of these subunits. The four subunits MED13, MED12, Cdk8 and CyclinC form a genetic, functional and physically separable module in yeast [Borggrefe et al., 2002]. This kinase module is greatly reduced in the B-Med, PC2 and CRSP complex but present in the larger TRAP and ARC complexes. Most recently, PC2 and CRSP/Med2 complexes lacking the MED1 subunit could be isolated [Malik et al., 2005, Taatjes and Tjian, 2004].

There is presently no high resolution X-ray structure of the complete Mediator available. However, low resolution electron microscopy data show an elongated overall structure for yeast, human and mouse Mediator [Dotson et al., 2000, Taatjes et al., 2002]. Yeast Mediator unfolds upon interaction with Pol II and adopts an elongated form that reaches around the polymerase [Asturias et al., 1999]. In this extended form of Mediator, the characteristic domains, head, middle and tail, are clearly visible. These EM studies suggest the head and middle module as Pol II interaction surfaces. EM analysis of the human complex together with various activators, Pol II CTD and the kinase module [Naar et al., 2002, Taatjes et al., 2002, Taatjes et al., 2004] showed a similar overall architecture of human and yeast Mediator. The data reasoned for conformational changes dependent on the binding of the activators, the CTD and the kinase module. The relevance of these structural changes on transcription initiation is unclear. However, it has been speculated that they facilitate PIC assembly as well as promoter clearance. The first high resolution data for parts of the yeast Mediator could be obtained by X-ray crystallography of the MED7/MED21 heterodimer of the middle module [Baumli et al., 2005]. This hetero dimer forms a long helical coiled-coil like structure that spans 110 Å in length. A hinge region leads to a change in the position of the C-terminus of 10 Å. This movement might account for at least

some of the different conformations observed in the EM structures.

1.4.3 Transcription regulation by Mediator

The molecular details of the regulation of RNA polymerase II transcription by Mediator are not yet completely understood. It involves complicated interaction network between Mediator, transcriptional activators, GTF's, cofactors and RNA polymerase II. A key function of Mediator is probably the recruitment of Pol II to promoter regions. Mediator was found to be associated with Pol II in solution [Kim et al., 1994, Meyers, 2000, Taatjes and Tjian, 2004] suggesting simultaneous binding to promoters [Thompson et al., 1993]. This was seen in some cases [Hatzis, 2002, Metivier R., 2003] however in other studies Mediator binds to promoters first [Cosma et al., 2001, Park et al., 2001].

Mediator stimulates basal transcription in in reconstitutes yeast systems [Kim et al., 1994] as well as human systems [Mittler et al., 2001, Baek et al., 2002, Wu SY., 2003]. B-Med, a small Mediator complex lacking the Cdk8 and MED16 module but containing MED26 proofed to be essential in crude systems for basal transcription [Mittler et al., 2001]. The strong effects on transcription were frequently seen with small Mediators like CRSP, PC2 and B-Med [Naar et al., 1999, Kretzschmar et al., 1994b, Mittler et al., 2001]. These complexes have been found to bind stronger to Pol II than large Mediator complexes [Naar et al., 2002]. Indeed, activation could directly relate to binding to the CTD of Pol II, cleavage of CTD does not inactivate the function of Pol II but it eliminates the basal and the activation function of Mediator [Myers et al., 1998]. TFIIH plays a key role in the regulation of the activity of Pol II. Mediator binds directly to TFIIH [Sakurai H., 2000, Giot l., 2003] and the Cdk7-Cyclin H pair of TFIIH is a subject of Mediator control. Mediator activates the kinase function of Kin28 the yeast Cdk7 homolog. Additionally, Cdk8 phosphorylates the Cyclin H and thereby inhibits the associated Cdk7 kinase.

Towards the question how Mediator is recruited by activators, several knock out cell lines have been generated. The pathway involving nuclear hormone receptors like the thyroid hormone receptor (TR) uses MED1 as direct interaction partner.

The activation function 2 (AF2) of TR binds to the Leu rich motif (LxxLL) of the MED1 subunit to recruit the complete complex [Ito et al., 2000]. In MED1$^{-/-}$cells, even though the complex is intact, nuclear hormone receptor functions are impaired. However, normal activation function is observed with other activators like VP16 and p53, that interact with different Mediator subunits [Ge et al., 2002, Malik et al., 2004]. MED23$^{-/-}$embryonic stem cells show a defect in the function of E1A and of Elk-1 [Boyer et al., 1999, Stevens et al., 2002]. Both of these factors were shown to bind directly to MED23, thereby recruiting Mediator to the target promoter. Similarly, the MED15 subunit is required in Xenopus embryos for the function of SMAD2-SMAD4 and SMAD3-SMAD4 complexes in the transforming growth factor-β and the nodal pathways [Kato et al., 2002]. Interactions of VP16 with MED17 [Ito et al., 1999] and MED25 [Mittler et al., 2003, Yang et al., 2004] have been demonstrated. All of these interactions lead to a recruitment of Mediator to the target promoter and hence positioning it in the vicinity of the PIC. They also allow for targeting of activator-specific distinct Mediator complexes to target promoters as well as for subtle conformational changes in Mediator, resulting from interactions with distinct activators, that could confer activator specific properties.

1.5 Chromatin

The DNA in a cell does not exist in a bare form, but is rather bound to small basic proteins called histones. This nucleoprotein material is termed chromatin. There are five types of histones H1, H2A, H2B, H3 and H4. The basic unit of chromatin in eucaryotes consists of 200 bp of DNA wrapped around a protein octamer formed by H2A, H2B, H3 and H4 [Chambon, 1978, Kornberg and Lorch, 2002]. In a nucleosome, 146 bp of DNA are wrapped twice around the protein core. The remaining DNA serves as a linker, and contributes to the flexibility of the chromatin fiber. In this way, the chromatin fiber forms a beads on a string structure.

The flexibility of DNA enables not only the formation of nucleosomes but also higher-ordered structures. Nucleosomes are the first stage in the condensation of DNA. By winding the DNA around the histone octamer, the linear length of the

DNA gets reduced. The 200 bp of DNA with a linear length of about 68 nm are thereby packed onto a 10 nm particle. At the next step of condensation, histone H1 plays an important role. Histone H1 binds the linker DNA in between two nucleosomes. There is only one histone H1 per nucleosome compared to two copies of each core histone. The function of histone H1 is to bind to histone H2A and by doing so, to tether the nucleosomes together. In the second stage of chromosome compaction, the nucleosomes themselves form a helical structure resulting in a 36 nm fiber. These 36 nm fibers can then be folded into even higher-ordered chromatin loops of about 300 nm diameter as well as in the 700 nm chromosomes.

Packing DNA into nucleosomes is relevant to gen regulation. If packed into chromatin, DNA is no longer freely accessible for many transcription factors. Enzymes that allow tor the regulation of DNA accessibility introduce modifications at the histones or remodel nucleosomes in an ATP dependent manner [Strahl and Allis, 2000, Becker and Horz, 2002]. In fact, many transcription cofactors have been identified that modify histone tails, linking histone modification directly to transcription [Bannister and Kouzarides, 1996] Common postranslational modifications are acetylation, methylation, phosphorylation and ubiquitination. For many of these modifications the precise residue that gets modified has been determined. Genes that contain histone tail modifications are recognized by different transcription cofactors, a histone-code hypothesis was proposed [Strahl and Allis, 2000, Jenuwein and Allis, 2001]. The theory implies individual changes and/or that specific combinations of histone modification provide binding sites for different effector proteins that translate this code into different binding platforms for transcription factors. For example, hyperacetylation of histone H3 and H4 correlate with actively transcribed genes, whereas hypoacetylation of these histones marks repressed genes [Eberharter and Becker, 2002]. Additionally, correlations to active transcription could be made for methylation of the histone H3 at K4, K36 and K79, whereas methylation of histone H3K9 is found to mark repressed genes [Schubeler and Turner, 2005].

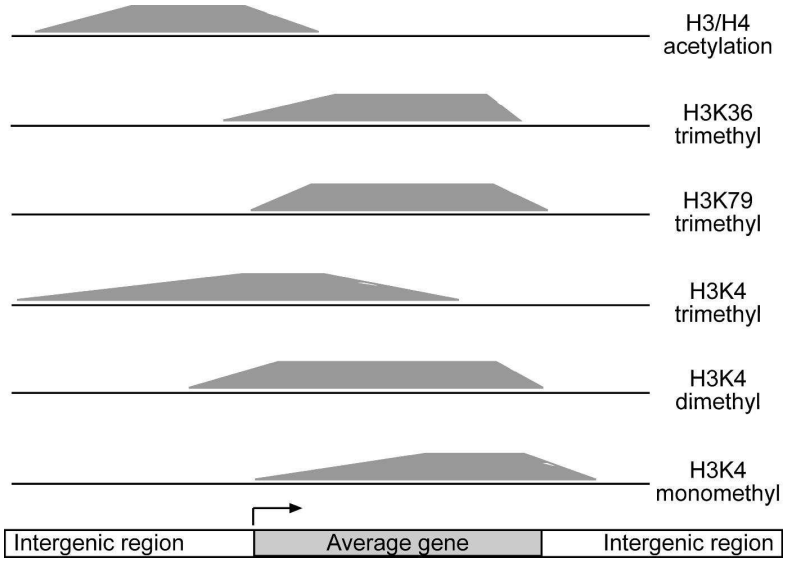

Figure 5: Distribution of histone modifications across a typical yeast gene. (Adapted from [Schubeler and Turner, 2005]).

When assessing the genome wide distribution of modified histones in yeast, one can show that different modifications predominate at the beginning, middle and the end of genes. Acetylated histones peak at the beginning of genes, whereas the distribution of methylated histones depends on the extent (mono-, di-, tri-) of methylation and the lysine that is methylated. Histone H3K4 trimethylation predominates at the beginning of genes and correlates with acetylation of histones whereas dimethylated histone H3K4 is enriched towards the middle and the monomethylated form is most abundant at the end of genes. Methylation of histone H3K36 is seen at the beginning of a gene and gradually increases towards the middle. Methylation of histone H3K79 is restricted to the actual gene, and is not found in intergenic regions [Schubeler and Turner, 2005].

1.5.1 Histone modifying enzymes

HATs and HDACs are the enzymes responsible for acetylation and deacetylation of histone tails. The histone acetyl transferases (HATs) can be grouped into three families [Marmorstein and Roth, 2001].

(i) The GCN5 related N-acetyl-transferases (GNAT) acetylate preferentially histone H3. They contain a C-terminal Bromodomain that binds to acetylated histones. Members of this family are GCN5 (yeast), Elp3 (yeast), PCAF (human) and TFIIIC (human).

(ii) The MYST family preferentially acetylates histone H4. Members contain a Chromodomain that binds to RNA and methylated histone tails. Examples of this family are Sas2 (yeast), Sas3 (yeast, NuA3 complex), MOZ (human) and Tip60 (human).

(iii) The CREB binding protein (CBP/p300) not only acetylates histones, but can also use other proteins as substrates (for example, p53, TFIIE and TFIIF). The CBP and p300 proteins contain a central Bromodomain, several Zinc fingers and two independent domains which can be bound by several transcriptional activators. The deletion of either CBP or p300 in mouse is lethal.

The histone deacetylases (HDACs) are counter-players of the HATs in that they reverse the activating effect of histone acetylation. There are three classes of HDACs.

(i) The class I HDACs comprise HDAC1 / 2 / 3 and 8. They share a highly conserved catalytic domain and their activity is closely linked to nuclear hormone receptors. HDAC1 and HDAC2 are part of the Sin3 and the NuRD/Mi2 complexes, respectively.

(ii) The class II HDACs comprise HDAC 4 to 7 and are poorly characterized. (iii) The class III HDACs comprise the yeast Sir proteins and the mammalian SIRT 1 to 7 proteins. Their deacetylase function is, at least *in vitro*, NAD^+dependent.

Histone methyltransferases (HMTs) modify histone tails at either arginine or lysine residues. Arginines can be either mono- or di-methylated by the protein methyltransferases (PRMT1 to 5). Lysines can be mono-, di- and tri-methylated by the HMTs. With the exception of Dot1, all the Lysine-HMTs contain a conserved SET domain [Feng et al., 2002]. Although the methylation of histones is chemically stable, recently the first histone demethylase (LSD1) that removes the methyl groups from were discovered [Shi et al., 2004, Shi et al., 2005]. The ex-

istence of demethylases was hypothesized for a long time, because the inhibitory function of some lysine methylations needs to be overcome in order to allow transcription. Histone replacement was discussed as an alternative possibility to remove lysine methylation from histone tails.

ATP-dependent Chromatin remodeling complexes subject nucleosomes to conformational remodeling in addition to the covalent modifications
[Becker and Horz, 2002]. The mobilization of nucleosomes involves the ATP dependent breaking and reforming of histone-DNA contacts. To date, several different remodeling complexes have been identified. All of these complexes contain an ATPase subunit along with additional subunits that affect regulation, efficiency and specificity. These chromatin remodeling complexes are divided into three groups, depending on the ATPase activity: (i) Swi/Snf, (ii) ISWI and the (iii) Mi2 group [Becker and Horz, 2002].

1.6 EBV plasmids

The Epstein-Barr virus (EBV) is an γ-Herpesvirus, that infects mostly human B-cells but can also infect human epithelial cells. Under normal circumstances, EBV behaves like a harmless parasite, but it also associates with different forms of cancers like Hodgkin-Lymphomas or T-Cell-Lymphomas [Epstein and Kaplan, 1978, Baumforth et al., 1999]. The Epstein-Barr virus does have a dual life cycle with a lytic and a latent phase [Bornkamm and Hammerschmidt, 2001]. After infection, the linear genome of EBV circularizes and remains as an episome in the cell nucleus [Hurley and Thorley-Lawson, 1988]. This episome remains stable during the latent phase of the life cycle and is passed on to the daughter cells. The latent replication of the EBV episome starts from the origin of replication (oriP). A prerequisite for replication of the EBV episome is the presence of EBNA1 that binds to the oriP region. These two components are sufficient for specific replication of the episome.

The EBV nuclear antigen 1 (EBNA1) is an transcription activator. It controls together with EBNA2 the expression of its own and further latent EBV genes

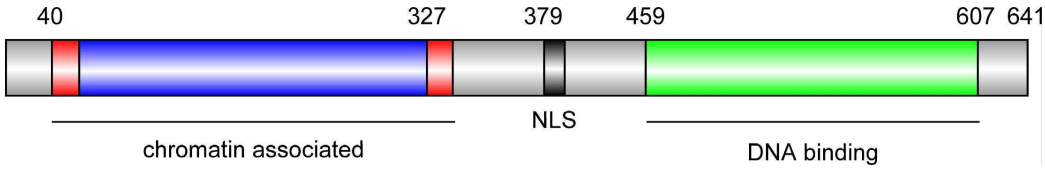

Figure 6: Structure of the viral EBNA1 protein. The C-terminal domain of EBNA1 contains the dimerization and DNA binding domain that binds specifically to the oriP region. The N-terminal transactivation domain consists of two arginine rich regions that are involved in the replication from the oriP region. The arginine rich regions are separated by long but non essential gly-ala repeats.

[Bornkamm and Hammerschmidt, 2001]. The binding of EBNA1 to the oriP is mediated by its C-terminal dimerization domain and its DNA binding domain (Fig.6). The crystal structure of the EBNA1 DNA binding domain is known. Binding to the oriP sequence occurs as a dimer [Rawlins et al., 1985]. To couple the EBV episome to the host genome, EBNA1 binds with its N-terminal domain to the host genome, connecting the two genomes [Mackey and Sugden, 1999b, Leight and Sugden, 2000, Kapoor and Frappier, 2003]. The EBV episome remains bound to the metaphase chromosomes during the cell cycle and ensures an evenly distribution of the episome [Leight and Sugden, 2000].

The oriP is a 1.8 kb large DNA stretch that consists of two elements, the family of repeats (FR-element) and the dyad symmetry (DS-elements) [Yates et al., 1984, Yates et al., 2000] (Fig.7). The FR-element contains 20 repeats of the EBNA1 binding motive and is most important for the episomal stability of the plasmid. The second functional element, the DS-element is approximately 1 kb away from the FR-element. The DS-element is 120 bp in length and harbors four EBNA1 binding sites in a pairwise arrangement. The DS-element is crucial for replication. Deletion or changes in the spacing between the EBNA1 binding sites leads to the loss of replication [Yates et al., 2000].

Exploiting the properties of the EBV episome by combining the expression of the EBNA1 gene and the oriP on one plasmid allows the generation of episomally stable cell lines [Mackey and Sugden, 1999a]. The advantage of such a system is that the generation of a stable cell line is rather quick and straight forward. Copy

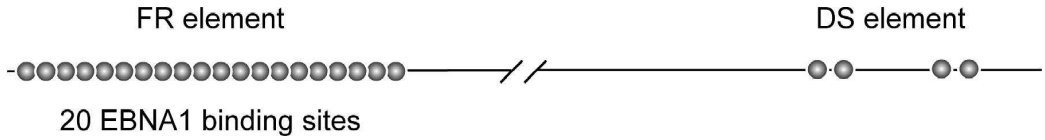

Figure 7: The latent origin of replication, oriP. OriP contains two functional elements, the family of repeats (FR element) and the dyad symmetry (SD elements). Both elements contain EBNA1 binding sites. The FR element is important for the episomal stability of the plasmid, the DS element is crucial for replication.

numbers are around 5 to 20 copies per cell, which is considerably less than in transient transfection assays, resembling in this respect the situation of a chromosomal gene more closely. A second advantage is the assembly of nucleosomes on this plasmids *in vivo*. Therefore they can also be used as a model system for chromatin related events.

1.7 Aims and scope of this work

The aim of this study was to gain further insight into transcription initiation mediated by VP16. A specific objective was the establishment of an inducible VP16 system based cell lines harboring stably replicating (EBV-)based plasmids. Most systems used for studying transcription initiation in mammalian cells so far are either *in vitro* systems or make use of endogenous genes. What was missing so far was an abstract, conditional *in vivo* model system that allows to verify biochemical knowledge about a defined activator in a physiological context. *In vitro* systems are useful to unravel molecular processes, but they usually simplify the situation and in an extreme situation may even lead to missinterpretations, because of non-physiological factor concentrations. The few inducible *in vivo* systems exploit hormone inducible endogenous activators that can act rapidly. However in using hormones, secondary effects have to be taken into account. The use of an exogenous DNA binding domain together with an exogenous transactivator has the advantage of . The aim of this study was a time-resolved analysis of the transcription initiation process using ChIP analysis. Especially the function of Mediator and its crosstalk to other transcription cofactors were in the focus of the investigation. One central aspect was the characterization of the VP16 specific MED25-containing (A-Med) complexes *in vivo* as well as *in vitro*.

A second aim was the biochemical characterization of the newly identified Mediator subunit MED25. Its role in forming a functional preinitiation complex and its molecular mode of interaction with VP16. Towards this goal first steps in understanding how activators contact Mediator on a structural level were planed in collaboration with P. Cramer.

2 Materials and Methods

2.1 Materials

2.1.1 Instruments

Acrylamide gel electrophoresis	Amersham / Hoefer / BioRad
Agarose gel electrophoresis	BioRad
Analytical balance	AE 100 and 163, Mettler
Autoradiography cassette	Amersham, Kodak
Centrifuges	Avanti, Beckman Multifuge 3L-R, Heraeus, 5417 / 5415R, Eppendorf
Confocal light microscope	TCS SP2, Leica
Developing machine	Curix60, Agfa
Electroblot, semi-dry	BioRad
Geigercounter	LB122, Berthold
Gel drier	GD2000, Hoefer
Heatingblock	Thermomixer compact, Eppendorf
Homogenizer	Douncer, Wheaton
Incubator	WJ311, Forma Scientific Unequip, Unitherm B6200, Heraeus
Instant Imager	Packard
Light microscope	Axiovert 25, Zeiss
PCR-Thermocycler	GeneAmp 2400, Applied
pH-Meter	Calimatic 760, Knick
Photometer	GeneQuant Pro, Amersham
Rotors	JA10, JA25-50, SW41, SW28, Beckman
Sonifier	W250 and 250-D, Branson
Ultra-centrifuges	L7, L8-M, Heraeus
UV-Illuminator	Bachofer

2.1.2 Chemicals and biochemicals

Acetic acid (p.a.)	Roth
Acrylamide/Bisacrylamide 30% (Rotiphorese Gel 30)	Roth
Acrylamide 30% / 40%	Roth
Agarose	Gibco
Ammonium persulfate (APS)	Merck
Ammonium sulfate	Merck
Ampicillin	Roth
Aprotinin	Sigma
Bacto Agar	Difco
Bacto Trypton	Difco
Bacto Yeast Extract	Difco
Benzamidine	Sigma
Bisacrylamide 2%	Roth
Boric acide	Roth
Bradford reagent	BioRad
5-Bromo-4-Chloro-3-indolyl-phosphate (BCIP)	Peqlab
Bromphenol Blue	Sigma
BSA	Roche
Caesiumchlorid	Sigma
Calciumchloride	Merck
Calciumhydrogenphosphate	Merck
Calciumhydroxide	Merck
CHAPS	Sigma
Chloroform	Merck
Coomassie brilliant blue R-250	Sigma
DAPI	Sigma
Deoxycholat (DOC)	Sigma
Dimethylsulfoxide	Sigma
Dithiothreitol (DTT)	Roth
Doxycycline	Sigma
DMEM medium	Gibco

dNTPs	Roche
Ethanol	Sigma
Ethanolamine	Sigma
Ethidium bromide	Sigma
Ethylendiamintetraacetate disodium salt (EDTA)	Merck
Fetal calf serum (FCS)	Gibco
Fish DNA	Sigma
Fish gelatine	Sigma
Glucose	Merck
Glykogen	Sigma
Glycerol	Roth
Glycine	Roth
Hepes	Biomol
Histogel mounting medium	Linaris
IGEPAL CA630 (NP-40)	Sigma
IPTG	Roth
Isoamyl alcohol	Merck
Isopropanol	Merck
Leupeptin	Roche
Lithiumchlorid	Sigma
Magnesiumchloride	Merck
β-Mercaptoethanol	Sigma
Methanol	Merck
Milk powder	Heirler, Roth
Mimosine	Sigma
N-Laurylsarcosin	Sigma
Nitro-blue-tetrazolium (NBT)	Peqlab
Paraformaldehyde	Roth
Penicillin-Streptomycin	Invitrogen
Phenol/Chloroform	Roth
Phenylmethylsulfonfluoride (PMSF)	Biomol
Phorbolmyristylester (PMA)	Roche
Ponceau S	Sigma

Protein G-Sepharose	Amersham
Protein A-Sepharose	Amersham
Radiochemicals	APB
RPMI 1640 medium	Gibco
Sodium azide	Sigma
Sodium borate	Roth
Sodium carbonate	Merck
Sodium chloride	Roth
Sodiumdodecylsulfate (SDS)	Merck
Sodium fluoride	Sigma
Sodium hydroxid	Merck
Sucrose	Sigma
Tetramethylethylendiamin (TEMED)	Sigma
Thymidine	Sigma
Trishydroxidimethyl-aminomethan (Tris; p.a.)	Sigma
Triton X-100	Sigma
Trypsin-EDTA	Gibco
Urea	Roth
Xylene cyanole	Fluka

2.1.3 Additional material

Disposable plastic material	Greiner, Nunc, TPP, Falcon
Dialysing tubes (Viking, MWCO 15 kDa)	Roth
ECL Western Blot Kit	NEN
Film X-OMAT, BioMax	Kodak
Gel Drying Kit	Promega
GFX Gel Band Purification Kit	Amersham
Luciferase Kit	Promega
mRNA Kit	Qiagen
Nitrocellulose membrane	BioRad
Nucleobond AX Plasmid DNA Kit	Machery & Nagel
Paramgnetic beads	Promega

RT-PCR Kit	Invitrogen
Siliconized Plastic tubes	Sorenson
Silver Staining Kit	PlusOne, Amersham
Sterilfilter	Roth
Whatman 3MM Paper	Whatman
TNT Kit	Promega

2.1.4 Enzymes

Calf intestine phosphatase	Fermentas
Klenow Fragment	Fermentas
Lysozym	Sigma
Pfu Polymerase	Promega
Proteinase K	Roche
Restriction enzymes	NEB or Fermentas
RNase A	Roche
T4 DNA ligase	NEB or Fermentas
T4 polynucleotide kinase	NEB
Taq polymerase	Fermentas

2.2 General buffers

BCx-buffer
20 mM Tris-HCl, pH 7.3 RT
0.2mM EDTA
20% (v/v) Glycerol
x mM KCl

PBS (phosphate-buffered saline)

10x stock solution, 1 liter:	Working solution:
80 g NaCl	137 mM NaCl
2 g KCl	2.7 mM KCl
11.5 g $Na_2HPO_2 \bullet 2H_2O$	4.3 mM $Na_2HPO_4 \bullet 2H_2O$
2 g KH_2PO_4	1.4 mM KH_2PO_4

TBE (Tris/borate/EDTA) electrophoresis buffer

10x stock solution, 1 liter:	Working solution:
121 g Tris base	100 mM Tris
61.8 g boric acid	100 mM boric acid
40 ml 0.5 mM EDTA, pH 8.0	2 mM

TBS (Tris-buffered saline)

10x stock solution, 1 liter, adjust pH to 7.6:	Working solution:
121 g Tris base	100 mM Tris-HCl, pH 7.6
87.6 g NaCl	150 mM NaCl

TGS (SDS electrophoresis buffer)

10x stock solution, 1 liter:	Working solution:
30.2 g Tris base	12.5 mM Tris
144 g Glycine	192 mM Glycine
10 g SDS	0.1% SDS

TE (Tris/EDTA) buffer
10 mM Tris-HCl, pH 7.3 RT
1 mM EDTA, pH 8.0

2.3 Cloning

2.3.1 Plasmids

Plasmid		cloned
pTU10	pREP4-7x tetO-Vβ8.1-Luc	this work
pML53	pEBNA-SVP-GL-EμCAG-rtTA	G. Bornkam
pTU6	pTet-On	Clontech
pTU7	pTRE2-Luc	Clontech
pTU14	pET21b- $ACID_{345\text{-}548}$	this work
pTU17	pET21b- $ACID_{345\text{-}560}$	this work
pTU15	pET21b- $ACID_{393\text{-}548}$	this work
pTU18	pET21b- $ACID_{393\text{-}560}$	this work
pTU16	pET21b- $ACID_{426\text{-}548}$	this work
pTU19	pET21b- $ACID_{426\text{-}560}$	this work
	pGEX-VP16	T. Stühler
	pGEX-VP16H1	T. Stühler
	pGEX-VP16H1mt	T. Stühler
	pGEX-VP16H2	T. Stühler
	pGEX-VP16H2mt	T. Stühler
pPHSF21	pGEX-$CBP_{1\text{-}1098}$	
pPHS125	pGEX-$CBP_{1098\text{-}1710}$	
pPHS121	pGEX-$CBP_{1678\text{-}2441}$	
pLB60	pCI-Flag-$MED25_{1\text{-}2189}$	L. Berti
pLB84	pCI-Flag-$MED25_{1\text{-}870}$	L. Berti
pLB96	pCI-Flag-MED25ΔACID	L.Berti
	pGL2-MRG5	M. Meisterernst
pPHS54	RcCMV-βGal	

Table 7: Plasmids used in this work.

2.3.2 Plasmid generation

The plasmid pTU10 was cloned as follows. The parental vector pPH273 (pREP4-Vβ8.1-Luc) was cut with the restriction enzyme XhoI for 2 hours at 37°C. After this time, the phosphate groups were removed by Calf Intestine Alkaline Phosphatase (CIAP; Fermentas MBI, Cat. No. EF0341) according to the manufac-

turers protocol. The linearized vector was purified over an 1% agarose gel and recovered using the GFX DNA and Gel Band Purification Kit (Amersham, Cat. No. 27-9602-01). The 7x tetO insert was generated by PCR, using the primers 7xTetO_5 and n7xTetO_3 together with the pTRE2-Luc vector as template. The ~400 bp PCR product was cut with the restriction enzyme XhoI and purified over an 1% agarose gel. Vector backbone and insert were ligated in a 1:5 molar ratio using T4 DNA ligase (Ferments MBI, Cat. No. EL0015) according to the manufacturers protocol. Positive clones verified by restriction digest with XhoI and sequencing.

The plasmids pTU14 to pTU19 were cloned as follows. The parental vector pET21b was linearized with the restriction enzymes NdeI and XhoI for 2 hours at 37°C. The linearized vector was purified over an 1% agarose gel and recovered using the GFX columns. The inserts were generated by PCR, using the primers CID345 and CID548 for pTU14y; primers CID345 and CID560 for pTU17; primers CID393 and CID548 for pTU15; primers CID393 and CID560 for pTU18; primers CID426 and CID548 for pTU16; primers CID426 and CID560 for pTU19 for all PCR reactions, the vector pLB60 was used as a template. The ~360 to 650 bp PCR fragments were cut by the restriction enzymes NdeI and XhoI and purified over an 1% agarose gel and recovered using GFX columns. Vector backbone and insert were ligated in a 1:5 molar ratio using T4 DNA ligase according to the manufacturers protocol. Positive clones were verified by restriction digest.

2.3.3 PCR primers

Primer	Sequence	Application
7xTetO_5	GCC CTT TCG TCT CGA GTT TAC	cloning
n7xTetO_3	TAT CTC GAG CTC GAC CCG GGT ACC	cloning
CID345	CCT GCT TCC CAT ATG AGT CTG	cloning
CID393	CCC TCG GTC ATA TGC AGT CAG T	cloning
CID426	CAC CAA GCT GCA TAT GTC ACT GC	cloning
CID548	CAG CTT CTC GAG CTG GAC CT	cloning
CID560	GCC TGC TCG AGC CCC ATT C	cloning
bio-pGL2	GCA TTC TAG TTG TGG TTT GTC CAA	solid phase template
Luc_bio_dw	GCC GGG CCT TTC TTT ATG TT	solid phase template
159_for	TGC CAA CTA TAT CCA TCT GCA CC	ChIP
159_rev	GAG TGG TAA ACT CGA GTC TAA CT	ChIP
Luc-3a	TTC ATA GCT TCT GCC AAC CGA	ChIP
Luc-5a	AAT GGA AGA CGC CAA AAA CAT	ChIP
OriP_f	TGA TAC CCA GTA GTA GAG TGG	ChIP
OriP_r	CAG CAG GAA AAG GAC AAG CAG	ChIP
TRE1	AGG CGT GTA CGG TGG GAG GCC	ChIP
TRE2	AGG CTG GAT CGG TCC CGG TGT	ChIP

Table 8: PCR primers used in this work.

2.4 Cell culture

2.4.1 Cell lines

HeLa	Human epithelial cell line, cervical carcinoma, adherent.
HeLa Tet-On	HeLa cell line expressing the Tet-VP16 transactivator (Clontech)
HeLa Vβ8.1	HeLa Tet-On cell line carrying the episomal plasmid pREP4-7x tetO-Vβ8.1-Luc
HeLa CMV	HeLa cell line carrying the episomal plasmid pEBNA-SVP-GL-EμCAG-rtTA
HeLa S3	HeLa cells adapted to growth in suspension
Jurkat	Human T lymphoblastoid cell line, grow in suspension

Table 9: Cell lines used in this work.

2.4.2 Growth conditions

HeLa adherent cell lines were grown in Dulbecco's modified Eagle medium (DMEM plus 4500 mg/ml glucose, L-Glutamine, without pyruvate. Gibco Invitrogen, Cat. No. 11971-025) supplemented with 10% fetal bovine serum (FCS; Gibco Invitrogen, Cat. No. 10270-106) and 1% Penicillin-Streptomycin (Gibco Invitrogen, Cat. No. 15140-122). The adherent cells were grown in culture dishes ranging from 15 and 10 cm down to 6 and 12 well plates (Nunc, Cat. No. 157150, 150350, 140675, 150628) in a tissue culture incubator under 5% CO_2 and at 37°C. Confluent cells were detached from the plates by the aid of 0.25% trypsin and 0.2% EDTA (Gibco Invitrogen, Cat. No. 25050-014) and seeded to a new plate in the ratio of 1:4.

HeLa S3 and Jurkat suspension cells were grown in RPMI 1640 medium, L-Glutamine (Gibco Invitrogen, Cat. No. 21875-034), supplemented with 10% FCS and 1% Penicillin-Streptomycin. The suspension cells were grown in 25 to 175 cm^2 cell culture flasks. When the cells reached a density of 0.8-1x10^6 cells/ml, they were diluted to a new concentration of 0.2-0.4 cells/ml.

Freezing of cells (5x10^6 cells/aliquot) was done in 90% FCS and 10% (v/v) dimethyl-sulfoxide (DMSO). The cells were resuspended in 1 ml aliquots and placed into a freezing box. This box was placed at -80°C for 24 hours to freeze the cells. The frozen aliquot's were stored in a liquid nitrogen tank.

Thawing of cells was done in a 37°C water bath. The cells were resuspended in 10 ml pre-warmed medium and transferred to the culture plate or flask.

2.4.3 Transfecting cells

Calcium phosphate transfection The day prior transfection, the adherent cells were seeded in 6-well plates at 25% confluency. 3 μg of DNA/well was brought to 22 μl with 10 mM Tris-HCl pH 7.5 and 3 μl of a 2.5 M $CaCl_2$ were added. While gently vortexing, 25 μl of 2x HBS added were drop wise. The 100 μl calcium phosphate-DNA precipitate was drop wise distributed onto the cells in complete medium. The plates were incubated over night in the incubator at 37°C under a 5% CO_2 atmosphere.

2x HBS:

	for 50 ml:
50 mM HEPES	0.6 g
280 mM NaCl	0.82 g
1.5 mM Na_2HPO_4	10.6 mg
pH with NaOH to pH 7.05 at RT	

PolyFect transfection Transfection of adherent cells with PolyFect transfection reagent (Qiagen, Cat. No. 301105) was conducted following the manufacturers protocol. In short, for each well of an 6-well plate, 1.5 μg DNA was diluted in 100 μl of DMEM without FCS or other additives before adding 12 μl of PolyFect reagent. The Polyfect-DNA complexes were diluted with 600 μl of complete Medium and distributed to the cells. The PolyFect reagent is toxic for the cells, therefore the medium has to be changed the following day.

2.4.4 Luciferase assay

Cells monitored in a luciferase assay were normally harvested 12 to 24 hours after transfection or induction. The assay is sensitive enough to assay 2x 10^4 cells from 12 well plates. In this case the cells were lysed on the plate in 100 μl of 1x Lysis buffer (Promga, Passive Lysis Buffer 5x, Cat. No. E194A). The lysate was collected in a Eppendorf tube and centrifuged at full speed for 10 minutes to clear the lysate. 20 μl were used for the luciferase assay (Promega, Luciferase Assay System, Cat. No. E1501).

In the case of transfected cells, a vector encoding β-Galactosidase (β-Gal) was cotransfected to normalize for transfection efficiency. In the β-Gal assay, 20 μl of the cell lysate were incubated together with 180 μl of substrate solution for 1 hour at 37°C. After this time, the absorption at 420 nm was measured in a 96 well plate reader (BIO-TEK INSTRUMENTS INC., EL800 Universal Microplate Reader).

In the case of stable cell lines, were no vector encoding β-Galactosidase could be cotransfected, luciferase values were normalized to total protein. For this, the protein content of the cell lysate was determined using an Bradford assay Kit (Bio-Rad, Protein Assay, Cat. No. 500-0006). In short, 1 μl cell lysate were mixed with 199 μl Bradford reagent (1x) in a 96-well microtiterplate and after an incubation time of 10 minutes the absorbance at 595 nm was determined in a plate reader (BIO-TEK INSTRUMENTS INC., EL800 Universal Microplate Reader).

2.5 Chromatin immunoprecipitation (ChIP)

To prepare the crosslinked material used in a ChIP experiment 1x10^8cells were grown in 15 cm cell culture plates, for HeLa cells this are 10 plates. To crosslink the cells formaldehyde (Roth, Cat. No. 4979.1) was added directly into the cell culture medium to a final concentration of 1% (0.54 ml for 20 ml medium) and incubated for 10 minutes at RT. To ensure that the formaldehyde was distributed evenly, the plates were put on a rocking platform. After exactly 10 minutes the crosslinking reaction was blocked by addition of 3.5 ml 2 M Glycine (125 mM final). The excess of formaldehyde reacts with the primary amid group of the

Glycine and therefore quenches the crosslinking reaction. The cells were then washed twice with ice cold PBS (see Tab.2.2). A police rubberman was used to harvest the cells from the plates. Collect the cells in a 50 ml Falcon tube.

The cell pellets were resuspended in 10 ml sonification buffer and transfered to a 15 ml Falcon tube. Prior to sonification, glass beads were added (Sigma, Cat. No. 9-1145) to support mixing in the suspension. The sonification duty-cycle was 30% Output, 15s On, 45s Off for a total time of 5 minutes using a Branson 250-D sonifier equipped with a Microtip. All the sonification was done in a ice water bath at all time. After the sonification the efficiency of the DNA shearing was monitored. For this a 20 μl sample was incubated with 1 μl of μg/μl of Proteinase K (Roche, Cat. No. 3-115-828) and 2 μl SDS (10%) for 1 hour at 56°C. The DNA was loaded on a 1% agarose gel with the majority of the sheared DNA being in the range of 500-750 bp. The glass beads were collected by centrifugation for 15 minutes at 3500 rpm (4°C) in a Heraeus Multifuge 3 L-R. The supernatant was collected in a new 15 ml Falcon tube and 100 μl 20% Sarcosyl (Sigma, N-Lauroyl-Sarcosine, Cat. No. L-5777) together with 5,67 g CsCl (ICN Biochemicals, Cat. No. 160041) were added. The solution was transferd to an ultracentrifugation tube (Beckman, Cat. No. 331372) and carefully balanced. The samples were centrifuged in an SW40 rotor at 38.000 rpm for 36-48 hours at 18°C.

After centrifugation, the gradient was harvested by puncturing the tube at the bottom and collection of 1 ml fractions. These fractions were analyzed on an agarose gel. The first fraction contains normally only very small DNA fragment and the last fractions mainly super crosslinked DNA, which were omitted. The other fractions, usually fractions 2 to 8 were pooled and dialyzed against TE containing 5% glycerol (see Tab.2.2) for two hours, followed by buffer exchange and dialysis over night. The dialyzed samples were centrifuged in a Heraeus Multifuge 3 L-R for 15 minutes at 5000 rpm which cleared the lysate of precipitates. The supernatant was incubated with 0.5 ml Protein G Sepharose beads (Amersham, Protein G Sepharose Fast Flow, Cat. No. 17-0618-03) for 2 hours at 4°C to avoid unspecific binding during the immunoprecipitation reaction. To prepared the beads, they were rinsed in water and equilibrated in PBS containing 1 mg/ml BSA. After pre-clearing, the DNA content of each sample was measured in an Eppendorf

BioPhotometer and aliquoted to 2 OD_{260} units. These aliquots were frozen in liquid nitrogen and stored at -80°C.

For the immunoprecipitation one aliquot together with 2-5 μg antibody and 2 μg fish sperm DNA (Serva, Cat. No. 18580) were incubated in 1 ml 1x NET buffer over night at 4°C on a rotating wheel. The fish sperm DNA was resuspended in TE and sonicated to the same size as the samples (500 - 750 bp) and was treated to be single stranded by boiling for 10 minutes and subsequent cooling on ice. The next day, the samples were centrifuged for 15 minutes at 13'000 rpm (4°C) in an Eppendorf Centrifuge 5415 R to clear the IP from possible precipitates. 40 μl Protein G or Protein A Sepharose beads (depending of the antibody) together with 40 μg BSA (Sigma, Bovine Serum Albumin, Cat. No. A3294) were prepared in a new pre lubricated Eppendorf Tube (Sorenson, Cat. No. 11720). The cleared samples were incubated with this beads for 4 hours at 4°C on a rotating wheel. After this time the beads were collected by centrifugation at 500 rpm in an Eppendorf centrifuge 5415 R at 4°C. During the washing procedure, the samples were kept on ice. The beads were washed twice with Wash-Buffer I followed by two washes with Wash-Buffer II (depending on the antibody, see Tab. 10) and once with Wash-Buffer III. After two final washes with TE, the protein-DNA complexes were eluted in 150 μl of TE + 1% SDS. These eluates were incubated over night at 65°C to reverse the crosslink. In the morning, 1 μl of 15 μg/μl of Proteinase K (Roche, Cat. No. 3115 828) was added and the samples were incubated for 1 hour at 56°C.

The DNA was recovered by Phenol-Chloroform extraction and ethanol precipitation. 150 μl of Phenol/Chlorophorm/Isoamylethanol (Roth, Roti-Phenol/C/I, Cat. No. A156.1) were added to the samples. They were vortexed and centrifuged for 10 minutes at full speed, the aqueous upper phase was collected in a new tube. For the ethanol precipitation 9 μl 5M NaCl (final 300 mM) and 10 μg glycogen (Sigma, Cat. No. G-1765) together with 380 μl 100% ethanol were added and the samples were incubated for at least 2 hours at -20°C. After 30 minutes centrifugation at full speed (4°C) the DNA pellet was washed with 70% cold ethanol. The clean DNA was air dried for 15 minutes and then resuspended in 40 μl of TE.

The PCR reaction to visualize a specific DNA fragment was done according to

standard procedures. In short, 50 μl reactions were prepared, 4 μl template was used and the products were labeled with radioactive α-^{32}P-dCTP (Amersham, 3000 Ci/mmol, Cat. No. AA0005). To visualize the amplicon by autoradiography the PCR product was separated on a native 6% poly acrylamide Gel.

PCR standard reaction:	Template	4 μl	
	10 x PCR-buffer	5 μl	
	$MgCl_2$	3 μl	
	Primer 1 (10pmol)	1 μl	
	Primer 2 (10pmol)	1 μl	
	dNTPmix (2mM)	0.4 μl	
	α-^{32}P-dCTP	0.1 μl	
	Taq polymerase	0.5 μl	
	H_2O	35 μl	
Reaction duty cycles:	95°C	5 min	
	95°C	0.5 min	
	58°C	0.5 min	
	72°C	0.5 min	go to step 2, 26 repetitions
	95°C	0.5 min	
	58°C	0.5 min	
	72°C	5 min	
	4°C	pause	
Native 6% poly acrylamide gel:	5 x TBE	10 ml	
for 50 ml	Gel40 (1:19)	8 ml	
	H_2O	32 ml	
	10% APS	400 μl	
	TEMED	40 μl	

Sonification buffer:	50 mM Hepes pH 7.9	
	100 mM NaCl	
	1 mM EDTA	
	1% SDS	
	0.1% DOC	
	1 mM PMSF	
10x NET buffer:	550 mM Tris pH 7.4 RT	
	1.5 M NaCl	
	50 mM EDTA	
	5% NP-40	
Wash buffer I:	20 mM Tris pH 8 RT	
	150 mM NaCl	
	2 mM EDTA	
	0.1% SDS	
	1% Triton X-100	
Wash buffer II:	20 mM Tris pH 8 RT	
	250 - 500 mM NaCl	check antibody list for details
	2 mM EDTA	
	detergent	check antibody list for details
Wash buffer III:	10 mM Tris pH 8 RT	
	250 mM LiCl	
	1 mM EDTA	
	1% NP-40	
	1% DOC	

Antibody	Ref.	Provider	Washing buffer II
CBP	sc-369	Santa Cruz	Det.I, 500 mM NaCl
GCN5	sc-6303	Santa Cruz	Det.I, 500 mM NaCl
Pol II	sc-899	Santa Cruz	Det.II, 500 mM NaCl
TFIIB	sc-225	Santa Cruz	Det.I, 500 mM NaCl
TFIIH	sc-292	Santa Cruz	Det.I, 500 mM NaCl
TRAP220	sc-5334	Santa Cruz	Det.II, 500 mM NaCl
TRAP220	sc-8998	Santa Cruz	Det.II, 500 mM NaCl
TRAP150	sc-5378	Santa Cruz	Det.II, 500 mM NaCl
CDK8	sc-1521	Santa Cruz	Det.II, 500 mM NaCl
TRAP240		Santa Cruz	Det.II, 500 mM NaCl
CRSP77	sc-12453	Santa Cruz	Det.II, 500 mM NaCl
MED25	9C2	E.Kremmer	Det.II, 500 mM NaCl
PC4		Euro Gentech	Det.II, 500 mM NaCl
CPSF	sc-17289	Santa Cruz	Det.II, 500 mM NaCl
CstF-64	sc-16473	Santa Cruz	Det.II, 500 mM NaCl
Ac-H4	06-866	Upstate	Det.I, 500 mM NaCl
Ac-H3K9	06-942	Upstate	Det.I, 500 mM NaCl
trimethyl-H3K4	07-523	Upstate	Det.I, 500 mM NaCl
dimethyl H3K79	07-366	Upstate	Det.I, 500 mM NaCl

Table 10: Antibodies used in ChIP. Two different detergent (Det.) mixes were included in the Washing Buffer II: Det. I : 0.1% SDS, 1% Triton X-100; Det. II: 1% Triton X-100.

2.6 RT-PCR

HeLa Vβ8.1 cells (see. Tab.9) were grown on a 10 cm tissue culture dish to confluency (~ 4 x 10^6cells). Luciferase expression was induced by addition of 1 μg doxycycline (Sigma, Cat. No. D9891) per 1 ml of culture media. Total RNA was collected from HeLa Vβ8.1 cells using the RNAeasy Kit (Qiagen, Cat. No. 74104) according to the manufacturers protocol. The concentration of the eluted RNA from the spin columns was determined and 2.5 μg was used for the reverse transcriptase (RT) reaction. The RT reaction was done using oligo dT primers according to the manufacturers protocol, using the ThermoScript RT-PCR

Kit (Invitrogen, Cat. No. 11146-024). Quantification of the luciferase mRNA was done in a real-time PCR reaction using the SYBR-Green Kit (PE-Biosystems, Cat. No. 4304886) according to the manufacturers protocol.

2.7 Solid Phase assay

The templates for the solid phase assay were generated in a standard PCR reaction. The products were separated on a 1% agarose gel, the band running at 600 bp was recovered by using a Gel Band Purification Kit (Amersham, Cat. No. 27-9602-01) and the DNA concentration was determined. 18 μg of DNA template was coupled to 600 μl paramagnetic Streptavidin beads (Promega, Streptavidin MagneSphere, Cat. No. Z5481). 75 fmol DNA was coupled to 1 μl of beads. In short, 600 μl beads were washed 3 times in 1 ml 0.5 SSC, once in 1 ml PBS-BSA (0.5 mg/ml) NP-40 (0.05%) and twice in 1 ml WB-buffer. For the coupling the beads were incubated with 18 μg DNA template in 400 μl WB-buffer for 1 hour at RT in an Eppendorf shaker. The coupling efficiency was tested by analyzing supernatants in 1% agarose gels. Beads can be stored at 4°C for several days.

For the assembly reaction, 18 μl beads, corresponding to 1.35 pmol DNA template, 200 ng Gal4-VP16, 20 μl NE corresponding to about 160 μg protein and 70 μl Tx-buffer were incubated for 60 minutes at 28°C. After incubation, the beads were concentrated with a magnet and washed 3 times in 200 μl Tx-buffer. When nucleotides where added to the reaction, preinitiation complexes were preassembled on the template for 50 minutes. After this time the beads were concentrated with a magnet and washed once with 200 μl Tx-buffer. The beads were resuspended in 95 μl Tx-buffer and 5 μl 10 mM of the specific NTP or NTP mix was added and the reaction was incubated for additional 10 minutes at 28°C. The kinase inhibitor H8 was always added for the complete reaction. After the 10 minutes incubation, the beads were washed twice with 200 μl Tx-buffer and the bound proteins were eluted with 2x SDS loading buffer and separated on a SDS-PAGE mini-gel (see 2.9.8). Specific proteins were detected by Western blot analysis (see 2.9.11).

PCR reaction:
5 μl 10x PCR Buffer
3 μl $MgCl_2$
1 μl forward primer
1 μl reverse primer
5 μl dNTP mix
1 μl Taq polymerase

Tm 58°C; 26 cycles

Standard assembly reaction:
18 μl beads
20 μl NE
200 ng Gal-VP16
70 μl Tx-buffer
5 μl H_2O

0.5x SSC:
75mM NaCl
7.5 mM Na_3citrate•$2H_2O$
Adjust to pH 7.0 with 1 M HCl

WB-buffer:
50 mM Tris-HCl pH 8.0 RT
2 M NaCl
0.1 mM EDTA

Tx-buffer:
25 mM Hepes-KOH pH 8.2
4 mM $MgCl_2$
5 mM DTT
5% Glycerol
0.5 mg/ml BSA
100 mM KCl + K-Glut

2.8 Immunofluorescence

HeLa cells were used for immunofluorescence. The cells were seeded onto coverslips and transfected with PolyFect as described above. The preparation of the samples was done at room temperature. The cells were washed twice with PBS

(see Tab.2.2) and then fixed with 3.7% of paraformaldehyde/PBS (PFA/PBS) for 10 minutes. After two washes with PBS, the rest of the PFA/PBS was quenched with 20 mM glycine/PBS for 10 minutes. The cells were again washed twice with PBS and permeabilized with 1% Triton X-100/PBS for 15 minutes. After two washes in PBS, the cells were blocked with 0.2% fish gelatin/PBS (Sigma Cat. No. G-7765) for 20 min. The actual staining was carried out in a humid chamber. The humid chamber is a box containing on layer of thick Whatman paper moisten with water and a stripe of parafilm. Onto this parafilm one spots (50-100 μl) the dilutions of the primary antibodies. The coverslips were now transferred facedown to this humid chamber and incubated 60 minutes at 37°C. After this time the cells were put back to the 6-well plates for the washing, which is at least 3 times 10 minutes in PBS. Incubation of the secondary antibodies conjugated to the fluorescent dye was done as above except that it was carried out in the dark to prevent the dye from bleaching and the incubation time was only 30 minutes. After three washes with PBS, one can stain the DNA by DAPI. For this staining the cells were incubated with 1 μg/ml DAPI/PBS (SIGMA Cat. No. D9564) for 10 minutes. After a final wash (3 times with PBS), the coverslips were mounted facedown on a microscope slide in HistoGel mounting medium (Linaris). These preparations can be stored for up to 4 weeks at 4°C. The analysis of the samples was done with a Leica TCS SP2 confocal microscope. The microscope is equipped with a 63x objective and four lasers: (1) diode laser (405 nm) to excite DAPI, (2) Ar laser (458 nm, 488 nm and 514 nm) to excite FITC (green) and Cy3 (red), (3) He-Ne laser (543 nm) to excite Cy3 and (4) He-Ne laser (633 nm) to excite Cy5. To avoid cross-talk between the dyes, the lasers were set at an intensity such that no cross-talk appeared or if this was not possible by taking a sequential scan in which only one dye was excited at the time. The images were acquired using the built-in software from Leica.

primary antibody	**species**	**producer**	**dilution**
NC2 (4G7)	rat	E.Kremmer	1:10
MED25 (9C2)	rat	E.Kremmer	1:1
Flag (M2)	mouse	Sigma	1:400
HA (3F10)	rat	E.Kremmer	1:10
secondary antibody			
Cy3 anti-rat		Jackson Imm. Res.	1:400
FITC anti-mouse (515-095-062)		Jackson Imm. Res.	1:25 - 1:100
Texas-red anti-mouse		Jackson Imm. Res.	1:25 - 1:100

Table 11: Antibodies used for Immunofluorescence

2.9 Protein chemistry

2.9.1 Immunoprecipitation (IP)

When coupling antibodies to the appropriate agarose beads (either protein A or G coated), a final concentration of 1 mg/ml antibody on the beads is desirable (hybridoma supernatant, ~ 50 μg/ml). The antibody is incubated with the beads for 2 hours at room temperature on the roller. After this incubation, the beads were washed three times with PBS and three times with 0.2 M sodium borate (pH 9.0). The beads get resuspended in 10 beads-volumes of 0.2 M sodium borate (pH 9.0) and 5.2 mg dimethylpimilidate (DMP) per ml is added. Beads were incubated for 30 minutes at room temperature with gentle mixing. The reaction was stopped by washing the beads twice in 0.2 M ethanolamine (pH 8.0) followed by a 2 hours incubation in the same solution. At the end, the beads were washed with PBS and resuspended in PBS containing 0.02% Sodium Azide for storage at 4°C.

For the IP, 50 μl beads were equilibrated to BC150 buffer and 250 μl of NE was added and incubated for 4 hours to over night at 4°C on a roller. After the incubation time, the beads were washed 5 times with BC buffer containing up to 500 mM KCl. BC150 buffer was always used for the last two washes to equilibrate the beads lower salt concentration for better SDS-PAGE analysis. All steps were

performed at 4°C and all buffers were supplemented with fresh DTT (1 mM), protease inhibitors (200 mM PMSF, 100 mM Benzamidine, 2 μg/ml Leupeptine and 2 μg/ml Aprotinine).

2.9.2 GST-pulldown

A way to assay direct protein protein interaction is a GST-pulldown assay. For this assay, a GST fusion protein is immobilized on a Glutation Sepharose matrix, serves as a bait. The putative binding partner is offered as a recombinant protein. Binding was ultimately monitored by SDS-PAGE (see 2.9.8).

GST-fusion protein was bound to Glutation Sepharose beads as described in (see 2.9.5) and the concentration on the beads determined in an Bradford assay. Normally, 5 to 20 μg fusion protein were used for one pulldown. Recombinant protein was added in equimolar amounts, to block unspecific binding BSA was added in a 2 - 3 fold excess or alternatively crude *E.coli* lysate was used. The binding reaction was done in 400 μl BC150 buffer (see Tab.2.2) for 90 minutes at 4°C on a rotating wheel. The supernatant was removed and the the beads were normally washed with 100 volumes of BC150 supplemented with 0.02% NP-40, to apply more stringency use higher salt concentrations. Bound proteins were eluted directly in SDS-loading buffer and analyzed on an SDS-PAGE (see 2.9.8).

2.9.3 Urea dissociation assay

The immunoprecipitation with the MED15 antibody was done as just described (2.9.1). The IP was washed 5 times with BC150 buffer for the non urea treated sample. In all the other cases, 1 M-, 2 M- and 3 M urea washings, the beads were washed twice with BC150 buffer, followed by two washes of BC150 supplemented with the appropriate urea concentration and a final wash in BC150 buffer. Bound protein was eluted from the beads with 50 μl of 2 x SDS-loading dye and subjected to SDS-PAGE analysis.

2.9.4 Recombinant protein expression and purification

Recombinant proteins were expressed in *E.coli*, using expression plasmids encoding the desired protein in the BL21 *E.coli* expression strain. An overnight starter culture was diluted to an OD_{600} of 0.1 into 200 ml LB medium and grown at 37°C to an OD_{600} of 0.6 - 0.8 where the expression of the protein was induced by addition of 0.5 mM IPTG. To prevent the formation of inclusion bodies and to enhance the expression of full length protein, the *E.coli* culture was shifted to 30°C for 2 - 6 hours. In critical situations, even lower temperature in combination with longer expression time were applied. The cells were harvested by centrifugation for 15 minutes at 3.500 rpm (4°C). All the following purification steps are carried out at 4°C.

2.9.5 GST-Tag purification

GST-taged proteins were resuspended in 10 ml of lysis buffer and lysed by incubation with 10 mg of Lysozyme for 10 minutes and sonification. For the sonification the microtip and an output amplitude of 30% for a total time of 2 minutes with a repetitive cycle of 10 seconds On-time and 50 seconds Off-time was used. During the sonification, the samples were cooled in an ice-water bath. The lysate was cleared by centrifugation for 10 minutes at 10.000 g (4°C). In the mean time, 200 μl of Glutathione-Sepharose 4B (Amersham, Cat. No. 17-0756-01) were washed and equilibrated in Lysis-buffer. The lysate was incubated together with the beads for 90 minutes at 4°C on a rotating wheel to allow binding of the fusion protein to the matrix. The supernatant was removed and the remaining beads were sub sequentially washed with 100 volumes of BC2000 and BC150 buffer (for BCx buffers see Tab.2.2). To elute the immobilized fusion proteins, they were incubated for 10 minutes at 4°C in 500 of μl elution buffer. For quality control, an aliquot was analyzed in an Coomassie stained SDS-PAGE gel (see 2.9.8).

Lysis buffer:
20 mM Hepes pH 7.5
100 mM KCl
1 mM EDTA
10% Glycerol
0.1% NP-40
5mM β-Mercaptoethanol
add protease inhibitors before use

Elution buffer:
25 mM Tris-HCl pH 8.2 RT
100 mM KCl
10% Glycerol
0.1% NP-40
30 mM reduced Glutathione
add protease inhibitors before use

2.9.6 His-Tag purification

The purification of 6xHis-taged proteins was conducted very similar to the purification of GST-taged proteins. Lysis of the cells was essentially the same, except that a different Lysis-buffer was used. The cleared lysate was added to 200 μl washed and equilibrated TALON Metal Affinity Resin (DB Bioscience, Cat. No. 8901-2) and incubated for 90 minutes at 4°C on a rotating wheel. The beads were washed with 100 volumes of Lysis-buffer supplemented with 5 mM Imidazole to reduce unspecific binding. The immobilized fusion proteins were eluted by incubation for 10 minutes at 4°C in 500 μl lysis buffer supplemented with 200 mM Imidazole. The eluted proteins were analyzed on a Coomassie stained SDS-PAGE gel to ensure quality (see 2.9.8).

Lysis buffer:
20 mM Tris-HCl pH 7.5 RT
500 mM NaCl
10% Glycerol
0.1% NP-40
5 mM β-Mercaptoethanol
add protease inhibitors before use

2.9.7 *In vitro* Transcription and Translation (TNT)

In vitro coupled transcription and translation was used to label proteins with radioactive ^{35}S-Methionine. These proteins were offered in GST-pulldown assays as putative binding partners.

The Promega TNT Coupled Reticulocyte Lysate System (Promega, Cat. No. L4611) was used and the protocol of the manufacturer was essentially followed. In short, the following reaction was assembled and incubated at 30°C for 90 minutes.

TNT Lysate	12.5 μl
TNT Reaction buffer	1 μl
T7 Polymerase	0.5 μl
aa Mixture, minus Methionine	0.5 μl
^{35}S-Methionine	1 μl
RNasin	0.5 μl
DNA-template	1 μl
H_2O	8 μl

2.9.8 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Protein were separated on an SDS-PAGE using either a maxi-gel system from Hoefer or the mini-gel system from Bio-Rad. Depending of the size of the protein, either 10, 12 or 15% gels were used. T achieve better resolution and more efficient blotting for the bigger proteins, 170:1 acrylamide / bisacrylamide gels were used. For electrophoresis, proteins were mixed 1:6 with 6x loading buffer, heat denatured at 95°C and loaded onto the gel. Proteins were separated applying a current of 30 mA for the mini-gels and 50 mA for the maxi-gels. In both cases the running buffer was 1x TGS (see Tab.2.2). The maxi-gels were connected to a cooling system. For molecular weight determination, unstained marker was run in parallel (Bio-Rad, SDS-PAGE standards Low Range, Cat. No. 161-0304; Bio-Rad, SDS-PAGE standards High Range, Cat. No. 161-0303; MBI Fermentas, Protein MW Marker, Cat. No. SM0431). Following electrophoresis, proteins

were stained with Coomassie Brilliant Blue G250, silver, or subjected to Western blotting.

6x loading Buffer: 0.35 M Tris-HCl (pH 6.8 RT)
0.12 mg/ml Bromphenol blue
40 mM β-Mercaptoethanol

	15%	**12%**	**10 %**	**stacking gel**
GelA (Roth, Cat. No. 3037.1)	5 ml	4 ml	3.3 ml	0.85 ml
GelB (Roth, Cat. No. 3039.1)	0.43 ml	0.35 ml	0.28 ml	0.35 ml
1.5 M Tris-HCl (pH 8.8 RT)	2.5 ml	2.5 ml	2.5 ml	
1 M Tris-HCl (pH 6.8 RT)				0.63 ml
H_2O	1.87 ml	2.95 ml	3.72 ml	3.1 ml
10% SDS	0.1 ml	0.1 ml	0.1 ml	0.05 ml
10% APS	0.1 ml	0.1 ml	0.1 ml	0.05 ml
TEMED	10 μl	10 μl	10 μl	10 μl

	Low Range	**High Range**	**Protein MW Marker**
Myosin		200	
β-galactosidase		116	116
Phosphorylase b	97	97	
Serum albumin	66	66	66
Ovalbumin	45	45	45
Lactate dehydrogenase			35
Carbonic anhydrase	31		
Bsp98I			25
Trypsin inhibitor	21		
β-lactoglobulin			18
Lysozyme	14		14

2.9.9 Coomassie staining

For Coomassie staining of polyacrylamide gels, the gels were incubated with staining solution for at least 30 minutes on an slowly rocking platform. To visualize protein bands, the gels were destained overnight in destaining solution or in dH_2O. After destaining, the gels were scanned and then dried between cellophane film at RT.

Coomassie staining solution:	Methanol	400 ml
	Glacial acetic acid	100 ml
	Coomassie R-250	0.25 g
	H_2O	500 ml
Destaining solution:	Methanol	500 ml
	Glacial acetic acid	100 ml
	H_2O	400 ml

2.9.10 Silver staining

Silver was used as a more sensitive staining reagent using the Silver Staining-Kit (Amersham, Cat. No. 17-1150-01) protocol. In short, the gels were fixed in 2.5 % Acetic acid and 40% Ethanol for 30 minutes. After this time the gels were rinsed twice in dH_2O. Sensitization was done with 30% Ethanol, 0.125% Glutardialdehyde (w/v), 0.2% Sodium thiosulphate (w/v) for 30 minutes. The gels were washed 4 times for 5 minutes with dH_2O. The Silver reaction was done in 0.25% Silver nitrate solution and 0.014% Formaldehyde. The gels were washed 4 times for 1 minute with dH_2O. Finally the gels were developed in Sodium carbonate and 0.007% Formaldehyde. The reaction was stopped with EDTA-$Na_2 \bullet 2H_2O$.

2.9.11 Western

Protein separated by SDS-PAGE were transfered to a nitrocellulose membrane (Bio-Rad, Cat. No. 162-0115) using a Semi-Dry Transfer Cell (Bio-Rad, Trans-Blot SD). For protein transfer, the gels was sandwiched between gel-sized What-

man papers, soaked in transfer buffer and run for 30 minutes at 15 V (mini-gels) or 60 minutes at 15 V (maxi-gels). After transfer, the membrane was shortly stained with a Ponceau S solution to make the transfered marker visible. The membrane was then incubated for 1-12 hours in TBS containing 5% milk powder to reduce unspecific background binding. After blocking, the membrane was incubated for 1-12 hours with appropriate dilutions of the primary antibodies in TBS-T (1x TBS, 0,05% Tween) containing 1% milk powder (see Tab.2.2). To remove excess antibody, the membrane was washed 3 times for 15 minutes with TBS-T. Secondary antibodies (1:10'000 dilution) were applied for 30 minutes in TBS-T and after 3 times 15 minutes washes in TBS-T and a final rinse in dH_2O the antibodies were detected using the enhanced chemiluminescence detection system (Perkin Elmer, Western Lightning Cat. No. NEL105) according to the manufacturer's instructions and exposed on BioMax MR film (Kodak, Cat. No. 873 6936).

Transfer buffer: 190 mM Glycine (14.4 g)
50 mM Tris base (15 g)
0.05% SDS (0.5 g)
20% Ethanol (200 ml)
800 ml H_2O

3 Results

3.1 Transcription in an inducible VP16 system

3.1.1 Generation of a doxycycline inducible HeLa cell system

To study recruitment and crosstalk of various transcription factors upon transcription initiation, we are in need of a system that is able to fulfill several demands. Firstly, it has to be direct, meaning that the signal that turns the system on has to be unique and independent of many activators. Secondly, it has to be fast, which requires that the activator is translated before induction. Thirdly, monitoring of activation should rely on an easy read-out like luciferase activity. Fourthly, it should be genetically flexible, such that promoters and activators remain interchangeable.

A system based on the commercial Tet-On gene expression system meets essentially all of these requirements [Gossen, 1992, Gossen, 1994]. The activator, Tet-VP16 is readily available in the cell and upon induction with doxycycline binds exclusively to the reporter plasmid. This reporter consists of the tetracycline-response element (TRE). In our experimental setup, the TRE consisted of seven direct repeats of a 42-bp sequence containing the tet operator sequence (7x tetO) and is located just upstream of the minimal CMV promoter (CMV_{min}). The response of the system is fast. Janicki et al. (2004) showed that the Tet-VP16 transactivator binds to the 7x tetO sites within minutes and luciferase is measurable hours after induction [Cunningham, 1997].

A 300 bp PCR fragment containing the 7x tetO sites from the Clontec vector pTRE2 was integrated upstream of the rearranged T-cell receptor beta promoter Vβ8.1 (Fig.8A). The vector backbone, pRep4, is a commercially available vector from Invitrogen. This vector carries the EBV oriP and the expression cassette for EBNA1, together that ensured the stable episomal expression of the vector. The plasmid also carries the selection markers ampicillin (for selection in procaryotes) and hygromycin (for selection in eucaryotes). The reporter plasmid was transfected into a HeLa cell line carrying a chromosomal expression cassette for the Tet-VP16 fusion protein (Clontech). The reporter plasmids were transfected

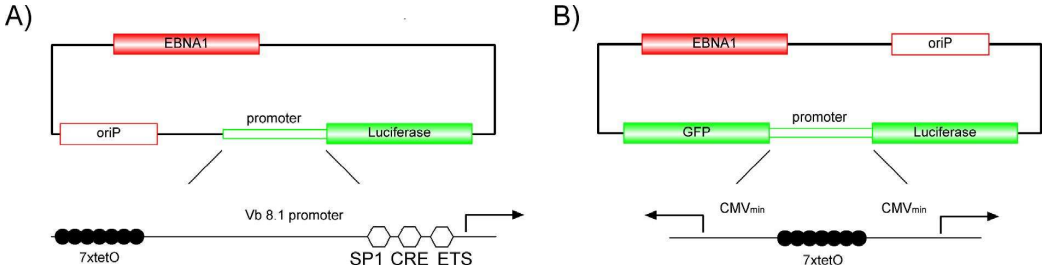

Figure 8: Reporter plasmids for the inducible VP16 system. A) pREP4 based plasmid carrying the expression cassette for the EBNA1 gene and the oriP region (red), both originating from the Epstein-Barr virus, to ensure episomally stable replication of the plasmid. The luciferase gene is under the regulation of the TCR promoter Vβ8.1 and the tet responsive element 7x tetO (green). B) Related vector with a different bidirectional CMV promoter. The minimal CMV promotes drive expression of the luciferase as well as the GFP gene. The vector also carries a constitutive active expression cassette for the transactivator Tet-VP16.

into HeLa cells. Transfected cells were selected for two weeks with hygromycin to generate stable episomal cell lines.

The functionality of the reporter was tested in transient reporter assays, in which a plasmid expressing Tet-VP16 (Tet-On; Clontech) and the newly cloned reporter (7x tetO Vβ) were cotransfected into HeLa cells. Addition of doxycycline to the media leads to binding of Tet-VP16 and expression of luciferase. Doxycycline can be used in a broad range, from 0.01 μg/ml to 10 μg/ml of media without being toxic for the cells. The doxycycline was titrated to find the optimal concentration (Fig.9B). The titration showed a 100-fold induction in the presence of 1μg/ml doxycycline. Higher concentration do not further enhance luciferase expression. Optimal working conditions were therefore 1μg/ml. Similarly, the stable cell line was tested for luciferase expression. The doxycycline concentration was again titrated from 0.1 μg/ml to 10 μg/ml media. Comparable to the transient situation, 1μg/ml of doxycycline proved to be the optimal working concentrations and was used for all further experiments (Fig.9A). One difference between the transient and the stable situation was reduced inducibility in the case of the later. Indeed, luciferase expression was about 20-fold higher in transiently transfected

cells compared with the stable system (compare Fig.9A,B).

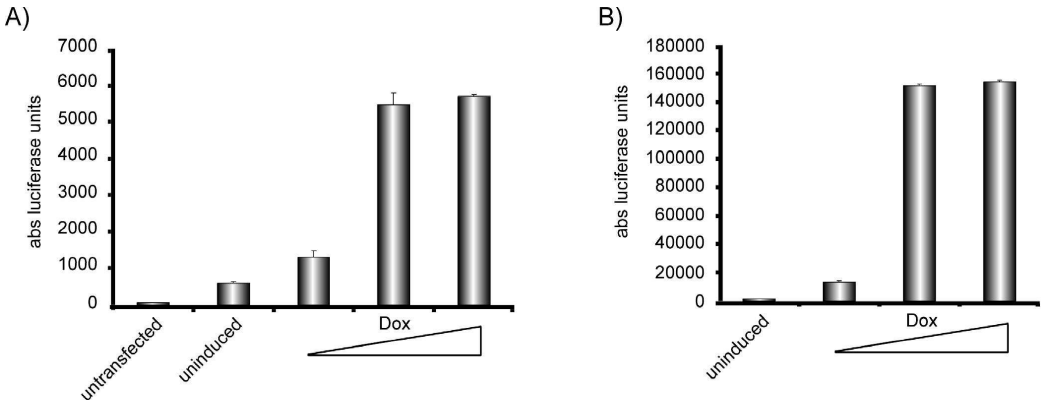

Figure 9: Inducibility of the 7x tetO Vβ luciferase reporter. A) Luciferase levels of the episomally stable cell line after 12 hours induction with doxycycline (Dox). B) In comparison the luciferase levels of the transiently transfected reporter plasmid. The Dox concentration were titrated for both situations (0.1, 1 and 10 μg/ml medium)

MED25 is a critical VP16 interactor in the Mediator complex [Mittler et al., 2003, Yang et al., 2004]. Notably, the N-terminal domain (NTD) of MED25 exhibits a strong dominant negative effect on VP16 activation in transient analyses. To test whether the new system was responsive to the NTD of MED25, the stable reporter cell line was transiently transfected with a plasmid expressing NTD, stimulated the cells with 1 μg/ml doxycycline and luciferase activity was monitored. A strong effect of NTD on luciferase expression would indicate that activation by VP16 is mainly mediated by Mediator and not by other VP16 interactors. Increasing amounts of the NTD expressing plasmids were transfected. The system proved to be responsive to NTD, in that a 50% drop of luciferase expression was measured. Indicating that also in the presence of a bona fide chromatin structure VP16 activation is MED25-dependent (Fig.10). That only a 50% repression of luciferase expression is measured in this system is probably due to the transfection efficiency which has been determined to be approximately 50%. In this case (of a 50% transfection efficiency), the maximum expected effect is some two-fold.

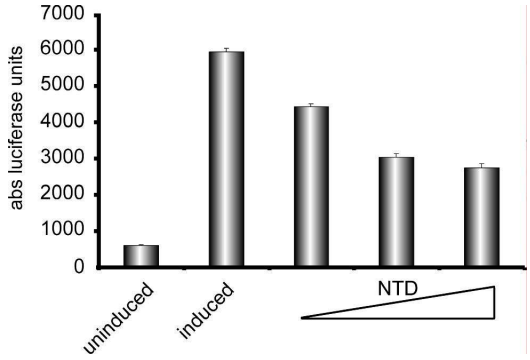

Figure 10: The NTD of MED25 represses VP16 activation. Episomally stable HeLa cells carrying the 7x tetO Vβ8.1 reporter were transiently transfected with increasing amounts of NTD (250, 500 and 1000 ng plasmid) Expression of luciferase was induced with 1 μg/ml doxycycline and measured by a luciferase assay.

Luciferase assays used above are relatively slow. The luciferase gene has to be transcribed and translated before it can be measured. Luciferase levels normally peak after 12 to 24 hours after induction. A faster readout is provided by the transcribed luciferase mRNA that was measured by real-time RT-PCR. Quantification of the reverse transcribed DNA was done by real time PCR. The expression of the luciferase gene was induced with doxycycline for 0, 5, 15, 30, 60 and 120 minutes and subsequently cells were lysed and total RNA was prepared. For reverse transcription reaction oligo dT primers were used, which leads to transcription of all polyadenylated mRNA. Subsequently, in the real-time PCR, primers specific for luciferase and actin were used. The actin levels served as a control to normalize the PCR reactions. Luciferase transcripts start to be detectable 15 minutes after doxycycline induction. Full levels of induction were reached after 30 minutes which then did not change significantly within the next 90 minutes (Fig.11).

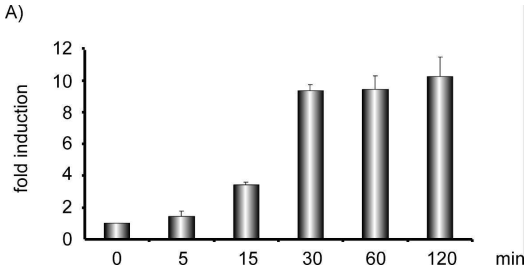

Figure 11: Luciferase transcripts are detectable only 30 minutes after inducing the Tet-On system by doxycycline. At the indicated time points after induction, RNA was prepared and analyzed for the amount of luciferase transcripts. The RNA amount of each time point was corrected to the amount of actin. The mean fold induction of three independent experiments is shown.

A commonly encountered problem with episomally stable Tet-On systems is that with time, they loose their inducibility. This loss of inducibility seems to be related to the packaging of the plasmid with nucleosomes [Pankiewicz, 2004]. Therefore, after some time, the Tet-VP16 can not overcome the inhibition exerted by the nucleosomes and inducibility is lost. To analyze transcription maintenance, the stable HeLa cell line harboring the 7x tetO Vβ reporter was cultured for eight weeks. Luciferase was measured in a weekly manner.

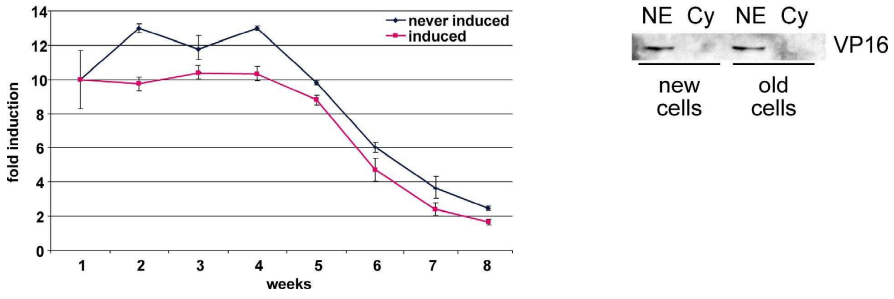

Figure 12: Longterm inducibility of the HeLa Vβ cell line. On the left hand side, the cells were cultured for eight weeks and the luciferase inducibility was measured. On the right hand side, the reduction of the inducibility is not due to lesser amounts of Tet-VP16 in the nucleus (NE: nuclear extract; Cy: cytoplasmic extract) in older cells as is shown by western blot analysis.

Newly stable cells showed an approximately 10-fold induction of luciferase. This induction level could be maintained for up to five weeks. After seven weeks, the inducibility dropped and remained low at a level of around 2-fold (Fig.12 left panel). This effect was not due to a loss of Tet-VP16 expression as a western blot against VP16 showed (Fig.12 right panel), nor to the loss of the reporter plasmid, since the cells were still grown under selection conditions and the basal luciferase values remained almost stable. This could be due to the packaging of the plasmid into chromatin and a relatively weak nucleosome permeability of the Tet-VP16 transactivator. An other reason might be that the episomes get silenced by methylation of histone H3K9, which would result in a heterochromatin like state of the luciferase gene. This possibility has to be tested in a ChIP experiment. Cells that lost the inducibility could be monitored for a higher histone H3K9 methylation status of the episome.

3.1.2 MED25 recruitment is VP16 dependent

MED25 is thought to be the primary target of VP16 in the Mediator complex [Mittler et al., 2003, Yang et al., 2004]. Evidence for this, stems from biochemical and functional *in vitro* data. Even more indicating were reporter assays where the overexpressed Mediator interaction domain exhibited a strong dominant negative effect on VP16-driven activation. Nevertheless, this is also a rather artifical system using highly overexpressed proteins and DNA reporters lacking bona fide chromatin. To ask the question whether VP16 is able to recruit MED25 to promoters *in vivo* we performed chromatin immunoprecipitation (ChIP) experiments, using the previously introduced HeLa 7x tetO Vβ8.1 cell line. Cells were induced with doxycycline (Dox) for 12 hours. Afterwards, the chromatin was crosslinked and compared to uninduced cells. The crosslinked chromatin was subjected to immunoprecipitation using antibodies directed against MED1, MED25 and RNA polymerase II (Pol II). Pol II serves in this experiment as a marker for transcription. In the uninduced situation, the Vβ8.1 promoter is only weakly transcribed in HeLa cells and only with bound VP16 is it possible to detect transcripts from this promoter (Fig.11). Consistently, before induction only background levels of Pol II are detectable on the promoter (Fig.13B). The situation changes after tran-

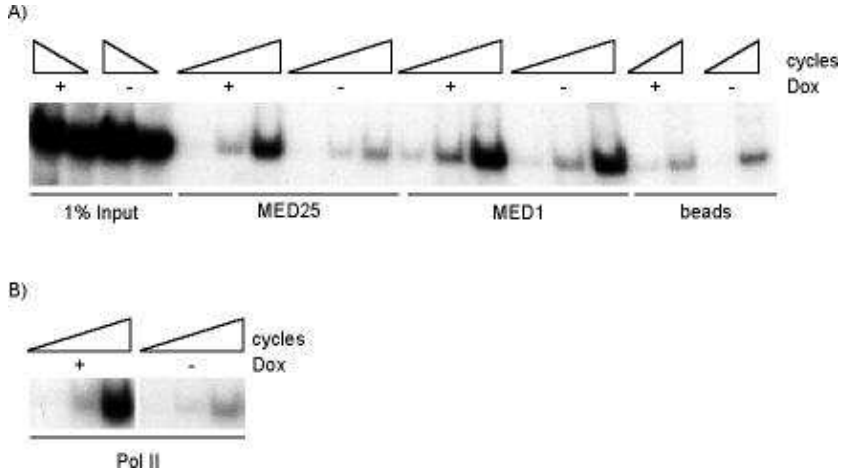

Figure 13: VP16-dependent MED25 recruitment to the Vβ8.1 promoter *in vivo*. A) ChIP analysis of MED1 and MED25 on the Vβ8.1 promoter. The episomally stable 7x tetO Vβ8.1 cells were induced for 12 hours with 1μg/ml medium Dox. The number of PCR cycles were 25, 27 and 29 to ensure analysis in the linear amplification range. B) ChIP analysis of Pol II from the same experiment served as a control for transcription induction.

scription is induced where an approximately 20-fold increase of Pol II occupancy is observed. In the uninduced situation MED25 is not detected at levels above background (Fig.13A). After addition of doxycycline however, an approximately 18-fold increase of MED25 was seen. This clearly indicates that MED25 can be recruited to promoters in an activator dependent manner *in vivo*. Interestingly, this is not seen for MED1 where levels remained unchanged upon induction, indicating heterogeneity on Mediator complexes or modules of Mediator. The preassembled Mediator complexes present at the promoter may change in subunit composition, or Mediator complexes lacking the MED1 subunit are recruited by VP16. For the second situation argues that Mediator complexes have been observed that lack the MED1 subunit [Taatjes and Tjian, 2004, Malik et al., 2005]. A third possibility is that free MED25 is recruited to the promoter. This can not be ruled out, there is evidence that some 50% of MED25 is not complexed in cell extracts [Blazek Erik, 2005].

3.1.3 VP16-dependent Mediator recruitment

Given that MED25 is recruited to the Vβ8.1 promoter in a VP16-dependent manner. It may indicate that the cofactor is incorporated into an already prebound complex. Alternatively, a MED25 containing but MED1 lacking Mediator is recruited by VP16. Both models would be compatible with the previous result. Incorporation of MED25 would lead to same MED1 levels but higher MED25 levels. Recruitment of a complex together with MED25 requires the existence of a MED1 deficient Mediator complex. To investigate this closer by ChIP analysis, recruitment of other Mediator subunits to the promoter were studied. In the light of the modular organization of Mediator, the antibodies for this experiment were chosen for their availability at this time. Only a few commercially available Mediator antibodies were reported to work in ChIP. However, the antibodies could be chosen in a way that every Mediator module (head, middle, tail and kinase) were targeted at least by one antibody. The antibodies used were the following: MED13 and Cdk8 are both subunits of the kinase module. The Cdk8 module is generally considered to have a repressive function within Mediator [Akoulitchev et al., 2000] even though in some reports this does not seem to be the case [Wang et al., 2005]. It was shown in pull-down assays that Mediator lacking the Cdk8 module binds to Pol II and that this Mediator complex is the transcriptionally active fraction in *in vitro* assays [Naar et al., 2002]. Therefore, the question arises whether this module would leave the initiation complex upon transcription induction. MED23 is a subunit restricted to higher eukaryotes that is located in the tail module where MED23, MED24 and MED16 form a submodule [Ito et al., 2002a, Stevens et al., 2002]. The mouse knock out of MED23 and MED24, show a greatly reduced activation of transcription by different activators, even though they are not directly targeted by these activators. Especially the MED16 subunit is considered to be indicative for Mediator complexes involved in activated transcription. The location of MED1 was annotated to the middle module of Mediator. The MED17 located in the head module was shown to bind directly to VP16 [Ito et al., 1999]. The location of MED25 is not known, although one can speculate that it is part of the head module, since the EM data show binding of VP16 in this region [Taatjes et al., 2002]. The antibodies finally used were

targeted against MED13, Cdk8, MED23, MED1, MED17 and MED25.

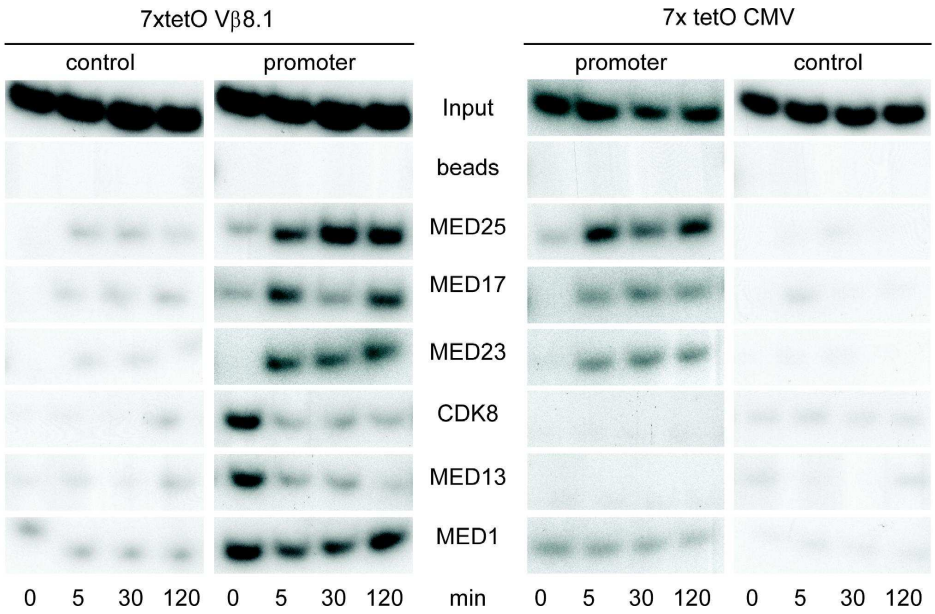

Figure 14: VP16-dependent recruitment of Mediator. ChIP analysis of MED25, MED17, MED23, Cdk8, MED13 and MED1. Tet-On HeLa cells harboring as stable reporter plasmid either the 7x tetO Vβ8.1 or the 7x tetO CMV promoters were crosslinked at the indicated timepoints after VP16 activation. Levels of MED25, MED17 and MED23 increase upon VP16 activation, whereas Cdk8 and MED13 levels decrease, MED1 levels remain invariant on the 7x tetO Vβ8.1 promoter. Increased levels of MED25, MED17 and MED23 are observed on the 7x tetO CMV promoter as well. No accumulation is seen for any of the Mediator subunits in the control region (oriP).

Tet-On HeLa cells carrying the 7x tetO Vβ8.1 or the 7x tetO CMV reporter plasmids (Fig.8) were crosslinked with formaldehyde 0, 5, 30 and 120 minutes after doxycycline induction. The timecourse was chosen to visualize possible subsequent recruitment steps of Mediator subunits. The induction of the system was monitored by measuring luciferase 12 hours after doxycycline addition. The autoradiogram of the ChIP gel shows the rapid recruitment of MED25 already 5 minutes after induction (Fig.14). No additional recruitment of MED25 was observed over the 120 minutes timecourse, arguing for an early steady state scenario. This result was seen for both promoters, the Vβ8.1 as well as the CMV reporter.

Following a similar kinetics, the two subunits MED17 and MED23 appeared at both promoters. Corecruitment of these three subunits argues for combined recruitment of the Mediator head and tail module. MED1 levels already present at the Vβ8.1 promoter before induction as seen before, remained constant. MED1 levels at the CMV promoter were slightly lower relative to MED25. The presence of MED1 at the Vβ8.1 promoter before induction seems unlikely to be an artefact, since the same result was obtained using a different MED1 antibody. Not only MED1 was found on the Vβ8.1 promoter but MED13 and Cdk8 of the kinase module as well. None of these two subunits were found at the CMV promoter at any time. It looks like the middle (assuming MED1 as a marker protein) and the kinase module (Cdk8, MED13) are present at the Vβ8.1 promoter, only after induction get components of the head (MED17) and of the tail module (MED23) recruited and the Cdk8 module is lost to form a transcriptionally active complex.

Figure 15: Vβ8.1 promoter sequence. Positions relative to the transcription start site are indicated by numbers. The consensus sequences are indicated below the potential binding sites for the different transcription factors.

Additionally it seem possible that a transcriptionally active complex is recruited that replaces the present complex, as would be seen in the case of the CMV promoter, no Mediator is present before induction and the transcriptionally active complex gets recruited as one. This might be due to the nature of the different promoters, the CMV promoter is a minimal viral promoter engineered for low background activity, whereas the Vβ8.1 promoter is an endogenous promoter including different potential activator binding sites [Halle, 1997]. Including sites for SP1, CRE, AML, ETS and LEF (Fig.15). Especially the binding site for CRE might account for some basal transcription activity.

It is not possible to decipher whether a complete transcriptionally active Mediator complex is recruited by VP16 or if a prebound transcriptionally inactive “core”

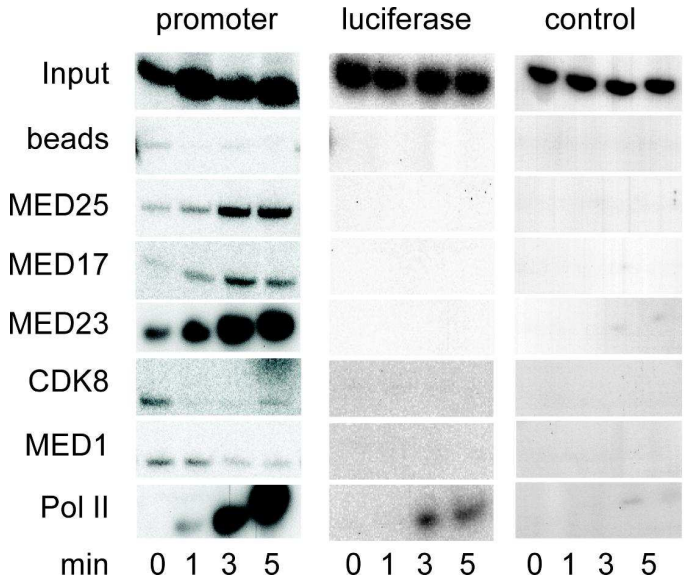

Figure 16: VP16-dependent recruitment of Mediator. Short term ChIP analysis of the Tet-On HeLa cells carrying the 7x tetO Vβ8.1 reporter plasmid. MED25, MED17 and MED23 are recruited within the first 3 minutes after VP16 activation. The Cdk8 subunit is lost at the same time, whereas MED1 levels remain unchanged.

Mediator gets "loaded" with head and tail module and thus activated by VP16. The presence of three Mediator subunits (MED1, Cdk8 and MED13) on the Vβ8.1 promoter before induction of transcription argues for the model that a core complex is prebound on the promoter. To shed more light into this aspect, a short term kinetic was applied. HeLa cells carrying the 7x tetO Vβ8.1 reporter plasmids were crosslinked with formaldehyde 0, 1, 3 and 5 minutes after induction. The ChIP analysis showed that MED17, MED23 and MED25 are perfectly corecruited in time, showing up approximately after 3 minutes of induction (Fig.16). Cdk8 leaves the promoter as the other three subunits appear and MED1 levels remain unchanged. A Pol II antibody was included into this experiment,demonstrating that Pol II is not prebound together with the "core" Mediator but is recruited after 3 minutes. Again, this perfectly correlates to the loss of the Cdk8 module, indicating that occupancy of the Cdk8 module and Pol II mutually exclusive. Consistent with an elongating Pol II, not Mediator but Pol II is found in the coding region.

In conclusion, the data showed that Mediator is recruited to promoters by VP16 *in vivo*. Moreover, the kinase module of Mediator is lost during transcription activation. Additionally, Pol II and active Mediator lacking Cdk8 enter the promoter with similar kinetics. There might be a possibility of dynamic changes in the subunit composition of Mediator upon transcription activation.

3.1.4 VP16-dependent chromatin modification

Histones can be modified by transcription cofactors. These histone modifications are a hallmark of transcribed genes. HAT activity leads to the decompaction of the chromatin whereas HDAC has a reverse effect. Histone acetylation as well as certain histone methylation may correlate to transcribed genes and are thought to form a code that leads to binding of transcription cofactors. For example, acetylated histone H4 and acetylated H3K9 as well as trimethylated histone H3K4 and dimethylated H3K79 are frequently found on actively transcribed regions [Jenuwein and Allis, 2001, Zhang and Reinberg, 2001, Kouzarides, 2002]. VP16 is known to interact with at least two different histone acetyltransferases, these are the CBP/p300 [Kraus et al., 1999, Ikeda et al., 2002] and the yeast SAGA complex [Utley et al., 1998]. The established inducible VP16 cell system provides a tool that allows to monitor the modification state of histones upon VP16 activation. Additionally the presence of the two HAT activities of CBP/p300 and hGCN5 on the promoter can be examined.

Tet-On HeLa cells carrying the 7x tetO Vβ8.1 or the 7x tetO CMV reporter plasmids were crosslinked with formaldehyde 0 and 30 minutes after doxycycline induction. The autoradiogram of the ChIP shows a VP16-dependent recruitment of both CBP/p300 and the hGCN5 histone acetyltransferases to the promoters (Fig.17A). Accumulation of both HATs is approximately equally strong on both, the Vβ8.1 and the CMV promoters despite the more distal position of the TRE on the Vβ8.1 promoter. The accumulation is restricted to the promoter region, no increased levels of CBP/p300 nor hGCN5 was visible in the coding or the control region. To monitor the histone modification state of the two promoters, antibodies directed against acetylated histone H4 and H3K9 as well as antibodies directed against trimethylated histone H3K4 and dimethlyated H3K79 were used

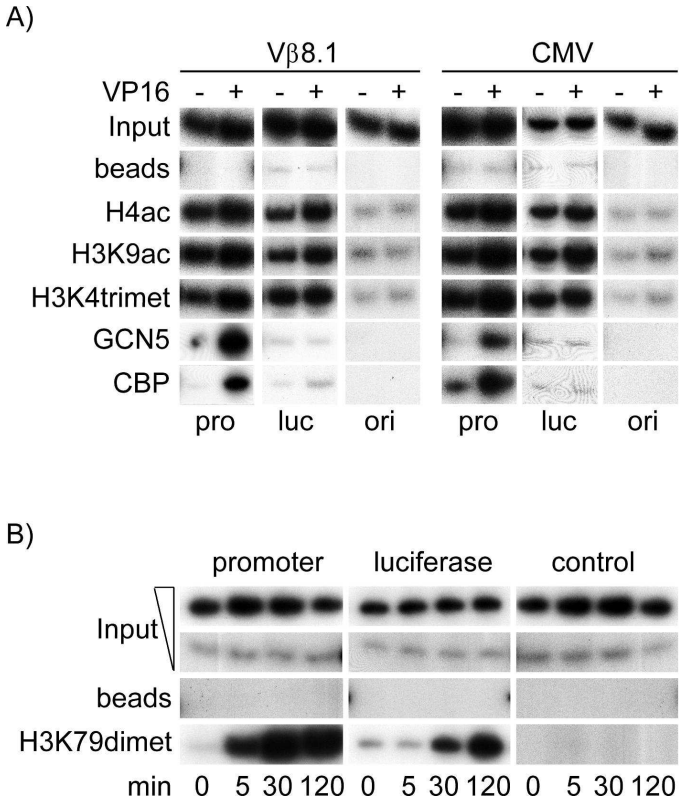

Figure 17: VP16-dependent histone modifications and recruitment of HAT activities. A) ChIP analysis, Tet-On HeLa cells carrying either the 7x tetO Vβ8.1 or the 7x tetO CMV reporter plasmids were crosslinked 0 or 30 minutes after VP16 activation. An increase of CBP/p300 and GCN5 but no change in the levels of histone modifications is seen. B) ChIP analysis, Tet-On HeLa cells carrying the 7x tetO Vβ8.1 reporter plasmid were crosslinked at the indicated time points after VP16 activation. A dramatic increase of dimethylated histone H3K79 is observed.

(Fig.17). Despite recruitment of CBP/p300 and hGCN5, no increase of acetylated histones were observed in the ChIP experiment. Histone H4 and the more specific H3K9 get only moderately acteylated upon VP16 activation at the promoter or the coding region (Fig.17A). This may be a consequence of the already high histone modification levels at the episomal reporter before VP16 activation. Additionally only a minor change of trimethlyated histone H3K4 was observed, which may be for the same reason. In contrast, a strong increase of dimethylated histone H3K79 of about 20-fold was seen on the promoter and the luciferase gene (Fig.17B). 5 minutes after induction, enhanced levels of K79 dimethylation were observed, which further increased 30 minutes after induction. In the coding region the observed accumulation of dimethylated K79 was delayed and was not yet visible 5 minutes after induction. The only known methyltransferase that is responsible for this modification is hDot1L.Interstingly, histone modifications are lower in the oriP control region. This finding is in accordance with reports from the Lieberman laboratory where they show that histone modifications spread over a large distance on the EBV episome [Chau CM. and Lieberman PM., 2004] and that the histones adjacent to the oriP region are the most heavily modified but not the oriP region itself [Zhou J., 2005].

In conclusion, a recruitment of the two HAT activities CBP/p300 and hGCN5 by VP16 *in vivo* occurs. Yet the system failed to detect increasing levels of acetylated histones H4 and H3K9. This could be due to increased HDAC levels as were seen in some preliminary experiments. However, increased levels of dimethylated histone H3K79 could be shown, a modification specific for actively transcribed genes.

3.1.5 VP16-dependent recruitment of GTFs and RNA processing factors

The first step in transcription is the formation of the preinitiation complex. It starts with the binding of TBP to the TATA box and ends with the recruitment of TFIIH that phosphorylates the CTD of Pol II leading to promoter clearance of RNA polymerase II. To monitor PIC formation, antibodies directed against TFIIB and TFIIH were used. TFIIB acts in an early step of PIC assembly, whereas TFIIH marks the endpoint of PIC formation. PIC formation in activated transcription is not only

supported by Mediator but also by other positive cofactors like PC4. PC4 was first characterized as a component of the USA fraction were it positively influences activated transcription [Ge and Roeder, 1994, Kretzschmar et al., 1994a]. PC4 is a direct interactor of VP16, it is involved in PIC assembly and was shown to bind to DNA, hence PC4 was chosen for this experiment.

Transcription stops at the termination signal and the nascent mRNA is cleaved and polyadenylated at the polyA signal (AAUAAA). The factors involved in this process are the cleavage and polyadenylation factor (CPSF) that acts together with the cleavage stimulatory factor (CstF) and the cleavage factors I and II (CF I, CF II). The inducible VP16 cell system gave us the possibility to take a closer look not only into PIC formation but also into the connection of transcription initiation and transcription termination. The Pol II and MED25 antibody were included to monitor Pol II and Mediator recruitment and act as a positive control.

Tet-On HeLa cells carrying the 7x tetO Vβ8.1 or the 7x tetO CMV reporter plasmids were crosslinked with formaldehyde 0 and 30 minutes after doxycycline addition. Mediator, TFIIB, TFIIH and Pol II are corecruited to the promoters and form a complete PIC as would be expected (Fig.18A). Whereas Mediator, TFIIB and TFIIH were seen at promoters but not in the coding and the control region, Pol II was also observed on the luciferase gene, which is indicative for ongoing transcription. PC4 and CPSF are also recruited by VP16 to the promoter, establishing a connection between transcription initiation and mRNA polyadenylation. Notably, PC4, CPSF and CstF64 were corecruited at a later timepoint than Mediator and Pol II (Fig.18B). Whereas Mediator and Pol II appeared within minutes (Fig.14), PC4, CPSF and CstF64 appeared between 5 and 30 minutes after induction at the promoter.

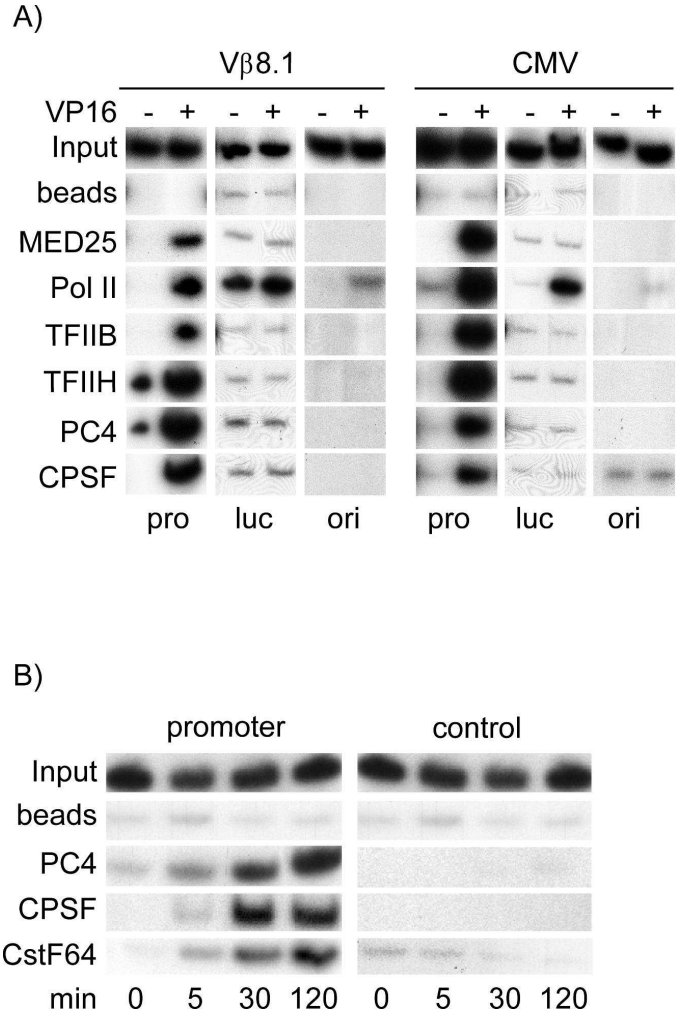

Figure 18: VP16-dependent recruitment of general transcription factors and polyadenylation factors. A) ChIP analysis, Tet-On HeLa cells carrying either the 7x tetO Vβ8.1 or the 7x tetO CMV reporter plasmid were crosslinked 0 or 30 minutes after VP16 activation. Of all the factors is only Pol II found in the coding region, the other factors are exclusively recruited to the promoter. B) Time-course ChIP analysis, Tet-On HeLa cells carrying the 7x tetO Vβ8.1 reporter were crosslinked at the indicated timepionts after VP16 activation. PC4 together with the polyadenylation factors CPSF and CstF64 were recruited at a later stage during transcription initiation than the other transcription factors (Fig.14 and Fig.17).

There are reports that show an interaction between PC4 and CstF64 [Calvo O., 2001, Calvo O., 2005], therefore a concerted action of at least PC4 and CstF64 seems possible. By the dual role of PC4 to facilitate PIC formation and in binding to the polyadenylation machinery, it might contribute to the link between transcription initiation and transcription termination.

The data show the *in vivo* recruitment of components of the preinitiation complex as well as of the polyadenylation machinery. Recruitment of GTF's and Mediator is restricted to the promoter regions. The corecruitment of PC4, CPSF and CstF64 might point towards a bridging function of PC4 between the initiation complex and the polyadenylation machinery. However, these data alone can not prove this possibility directly. Instead, they could also be consistent with a role of CPSF/CstF in a later phase of transcription. In addition, the late recruitment of PC4 might also indicate a function involved in late transcription initiation or even elongation. Notably, luciferase mRNA was only detected some 15 minutes after VP16 activation (Fig.11). This might reflect a delay in formation of full length transcripts but could also reflect the time request for splicing and RNA export.

3.2 *In vitro* PIC assembly

The ChIP technology is a valuable tool to study chromatin related molecular processes *in vivo*. However, it is inefficient to decipher molecular processes on genes. For this, *in vitro* systems are expected to be more efficient. For example transcription initiation complexes may be selectively formed and analyzed in vitro which then become converted into elongating complexes only upon addition of nucleotides.

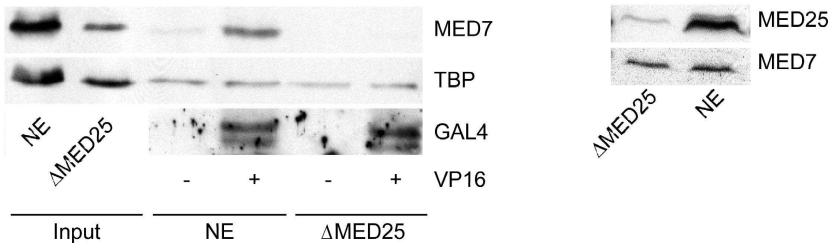

Figure 19: A MED25 containing Mediator complex (A-Med) is necessary for VP16-dependent recruitment of Mediator. Left panel, in a solid phase assay, biotinylated promoter DNA was immobilized on streptavidin coated beads. In the presence of VP16 Mediator was recruited from nuclear extracts (NE) but not from MED25-depleted nuclear extracts (ΔMED25). Right panel shows a control for the MED25 depletion.

Towards a solid phase assay, biotinylated DNA fragments were immobilized on streptavidin coated paramagnetic particles to assemble a preinitiation complex (PIC). The DNA fragment used contained the promoter fragment MRG5 [Meisterernst et al., 1991], that consists of the HIV core-promoter sequence and the adenovirus major late (ML) initiator under the regulation of 5 Gal4-binding sites. It has previously been shown that cell extracts contain both MED25-Mediator (A-Med) and other Mediator complexes lacking this specific subunit [Mittler et al., 2003]. It was first analyzed whether Gal-VP16 dependent recruitment of Mediator is observed in a MED25-depleted nuclear extract (ΔMED25). Recruitment of Mediator was monitored by a MED7 antibody. TBP was used as a control because this factor does not bind efficiently to Mediator [Mittler et al., 2003]. In a normal nuclear extract (NE), a Gal-VP16 dependent recruitment of MED7 is

indeed seen (Fig.19) whereas in a MED25-depleted nuclear extract no Gal-VP16 dependent Mediator recruitment could be observed. Highlighting again that a MED25-containing Mediator complex (A-Med) is indeed crucial for Gal-VP16 dependent recruitment. In both nuclear extracts, TBP binds to the DNA regardless of the presence of Gal-VP16 (Fig.19).

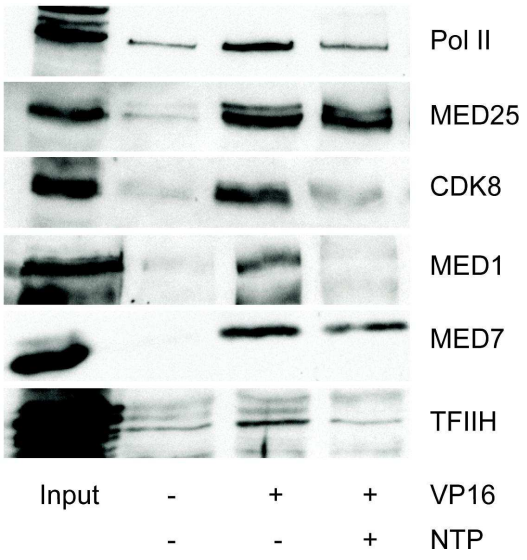

Figure 20: Addition of nucleotides (NTP) releases the kinase module as well as the MED1 subunit form the Mediator complex. In a solid phase assay, biotinylated promoter DNA was immobilized on streptavidin coated beads. In the presence (+) VP16, Mediator, together with Pol II and TFIIH are recruited to the Promoter. Addition of 0.5 mM of nucleotides releases Cdk8 and MED1 from the bound Mediator complex.

From the ChIP analysis it was predicted that the kinase module of the Mediator is not recruited by Tet-VP16. Additionally, MED1 should not be enriched, whereas other Mediator subunits should. To examine this, proteins retained by the immobilized template after addition of Gal-VP16 were analyzed by SDS-PAGE and subsequent immunoblotting. In the absence of the activator, moderate levels of Pol II and of the general transcription factor TFIIH, but not of Mediator could be detected (Fig.20). Addition of Gal-VP16 to the immobilized template significantly enhances Pol II and TFIIH levels. Under these conditions the Mediator

subunits MED1, MED7, MED25 and Cdk8 accumulate at the promoter. This underlines the need of an activator for Mediator binding, but raised questions about Cdk8. After addition of nucleotides (NTP), MED7 and MED25 are retained at the template. The other two Mediator subunits MED1 and Cdk8 are lost from the initiation complex. Pol II and TFIIH levels are reduced to the same level before addition of the activator. The loss of Pol II after addition of nucleotides is expected, since under these conditions the CTD of Pol II becomes phosphorylated and Pol II clears the promoter. Retention of TFIIH and Mediator at the promoter is observed and resembles most likely a scaffold complex as was discussed in yeast. The scaffold complex is thought to facilitate transcription reinitiation. The Mediator complex that is retained at the promoter lacks the kinase module that is a hallmark of the small, transcriptionally active Mediator complexes (PC2, CRSP, B-Med). Additionally, it lacks the MED1 subunit as well. Recently, Mediator complexes lacking MED1 have been described as PC2-like complex by the Roeder laboratory and the CRSP-2 complex by the Tjian laboratory. The VP16 specific Mediator (A-Med) is similar to these two complexes, it remains to be shown whether the MED25 subunit is specific for VP16 targeted Mediator complexes only. An interesting point however is that a complete TRAP-like Mediator complex is recruited by VP16 and that the transition into the specific A-Med complex is NTP dependent.

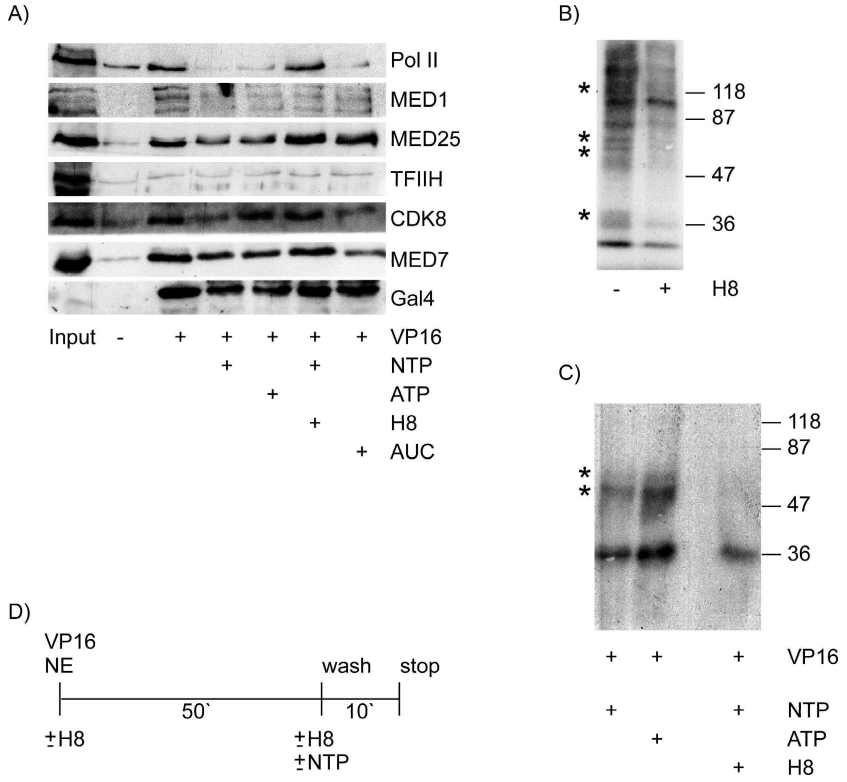

Figure 21: Phosphorylation-dependent remodeling of the Mediator complex within the preinitiation complex. A) Addition of ATP releases Pol II and MED1 from the PIC. However, phosphorylation alone still retains Cdk8 on the template. Phosphorylation in combination with ongoing elongation (+ AUC) releases Cdk8 from the PIC. B) Autoradiogram of proteins retained at the promoter in the situation were a complete NTP mix together with ^{32}P γATP was added to the reaction. Addition of H8 prevents phosphorylation of most proteins with the exception of a band of around 100 kDa. C) Autoradiogram of proteins lost from the PIC. After PIC formation, the complexes were washed and NTP, ATP and H8 together with ^{32}P γATP was added to the reaction. Released proteins from the PIC were subjected to TCA precipitation and phosphorylated proteins were visualized. Bands sensitive to H8 are marked by asterisks. D) Schematic overview of the experiment.

Addition of a complete nucleotide mix leads to elongation and run-off of Pol II. To address the question whether phosphorylation plays a role, ATP and the kinase inhibitor H8 were used. Again, addition of 0.5 mM NTPs to the reaction led to the

loss of the MED1 and Cdk8 subunit of Mediator. Treatment of the nuclear extract with the kinase inhibitor H8 for 50 minutes before addition of the NTPs, blocked the loss of Cdk8 and of Pol II (Fig.21C). This may indicate a role of phosphorylation in dissociation of Cdk8. Phosphorylation lead to a loss of MED1, but not the Cdk8 subunit, indicating that the loss of Mediator-Pol II interaction is not sufficient for the loss of the kinase module. It is unclear whether Mediator complexes of modules of it fall off the template alone or in combination with Pol II. Only after addition of ATP, UTP and CTP (AUC), a loss of Cdk8 could be observed. Indicating that in addition to phosphorylation, elongation seems to be necessary to remove the kinase module of the Mediator complex (Fig.21A). However there were exceptions when reduced levels of Cdk8 were already observed with ATP alone. This raises the question about which proteins become phosphorylated and whether they remain at the promoter or if they are lost during transcription activation (Fig.21B and C). To start answering this question, preliminary experiments were carried out. Together with ATP or a complete NTP mix, radioactive ^{32}P-γATP was added to the reaction to label phosphorylated proteins. Labeled proteins retained at the promoter were analyzed by SDS-PAGE and subsequent autoradiography (Fig.21B). There are several proteins that become phosphorylated. Most of this phosphorylation could be blocked by addition of H8, indicating their relevance in transcription activation as was shown before (Fig.21A). The phosphorylation of four proteins (marked with an asterisk) were sensitive to H8 and therefore the most likely candidates for being responsible in regulating open complex formation and remodeling of the Mediator complex. The identity of these proteins remains to be determined by other means, like masspectrometry. Labeled proteins released from the PIC were concentrated from the supernatant by TCA precipitation, separated on an SDS-PAGE and visualized by autoradiography (Fig.21C). Indeed, one double band at around 50 to 60 kDa shows up when either ATP or an NTP mix was added to the reaction. This double band is lost when the PIC was pretreated with the kinase inhibitor H8. About the identity of the protein can only be speculated, however the size is close to Cdk8 which often migrates as a double band according to Western blot analysis. Unfortunately, the recovered protein from the supernatant proved to be too little to be analyzed by Western blot.

3.3 MED25, a novel Mediator subunit

3.3.1 Cellular distribution of MED25

Scanning the MED25 sequence for targeting signals, using programs predicting the cellular localization, none of them could find a nuclear localization signal (NLS). To investigate this aspect, indirect immunofluorescence (IF) experiments were performed in adherent HeLa cells.

Exponentially growing cells were fixed and incubated with the MED25 monoclonal antibody to visualize the protein by confocal microscopy. Immunofluorescence of endogenous MED25 shows a predominantly nuclear but weak and diffuse staining. However the signal is only slightly stronger than the negative control (HA antibody) which renders the analysis somewhat ambiguous. As an

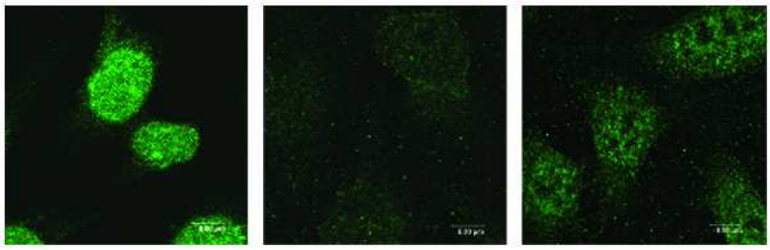

Figure 22: Endogenous MED25 is visualized by a monoclonal antibody (VC1 9C2) and a secondary anti-rat antibody conjugated to FITC (green). The MED25 signal is only slightly above the background signal of the HA antibody (negative control). In contrast to the NC2α signal as a positive control, that shows a strong nuclear staining.

example for a clear nuclear staining, the positive control (NC2) shows a strong nuclear signal (Fig.22). To further substantiate MED25 localization, HeLa cells were transfected with a Flag-MED25 expression vectors. The tagged protein can be detected by two different antibodies either directed against MED25 or against the Flag-tag. The overexpressed protein clearly localizes to the nucleus, only the nucleoli are excluded from the staining (Fig.23). The protein is evenly distributed and no preferential localization is evident (e.g. speckles). As expected, if

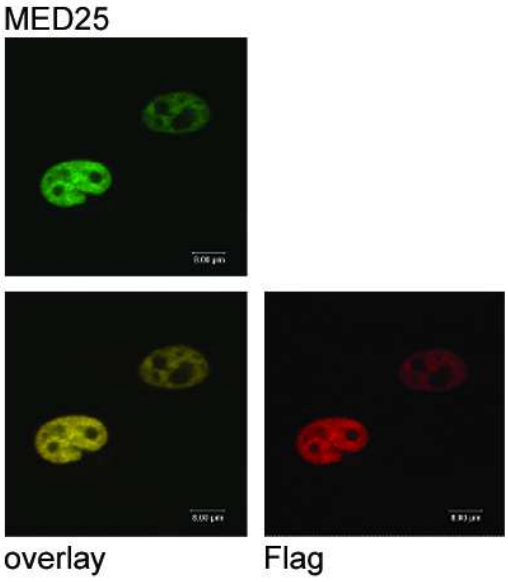

Figure 23: MED25 localizes to the nucleus. HeLa cells were transfected with Flag-MED25 and stained with antibodies against Flag (M2; red) and MED25 (VC1 9C2; green). The overlay shows a nice colocalization of the two independent antibodies.

the MED25 monoclonal antibody and the Flag specific antibody (M2) recognize solely the target protein, the signals colocalize perfectly.

3.3.2 PTOV1 shares some homologies with MED25

MED25 is not the only protein that carries an ACID domain. A second protein, termed prostate tumor overexpressed protein (PTOV1) carries two ACID domains and lies next to MED25 on chromosome 19q13.33. The 415 amino acid long PTOV1 protein consists virtually of the fusion of two ACID domains (Fig.24A). PTOV1 is overexpressed in prostate tumor cells. It was shown that it is a mitogenic protein that shuttles between the cytoplasm and the nucleus in a cell cycle dependent manner and promotes entry into the S phase [Santamaria A., 2003, Santamaria A., 2005]. Overexpression of PTOV1 strongly induces cell proliferation whereas depletion represses proliferation. To investigate the possibility that

MED25 is also shuttling between the cell compartments, nuclear extracts derived from synchronized Raji cells were monitored in Western blot analysis.

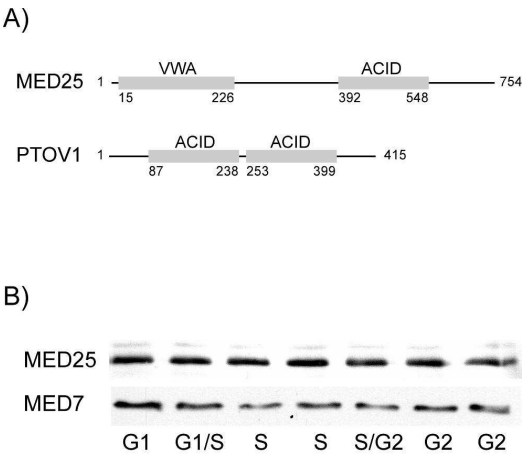

Figure 24: A) Cartoon of MED25 and POTV1 indicating the shared ACID domains in light grey. B) Westernblot analysis of MED25 expression during the cell cycle. Nuclear extracts from synchronized Raji cells were separated on a 12% SDS-PAGE gel. The MED7 subunit is used as a marker for Mediator. (MED7 3E12 rat monoclonal, 1:20 dilution in TBS-T 1% milkpowder; MED25 9C2 rat monoclonal, 1:20 dilution in TBS-T 1% milkpowder).

Exponentially growing Raji cells were harvested in an Elutriator. Cells in different phases of the cell cycle could be obtained, namely from the phases G1, G1/S, S, S/G2 and G2. Nuclear extracts were produced from each cell fraction (kindly provided by A. Schepers). Equal amounts of proteins were subjected to SDS-PAGE, blotted and probed with MED7 and MED25 antibodies. MED25 was found in all cell fractions and neither increased nor decreased in any of the fractions assayed (Fig.24B). MED7 serving as a control for the presence of Mediator in the extracts behaved in the same way.

In conclusion, the immunofluorescence of overexpressed MED25 showed a nuclear localization, indicating an efficient transport into the nucleus. Furthermore, localization of endogenous MED25 into the nucleus is independent of the phase of the cell cycle.

3.3.3 MED25 binds CBP *in vitro*

Immunoprecipitation of Mediator also precipitates CBP/p300 as was shown by Westernblot and masspectrometry [Stuehler Thomas, 2005]. It was shown that VP16 binds directly to CBP/p300 [Kraus et al., 1999, Ikeda et al., 2002] as it does to MED25 [Mittler et al., 2003, Yang et al., 2004]. To ask whether MED25 and CBP interact directly and to map the interaction surface within the proteins, a GST pull down assay was employed. CBP can be roughly divided into three regions.

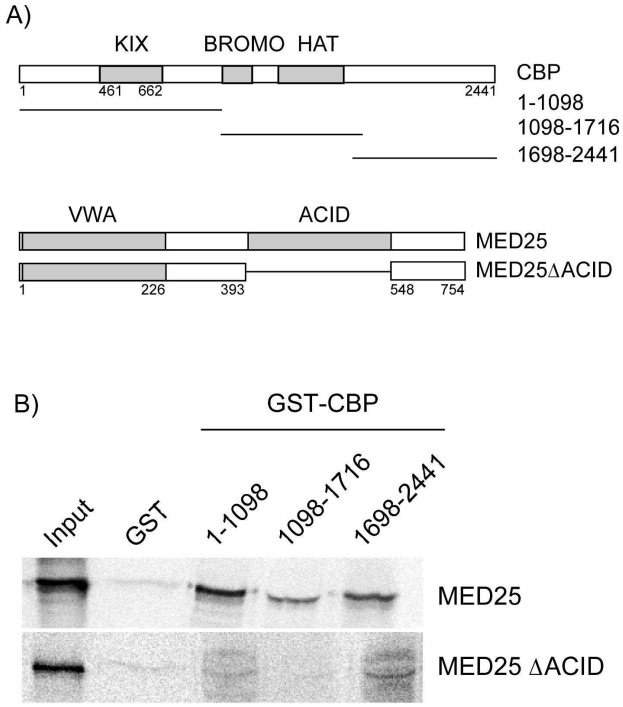

Figure 25: MED25 binds CBP *in vitro*. A) Structure of CBP and the used GST-derivatives are indicated as well as the MED25 constructs used in this assay. B) GST-pull down of ^{35}S-labeled MED25 proteins on a GST-CBP matrix. 20 μg of GST-fusion proteins were blocked with 60 μg of BSA and incubated with 5 μl of radioactive labeled MED25 proteins in BC150 buffer. Shown is the autoradiogram after separation of the proteins on an SDS-PAGE.

The N- and the C-terminal regions are known to bind to a myriad of transcription factors. The central region harbors the Bromo and the HAT domain (Fig.25A).

GST, GST-CBP$_{1\text{-}1098}$, GST-CBP$_{1098\text{-}1710}$ and GST-CBP$_{1678\text{-}2441}$ deletion constructs were purified and immobilized on a GST columns. MED25 full length and MED25ΔACID proteins were translated *in vitro* in a coupled transcription and translation reaction that allowed labeling with ^{35}S-Methionine (Fig.25A). The radioactive labeled MED25 proteins were incubated together with the GST-CBP deletion constructs or GST alone. The N-terminal region of CBP, GST-CBP$_{1\text{-}1098}$ and the C-terminal GST-CBP$_{1678\text{-}2441}$ bind the MED25 full length protein slightly stronger than the central region of CBP (Fig.25B).
The MED25ΔACID construct is not bound by any of the CBP deletion proteins, indicating an essential role of the ACID domain in binding CBP. Although, the question whether the ACID domain alone is sufficient for interaction with CBP remains open, the result would imply a simultaneous binding of VP16 to MED25 and CBP. VP16 binds to CBP via its H2 domain, which leaves the H1 domain free for Mediator interaction. For the future it would be interesting to see whether VP16-H1 coprecipitates CBP with MED25 at low stringency.

3.3.4 Differential urea dissociation of Mediator subunits

Using differential dissociation by urea, the Kim laboratory could demonstrate that yeast Mediator can be dissociated into subcomplexes [Sang Jun Han, 2001] [Jong Seok Kang, 2001], namely the Mediator tail/middle subcomplex and the head subcomplex. The association of the newly identified Mediator subunit MED25 to one of the subcomplexes is unknown. To address this issue, human Mediator from HeLa nuclear extracts was immobilized on anti-MED15 antibody agarose beads. The retained proteins were examined by Western blot analysis after extensive washing with urea-containing buffer. MED15 is the mammalian homologue of the yeast Gal11 subunit that localizes to the tail subcomplex of Mediator. The tail and middle subcomplex were shown to be the most resistant ones to treatment with urea [Han et al., 2001].

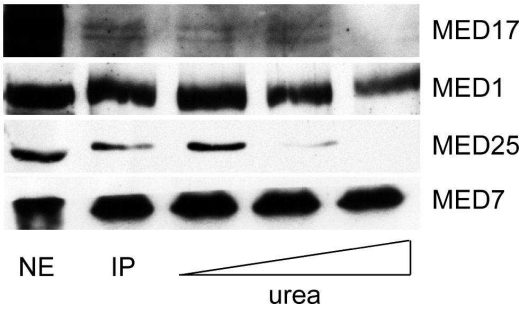

Figure 26: Differential dissociation of Mediator components by urea. Mediator was immobilized by MED15 antibody beads and washed with buffer containing 0M (IP) to 3M urea (urea). The remaining Mediator subunits on the antibody beads were subjected to SDS-PAGE followed by western blot analysis.

Mediator was immunoprecipitated using MED15 antibody columns. The immunoprecipitation was done under standard conditions using 150 mM KCl for precipitation and in the BC150 wash buffer (for detail see Materials and Methods 2.9.3). 2M urea washes removed most of MED25 from the MED15 immobilized beads. MED17, a subunit of the head subcomplex, was only removed after 3M urea washes (Fig.26). On the other hand, MED1 and MED7 were retained on the antibody beads even after 3M urea treatment. MED1 and MED7 are both subunits of the middle subcomplex. MED25 is among the first proteins that are lost from the complex, in accordance to the data from Kim and colleagues [Han et al., 2001] this would point towards a localization of MED25 within the head subcomplex of Mediator. However, the MED25-Mediator interaction might be weak and and only transient as was suggested from the Conaway laboratory [Sato et al., 2004]. If this is the case, MED25 could be washed away by urea independent of its respective subcomplex. However the finding that MED25 does not associate with the tail nor with the middle, but rather with the head subcomplex of Mediator would be in line with previous reports that VP16 crosslinkes to MED17 [Ito et al., 1999] and with electron microscopy data that locates Mediator bound VP16 to the head domain [Taatjes et al., 2002].

3.3.5 Towards the structure of the ACID domain

The activator interaction domain (ACID) of MED25 is a novel protein interaction domain. The human ACID domain binds strongly to the activation domain of the Herpes simplex virion protein 16 (VP16) [Mittler et al., 2003, Yang et al., 2004], and LANA-1 [Roupelieva, 2005]. The drosophila ACID domain binds to differentiation inducing factor (DIF) and to heat-shock factor (HSF) [Kim TW, 2004]. All of these activators are viral proteins. A cocrystal structure with VP16 at atomic resolution would allow us to study the interaction surface of the two proteins and the many existing mutations within the VP16 activation domain could be better explained. Additionally, from a pharmaceutical point of view this domain might act as a potent Herpes virus inhibitor. To learn more about the ACID domain we decided to determine the crystalstructure in collaboration with P. Cramer.

To determine the exact localization of the domain borders, several N- and C-terminal deletion constructs were generated. Six different constructs, spanning amino acids 345-560, 345-548, 393-560, 393-548, 426-560 and 426-548 were generated (Fig.27A). The N-terminal deletions were chosen to delete poorly predicted secondary structures and the C-terminal deletions to remove part of a polyproline stretch. First, all this constructs were expressed in *E.coli* and tested for solubility. The soluble fractions and the insoluble pellets were compared on an Coomassie stained SDS-PAGE gel in parallel with an uninduced cell aliquot. All proteins with the exception 426-560 and 426-548 of were expressed at high levels and were present in the soluble fraction.

Specific binding of the truncated domains to VP16 was investigated. Immobilized GST-VP16 H1 and H1mt were incubated with *E.coli* lysate containing the different ACID domains (amino acids 345-560, 345-548, 393-560 and 393-548). Binding to VP16 H1 was visualized on a Coomassie stained SDS-PAGE. As expected all proteins bound to H1 but not to H1mt (Fig.27B), and were therefore specific.

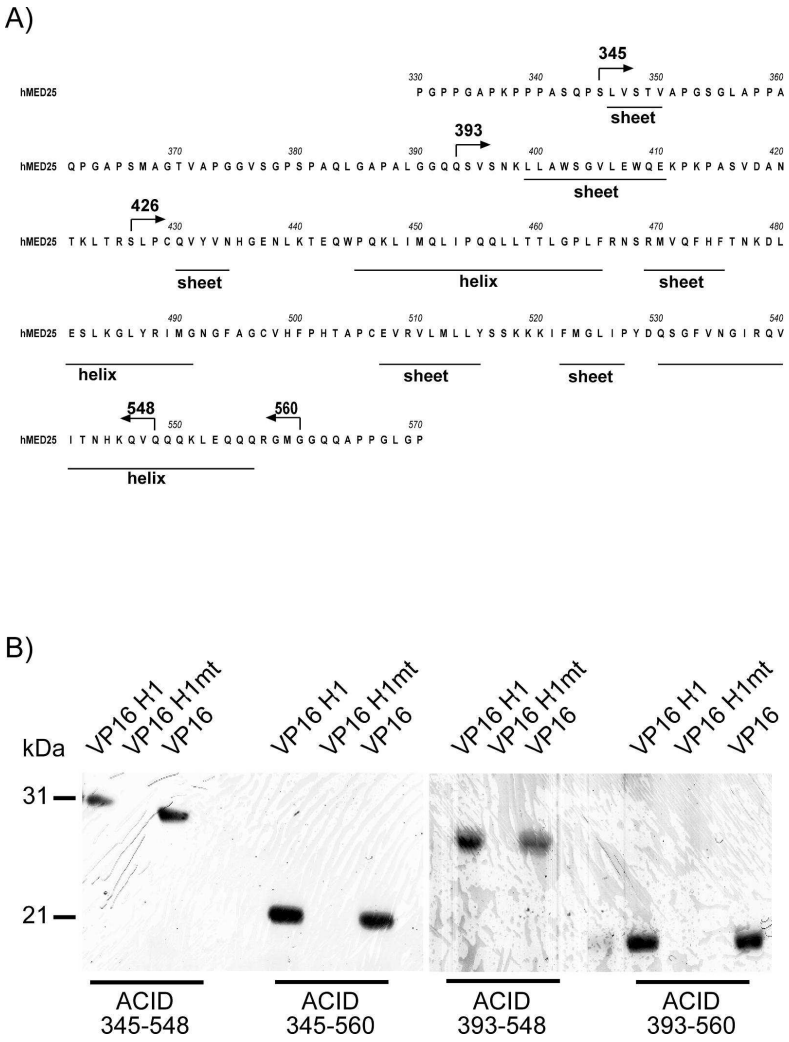

Figure 27: Primary sequence of ACID and specific binding of the ACID deletion proteins to VP16. A) Sequence of the human ACID domain. Depicted are the secondary structure prediction as well as the start and end points of the used deletion constructs. B) All of the deletion proteins bind to the full length VP16 activation domain (VP16), and the H1 activation domain (H1), but not to the mutated H1 activation domain (H1mt), indicating specific binding. Shown is a Coomassie stained 15% SDS-PAGE of eluted proteins.

Binding to GST-VP16 could also be seen when GST-VP16 was coexpressed together with the ACID constructs. *E.coli* were transformed with two different plas-

mids, one plasmid encoding for GST-VP16 and the second plasmid for a 6xHis-tagged ACID domain. The cells were lysed and the cleared lysate was subjected to either a Ni-column or a GST-column (for details see Materials and Methods 2.9.2; 2.9.5 and 2.9.6). When using a Ni-column to purify the ACID domain, GST-VP16 always copurified (Fig.28) and vice versa.

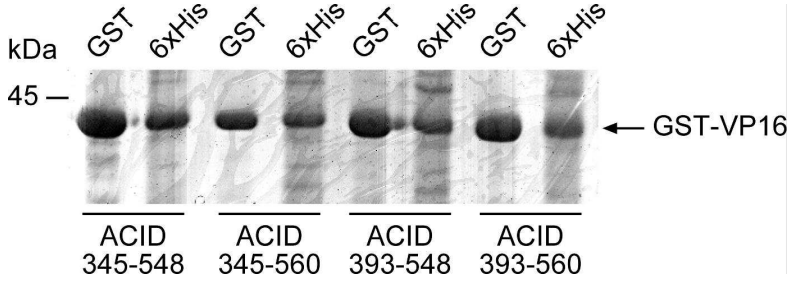

Figure 28: 6xHis-MED25 can be copurified with GST-VP16. *E.coli* expressing both 6xHis-MED25 and GST-VP16 were lysed and the proteins were purified either over a GST-column or a Ni-column, respectively. Using the Ni-column, GST-VP16 is copurified together with 6xHis-MED25. Shown is a Coomassie stained 12% SDS-PAGE.

To determine the exact domain borders of the ACID domain, purified proteins were incubated at 37°C together with Trypsin and subjected to limited protease digestion. Correctly folded domains show a higher resistance towards proteolysis than unfolded regions, thus trimming the domain to its minimal form. For trypsin treatment, 1μg of the protease was added to 20 μg of purified protein. Digests were done in BC150 buffer supplemented with $CaCl_2$to a final concentration of 4 μM. The mixture was incubated at 37°C for 1 min, 3 min, 10 min and 30 min. The reactions were stopped by addition fo SDS sample buffer and were heated immediately to 95°C for 5 min. For proteinase K treatment, 1 μl od dilutions of the proteinase (3 μg/μl, 0,3 μg/μl, 0,03 μg/μl, 0,003 μg/μl) were added to the protein samples. The mixtures were incubated on ice for 1 hour. All of the four deletion proteins tested proved to be stable during the limited proteolysis experiments. One exception was the protein spanning amino acid 345 to 560 which was degraded to some extent. (Fig.29). The experiment was repeated with proteinase K instead of Trypsin. Proteinase K has a broad specificity and cuts proteins randomly. The

outcome of this experiment was similar to the one with Trypsin. Again the protein spanning the region from amino acid 393 to 548 was the smallest stable fragment. For crystallization trials, this construct was used.

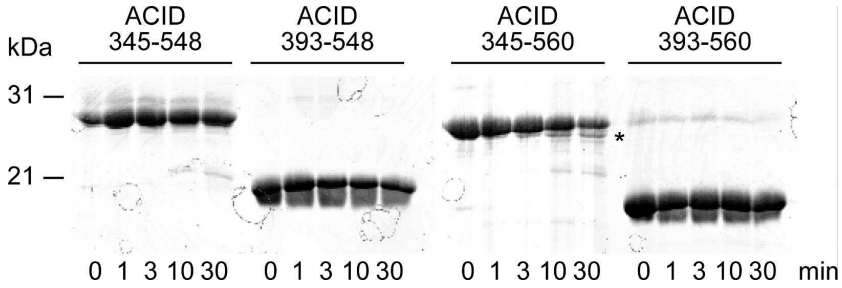

Figure 29: Protease protection assay to determine and verify the ACID domain borders. Purified deletion proteins were incubated with Trypsin for the indicated periods of time. The proteins were separated on a SDS-PAGE and stained with Coomassie for analysis. All the proteins are extremely resistant towards the protease, indicating a stabile folded protein domain. Degradation product is indicated by an asterisk.

4 Discussion

4.1 Establishment of an inducible VP16 model system

To study transcription initiation *in vivo* one is in need of a system that allows to turn on transcription of a specific gene of interest. In reporter assays, the effect of a protein on transcription is measured by the expression of the exogenous luciferase gene. The reporter plasmid (e.g. pGL2-MRG5) used in such an assay consists of a model promoter (e.g. HIV) that is under the control of exogenous activator binding sites (5x Gal). These reporter plasmids are transiently cotransfected together with a plasmid coding for the transactivator (e.g. Gal-VP16). The advantage of such a system is clearly that the effects of cofactors on transcription can be studied *in vivo*. However, to visualize transcription cofactors by ChIP analysis these transient systems are of no use. In most reports to date, transcription is studied on endogenous gene promoters by ChIP analysis. The signals to turn transcription on in these mammalian systems are based on hormones like estrogen [Metivier R., 2003], serum induction [Wang et al., 2005] and cell differentiation [Soutoglou and Talianidis, 2002] or galactose induction in yeast [Bryant, 2003]. Even if the gene promoter under investigation is the major target of these signals, secondary effects can not be excluded. In the system using serum induction for example, the *Egr1* and *Egr2* genes are not the only genes affected by the signal but over 20 other genes as well [Wang et al., 2005]. Therefore the use of an exogenous transactivator and signal to induce transcription of a model gene would be a nice system to study transcription initiation timely resolved in ChIP assays.

Conventional stable cell lines with integrated reporter genes into their genomes can produce positioning effects dependent on the locus of the integration. To circumvent this complication, EBV (Epstein-Barr Virus) based vectors that replicate episomally carrying a suitable promoter and a Tet-VP16 expression cassette allow induction of VP16-dependent transcription of the luciferase gene. This vector system has several advantages: (i) it was shown for these mini-episomes to be packed into chromatin [Chau CM. and Lieberman PM., 2004, Zhou J., 2005], (ii) the transactivator is unique and targets only one specific promoter (iii) the transactivator as well as the promoter are interchangeable with other activation domains

or promoter sequences.

The transactivator Tet-VP16 is a fusion protein of the DNA binding domain of the tetracycline repressor and the activation domain of VP16. The VP16 protein is a potent activator that controls the transcription of immediate early genes of the herpes simplex virus through interaction with host cell factors. The mechanism of transcription stimulation by VP16 has been studied extensively. VP16 promotes the enhancement of transcription rates and increase in the number of functional active promoters [Ranish et al., 1999]. It facilitates open complex formation [Jiang et al., 1994] and increases the processivity of Pol II [Yankulov, 1994]. The expression cassette for the luciferase gene, consists of a model promoter under the regulation of the tetracycline-response element (TRE) and the SV40 poly(A) signal. The TRE contains seven direct repeats of a 42-bp sequence (7x tetO) recognized by the DNA binding domain of the tetracycline repressor. Addition of doxycycline allows Tet-VP16 to bind specifically to the TRE and leads to the expression of luciferase. The model promoters used in this study were the T-cell specific promoter of the TCRβ locus, the Vβ8.1 and the minimal core of the CMV promoter (Fig.8). The functionality of the system was tested by monitoring luciferase expression (Fig.9 and 10 and by detection of luciferase mRNA by RT-PCR (Fig.11). The system is fast responding to doxycycline stimulation and luciferase mRNA could be measured 15 minutes after induction. Binding of Tet-VP16 is even faster as could be shown by D.L. Spector and colleges by real-time fluorescent microscopy [Janicki et al., 2004].

The inducibility of the Vβ8.1 cell line was monitored over the course of eight weeks (Fig.12). Over time, the cells lost their inducibility and after six weeks, hardly any luciferase could be measured anymore. This loss of inducibility is not due to the loss of Tet-VP16 as could be shown by a western blot against VP16. The levels of expressed VP16 is the same regardless of the inducibility of the cells. A loss of the reporter plasmid can also be ruled out, since the cells were kept constantly under selection pressure. The explanation might lie in the positioning of the nucleosomes together with the lesser nucleosome penetration potential of the Tet-repressor compared to the Gal-DNA binding domain [Pankiewicz, 2004] and the relatively weak transcription potential of the Vβ8.1 promoter.

Taken together, this newly established episomally stable vector system allows rapid activation of specific model promoters *in vivo*, and allows to study transcription in the context of chromatin.

4.2 Formation of the transcription complex *in vivo*

Induction of transcription requires the formation of the preinitiation complex (PIC) on the promoter, comprising the six general transcription factors TFIIA to F and Pol II [Berk, 1999]. This assembly process normally takes place at the core promoter, which includes defined sequence elements within the 35 bp upstream and downstream of the transcription start site in human and drosophila. These elements include the TATA element (TBP binding), TFIIB recognition element (BRE), Initiator element (INR) and Downstream promoter element (DPE)[Smale and Kadonaga, 2003]. The most prominent core promoter sequence is the TATA element which is bound by TBP that belongs to the 15 subunit TFIID complex. TBP binding promotes DNA bending and is the first step in transcription machinery assembly [Kim et al., 1993b]. Next, TFIIA binds and stabilizes the TBP/DNA complex [Geiger et al., 1996, Tan et al., 1996]. TFIIB directly contacts TBP and DNA [Nikolov D. and S., 1995]. Together they form the platform on which Pol II and TFIIF can assemble. After the recruitment of TFIIE and TFIIH, ATP dependent promoter opening can occur ([Hahn, 2004]). These basic mechanisms have been elucidated in various *in vitro* systems in the past [Orphanides, 1996]. Only in recent years it became possible by ChIP technology to investigate transcription machinery assembly *in vivo*. In an estrogen inducible *in vivo* system preinitiation complex formation was studied on the example of the estrogen receptor-αtarget pS2 gene promoter [Metivier R., 2003]. An ordered and interestingly, cyclical recruitment of cofactors could be observed.

In this thesis PIC formation was monitored *in vivo* by ChIP analysis of TFIIB, TFIIH, PC4, Mediator and Pol II (Fig.18). Not so much the formation of the PIC was in the main focus but the interaction and possibly crosstalk between the general transcription factors and transcriptional cofactors like Mediator and PC4. The cyclical recruitment of cofactors seen before could not be recapitulated in this

study. This might be due to the serum starvation and α-amanitin treatment that was used to synchronize the pS2 promoter. Such precautions were not made for the doxycycline inducible gene promoters Vβ8.1 and CMV. Therefore we have to assume that we monitor steady state levels of the transcription cofactors on the promoter. Recruitment of all the factors monitored seem to happen with the same kinetic with the sole exception of PC4 that appears at a later step in PIC assembly. The recruitment of TFIIB, TFIIH, Mediator and Pol II could not be separated from each other. However, changes in Mediator could be resolved and will be discussed in further details below.

4.3 VP16-dependent recruitment of MED25

In a biochemical screen for VP16 interacting proteins, MED25 could be characterized as the major interaction partner of VP16 in the human Mediator complex [Mittler et al., 2003, Yang et al., 2004]. Depletion of MED25 containing Mediator complexes (A-Med) leads to a loss of VP16 specific activation in *in vitro* transcription assays. Additionally, the N-terminal Mediator-binding domain of MED25 (NTD) was shown to act dominant negatively on VP16-driven transcription in reporter assays. It is noteworthy than no other activator tested nor basal transcription levels were affected by overexpression of NTD [Mittler et al., 2003]. This clearly argues for an activator specific function of A-Med. Using the inducible system, VP16-dependent recruitment of MED25 was demonstrated *in vivo* (Fig.13). This was shown on two independent model promoters, namely the Vβ8.1 and CMV. The two promoters vary in various aspects. On the Vβ8.1 promoter, MED25 levels increased about 20 fold, which correlated with increased levels of Pol II (Fig.13). In an immobilized template assay, VP16-dependent binding of Mediator to the MRG5 promoter could be demonstrated to depend on MED25 (Fig.19). This would argue that a distinct Mediator complex, A-Med is recruited to the promoter. However, it could also be that independently of Mediator free MED25 is recruited and becomes incorporated into the complex. It was shown that free flag-MED25 can be integrated into Mediator complexes during IP experiments to determine the minimal Mediator binding domain in MED25 [Mittler et al., 2003]. However, free MED25 could not complement for A-Med

function in an *in vitro* transcription assay so far which would rather be an indication that endogenous MED25 was exchanged with exogenous flag-MED25 instead.

As an unexpected finding in the ChIP experiments, MED1 was found to be present at the Vβ8.1 promoter even before the system was turned on. Addition of doxycycline did not change MED1 levels, as if no additional MED1 gets recruited together with A-Med. Only recently have Mediator complexes been characterized that lack MED1 [Taatjes and Tjian, 2004, Malik et al., 2005]. In an immobilized template assay, we could demonstrate the VP16-dependent recruitment of a Mediator complex containing MED1. However, addition of nucleotides lead to a loss of MED1 from the complex (Fig.20). This finding could explain why no change in MED1 levels could be observed in the ChIP assay. A similar finding was reported using p53 as an activator [Malik et al., 2005]. It looks like MED1, even though required by nuclear hormone receptors can be omitted by activators like VP16 and p53 [Malik et al., 2005]. In the same study, the authors even propose that most of human mediator lacks MED1. Taken together, these findings point towards a more dynamic model of Mediator in which Mediator can change its subunit composition depending on the activator.

The fact that MED1 was found to be present on the Vβ8.1 promoter even before induction of the system might be due to the structure of the promoter. Of the Vβ8.1 promoter of the TCRβ chain a 1kb fragment was used, including upstream and downstream sequences as well as several endogenous activator binding sites (Ets, CREB, SP1) (Fig.15). This explanation is plausible since the basal transcription levels from the Vβ8.1 promoter are higher than the one seen from the CMV promoter. The CMV promoter is a minimal viral promoter with high induction rates and low background activity, that is widely used in eucaryotic expression plasmids. In addition to that, the TRE in the Vβ8.1 promoter is located more distal to the transcription start site compared to the short minimal promoter sequence of the CMV promoter. Furthermore, the seven Tet-binding sites could be responsible for synergistic effects that could lead to a preoccupied Vβ8.1 promoter. However, this concern can be appeased, since the system is responsive to the dominant negative effect that NTD exerts on VP16 activation. Indicating that

VP16 dependent transcription initiation in this system is also mainly dependent on A-Med.

4.4 The A-Med complex

Human Mediator complexes have been isolated in several laboratories in independent lines of research using different activators or functional assays. All these complexes, ARC/CRSP, DRIP, PC2, SMCC and TRAP turned out to represent human Mediator complexes [Blazek et al., 2005, Malik and Roeder, 2005b]. These different complexes share a consensus set of subunits [Sato et al., 2004]. To date, 37 different subunits have been characterized in different organisms from which 22 are conserved in all eukaryotes [Bourbon et al., 2004]. The conserved subunits most likely form the core of Mediator. However, it is unclear whether the many human Mediator complexes are the product of the different purification strategies or if they are functionally distinct complexes.

Mediator is know to have a modular organization and can be divided into head, middle, tail and kinase modules. The tail module is formed by MED24, MED23 and MED16. It is likely that this three subunits form a bona fide submodule, since Mediator complexes isolated from mouse cells in which the genes encoding either MED24 [Ito et al., 2002b] or MED23 [Wang et al., 2001, Wang and Berk, 2002] have been knocked out are deficient for all three subunits. This submodule has been discussed to play a major role in integrating signals form different activators, even if they do not bind directly to any of the three subunits. For example, nuclear hormone receptors no longer activate transcription in reporter assays if the MED24 subunit is knocked out, even though MED1 and not MED24 is the direct interaction partner. In contrast to this, overexpression of the NTD of MED25 shows a dominant negative effect in reporter assay only for VP16, activation by nuclear hormone receptors are not impaired [Mittler et al., 2003]. In turn, activation by VP16 is impaired in the MED24 knock out cell, as well as activation by p53 and others [Ito et al., 2002a]. It seems that activators although bound to different Mediator subunits can transmit a signal within the complex from their respective point of interaction to the Mediator tail domain. This the-

ory is supported by expression profiling in yeast. This report shows that MED2 and MED18, both subunits of the tail, function downstream of the Cdk8 module [van de Peppel et al., 2005]. Interpreting the expression profile shows that med2Δ is completely dominant over cdk8Δ of cyccΔ, with no additional effects observed when deleting the Cdk8 subunit in med2Δ. Phosphorylation of MED2 was proposed as mechanism for the negative regulatory pathway within Mediator. Indeed, it was shown that MED2 can be phosphorylated *in vitro* [Hallberg et al., 2004] and *in vivo* [van de Peppel et al., 2005] by Cdk8. Furthermore, functional antagonism between the Cdk8 module and the tail (Med15, Med2, Med3), head (Med20, Med18) and middle (Med31) could be shown by expression profiling [van de Peppel et al., 2005]. Which is an indication that different subunits within the same complex can have opposite roles on the same genes. Neither MED2 nor MED3 have homologs in the mammalian Mediator and in turn, neither MED23 nor MED24 have a homologue in the yeast Mediator. Nevertheless, all of this subunits are located in the tail region of yeast and human Mediator, respectively and play a role in activated transcription. One might ask whether these subunits are at least the functional homologs of each other.

It seems reasonable to propose that the transcriptionally active A-Med complex contains the submodule formed by MED24, MED23 and MED16. Indeed, when using the MED23 antibody in ChIP assays, a strong accumulation of MED23 on the Vβ8.1 and CMV promoters was observed. One can think of two possible scenarios: 1) A large mediator complex is recruited to the promoter and gets subsequentially remodeled or 2) a smaller Mediator complex lacking the activating tail module that gets recruited independently of the complex. To answer this question a short-term ChIP kinetics was applied, resolving the first five minutes after VP16 activation (Fig.16). Even after this short time points following transcription induction it was not possible to dissect whether a complex or the different submodules were recruited. This might be due to the limitations of ChIP experiments. In ChIP, one always monitors the steady-state levels and not the transition-state levels that are of transient nature. To investigate this transition-states an *in vitro* assay is a more powerful tool. In an immobilized template assay a large Mediator complex (containing Cdk8) is recruited by VP16 to the MRG5 promoter *in vitro* (Fig.20), therefore one has to conclude that model 1 seems to be the more likely one.

During open complex formation in an immobilized template assay Mediator did change in its subunit composition (Fig.20). MED1 was one of the subunits that was lost. This is in support with the finding that VP16 activation does not lead to an accumulation of MED1 on the Vβ8.1 promoter nor on the CMV promoter seen in the ChIP assays. In recent publications, a sub-fraction of the CRSP complex (CRSP/Med2) [Taatjes and Tjian, 2004] could be purified missing MED1 and for p53 activated transcription a loss of MED1 could be demonstrated *in vitro* [Malik et al., 2005].

MED1 containing complexes are involved in activated transcription through nuclear hormone receptors. Knock out of MED1 leads to the loss of activation by nuclear hormone receptors such as TR and ER but not by VP16. Mediator complexes purified by MED1 are highly enriched in Pol II and interestingly, these complexes also contain MED25 [Xiaoting Zhang, 2005]. A question that arises is whether MED25 is found in all active Mediator complexes. There are many arguments against this hypothesis, among them that NTD only acts dominant negatively on VP16 but not on ER for example. Another argument against a general function of MED25 in transcription is that MED25 is only associated with a fraction of Mediator. However, the model gets some support from the ChIP result that shows VP16 dependent recruitment of MED23 *in vivo*, that forms the tail of Mediator which was shown to be crucial for all activators [Ito et al., 2002b]. Since the localization of MED25 within the complex is unknown it would be interesting to know whether MED25 contacts or influences the tail module. With regard to the low resolution EM structures for VP16 bound Mediator, it would be reasonable to predict the localization of MED25 in the head module of Mediator. However, given the structural flexibility of Mediator shown in these EM structures the presence of MED25 could introduce a Mediator conformation that allows for binding of the activating tail module. Since only VP16 makes direct contact to MED25 this could explain why only defects for VP16 activation could be observed when overexpressing NTD.

The main question remains how A-Med differs from other Mediator forms. One explanation might come from the observation that affinity purified Mediator with antibodies directed either against MED25 or MED15 copurify different accessory proteins as was shown by masspectromety [Stuehler Thomas, 2005]. It is striking

that in the MED25 IP many proteins involved in histone modification as well as RNA processing were copurified. These proteins were absent in the MED15 IP. In this light, it might be possible that A-Med establishes a link between the basal transcription machinery and histone modification as well as RNA processing.

4.5 The Cdk8 kinase module

Cdk8 is one of the best studied Mediator subunit, and several different models have been proposed for the mechanism of Cdk8 function. In mammalian cells, Cdk8 was shown to phosphorylate the CTD of the largest subunit of Pol II and thus activates transcription. Additionally, Cdk8 has a negative regulatory function by phosphorylation of the Cyclin H subunit of the general transcription factor TFIIH. This phosphorylation represses both the ability of TFIIH to activate transcription and its CTD kinase activity[Akoulitchev et al., 2000]. Cdk8 has also been implied in regulating the turnover of the Notch enhancer complex at target genes [Fryer, 2004]. Mastermind binds directly to Cdk8 and recruits it to the Notch receptor intracellular domain (ICD). Cdk8 promotes phosphorylation of Notch ICD that leads to PEST-dependent degradation and therefore to negative transcription regulation. A similar model was proposed in yeast, where Cdk8 (Srb10) inhibits filamentous growth in cells growing in rich medium by phosphorylation of Ste12 and thus decreasing its stability [Nelson C, 2003]. Positive roles for Cdk8 could be shown in yeast, where Cdk8 (Srb10) phosphorylates Sip4 during growth in non glucose media [Vincent et al., 2001]. Additionally, Cdk8 (Srb10) and Cdk7 (Kin28) were shown in yeast to have overlapping roles in promoting ATP-dependent dissociation of the preinitiation complex into the scaffold complex[Liu et al., 2004]. It was shown in mammalian cells as well as in *S.pombe* that Mediator binding to either Pol II CTD or to the kinase module is mutually exclusive [Naar, 1998, Spahr et al., 2000]. Studies of mammalian transcription showed that for some genes, Cdk8 is associated with inactive transcription complexes that have to be activated [Pavri et al., 2005]. PARP-1 was proposed to function as a specificity factor regulating the retinoic acid-induced switch of Mediator from the inactive to the active state in RAR-dependent transcription. However in a controversial study on the Egr1 promoter, no transition from inactive to active

Mediator was observed and Cdk8 localizes with the initiation complex of active genes [Wang et al., 2005].

In this work, the loss of the Cdk8 module could be shown *in vivo* (Fig.14) as well as *in vitro* (Fig.20). In ChIP experiments a loss of Cdk8 and MED13 was observed on the Vβ8.1 promoter after VP16 activation (Fig.14). The loss of the Cdk8 module upon VP16 activation *in vivo* is in line with the idea of derepression of Mediator function and follows most likely a similar mechanism as seen *in vitro*. This seems to be a fast process, Cdk8 was lost on the relatively weak Vβ8.1 promoter within the first 5 minutes after induction, on the strong CMV promoter it is not even detectable. The controversy of the *in vivo* results reported might to some extend account to what promoter was used for these investigations. In immobilized template assays Mediator, Pol II and TFIIH are recruited in a VP16 dependent manner to the promoter. Depletion of MED25 from a nuclear extract (ΔMED25) lead to a loss of VP16-dependent Mediator binding. This again underlines the crucial function of MED25 in VP16 activated transcription. In the absence of nucleotides, the recruited Mediator complex contained the Cdk8 module as well as all of the tested subunits; MED1, MED7 and MED25. In the presence of nucleotides however, a loss of Cdk8 and MED1 was observed. The other two Mediator subunits tested were less affected. The remaining of Mediator on the promoter is in line with studies in yeast that show the formation of a scaffold complex after transcription initiation that facilitates transcription reinitiation. Addition of ATP or a nucleotide mix renders the assembled preinitiation complex into the open complex form and Pol II can start transcribing. Therefore the observed loss of Pol II on the immobilized template is expected. In our analysis, the loss of Cdk8 and Pol II is dependent on a kinase, since the kinase inhibitor H8 is able to completely block both the loss of Cdk8 and Pol II (Fig.21). Whereas most of the phosphorylated proteins remained on the template, only few proteins were recovered from the supernatant. The most prominent one forms a double band at around 50 kDa that could be Cdk8. The loss of MED1 however is not blocked by H8, and is therefore probably not phosphorylation dependent.

Previously, A-Med was defined as a MED25 containing Mediator complex that is crucial for VP16-driven transcription activation *in vivo* and *in vitro*. Summarizing

these results here, a refined definition can be made. Active A-Med does not contain the Cdk8 module as was shown *in vivo* and *in vitro*. The ChIP experiments implied a fast dissociation of the kinase, only on the preactivated Vβ8.1 with its prebound Mediator complex a dissociation of Cdk8 could be shown but not on the CMV promoter. The dissociation of Cdk8 together with Pol II is dependent on nucleotides and can be blocked by the kinase inhibitor H8. Phosphorylation alone leads to open complex formation and the dissociation of Pol II but not of Cdk8 from Mediator.

4.6 VP16 effects on chromatin

VP16 binding let to the recruitment of the two histone acetyltransferases CBP and GCN5, the catalytic subunit of the human STAGA complex (Fig.17). This was observed on both, the Vβ8.1 and the CMV promoter. Surprisingly only moderate hyperacetylation of either histone H3K9 or histone H4 on both promoters as well as in the coding region was seen. One explanation might be that nucleosomes are already modified in the non activated state on the EBV reporters. These EBV vectors are basically nothing else than mini EBV episomes and it was shown for wild type as well as exogenous EBV episomes that their histones are modified [Chau CM. and Lieberman PM., 2004, Zhou J., 2005] [Chau CM. and Lieberman PM., 2004, Zhou J., 2005]. These modifications include histone acetylation and methylation and were shown to spread several kilo bases up- and downstream of the oriP region. The only region having less modifications on the episomes is the oriP region, which seems be looser packed by histones for better accessibility during replication [Chau CM. and Lieberman PM., 2004, Zhou J., 2005]. For a role of CBP argues the direct interaction with MED25 as was shown in an *in vitro* GST-pull down assay (Fig.25). This interaction with the ACID domain of MED25 is apparently possible through both the N- and C-terminal parts of CBP. Both VP16 and CBP interact through the ACID domain with MED25. It would be interesting to see whether this is a concerted interaction between the three partners. The H1 and H2 region of VP16 function through distinct pathways. The H1 region can function through Mediator and general transcription factors whereas the H2 requires CBP [Ikeda et al., 2002]. A second explanation for the

moderate hyperacetylation observed might be due to enhanced levels of HDACs on the promoter. Preliminary data suggest enhanced levels of HDAC1 and 2 on the Vβ promoter. If this would be the case, the net effect could result in only moderate hyperacetylation of either histone H3K9 or histone H4.

Certain histone methylation marker are correlated to actively transcribed genes [Jenuwein and Allis, 2001, Kouzarides, 2002]. These modifications are namely, histone H3K4 and histone H3K79, each existing in a mono-, di- or trimethylated form. Like the antibodies directed against acetylated histones, the histone H3K4 trimethylation did only moderately change upon VP16 activation. Again, this specific modification is already found in wild type EBV episomes to spread over a large distance [Chau CM. and Lieberman PM., 2004]. However the dimethyl modification of histone H3K79 showed a strong response to VP16 activation. The only known histone methyl transferase (HMTase) known responsible for this modification is Dot1L. Interestingly, Dot1L was among the peptides that coprecipitated specifically in a MED25 IP. Unfortunately, Dot1L escaped a direct identification in western blot analysis due to the lack of sensitive antibodies. To date, there is one publication that links Dot1L to leukemogenesis via MLL-AF10 [Okada, 2005]. However how Dot1L is targeted to active promoters is still unknown. An association with Mediator would nicely explain the correlation of histone H3K79 methylation with active genes.

4.7 A link of transcription to RNA processing via Mediator?

Affinity purified A-Med with an antibody directed against MED25 coprecipitates different accessory proteins as was shown by mass spectrometry [Stühler T., 2006]. Some of these proteins are involved in RNA processing. Namely all the subunits of the cleavage and polyadenylation complex (CPSF) and the cleavage stimulatory factor (CstF) were copurified in the MED25 IP. These data have not yet been confirmed by additional biochemical data. However in ChIP experiments members of both complexes could be detected at the model promoters after VP16 activation. Strikingly, CPSF1 and CstF64 were the only two proteins monitored, besides PC4, that arrived relatively late at the promoters. They were only

detectable 5 to 30 minutes after activation, whereas the other components of the general transcription machinery arrived within the first 5 minutes.

In the last years, numerous studies have indicated that transcription of mRNA precursors and subsequent processing (i.e. capping, splicing and polyadenylation) are closely connected and many reports support the idea that the CTD of Pol II coordinates these processing steps (reviewed in [Hirose, 2000, Proudfoot, 2002, Zorio, 2004]). Polyadenylation of mRNA precursors is a two-step reaction involving endonucleolytic cleavage of the pre-mRNA and synthesis of the poly(A) tail. In mammalian cells, the component of the polyadenylation complex include CPSF which recognizes the AAUAAA signal; CstF which bind to a GU-rich sequence downstream of the cleavage site and two cleavage factors CF I and CF II as well as poly(A) polymerase (PAP). In a yeast two hybrid screen CstF-64 was shown to interact with PC4 [Calvo O., 2001]. The interaction of this two proteins proofed to be evolutionarily conserved between yeast and man and providing evidence that this is important for efficient transcription elongation. Furthermore, Sub1 and Rna15, the yeast homologs of PC4 and CstF-64, respectively were shown to be recruited to promoters and are present along the length of several yeast genes [Calvo O., 2005].

Of interest here in this study is not only the fact that members of the 3‘ end formation complexes were found at a mammalian promoter but also the fact that they are recruited timely correlated with PC4. This finding provides evidence for a conserved mode of action of PC4 and CstF-64 between yeast and man. It is noteworthy that only after the recruitment of PC4 and CstF-64 increased levels of luciferase mRNA could be detected (Fig.18). Of additional interest is the circumstance that CPSF and CstF are coprecipitated by A-Med. This might indicate a role for A-Med not only in transcription initiation but also in efficient elongation or RNA processing by recruitment of CPSF and CstF.

4.8 Subcellular localization of MED25

MED25 is a newly characterized Mediator subunit that contains an N-terminal Mediator binding domain with homologies to the Von-Willebrand factor A domain

(VWA) and a novel C-terminal activator interaction domain (ACID). Only one other protein is known to carry an ACID domain, PTOV-1 (prostate tumor overexpressed protein 1). PTOV-1 consists of two repeated ACID domains (Fig.24A). The gene encoding PTOV-1 lies just adjacent to the MED25 gene on chromosome 19q13.33. PTOV-1 was not only shown to be overexpressed in prostate tumor cells but was also shown to shuttle between the cytoplasm and the nucleus in a cell-cycle dependent manner [Santamaria A., 2003]. Flotillin-1, a major lipid raft protein, gets translocated to the nucleus together with PTOV-1[Santamaria A., 2005]. Both proteins have a mitogenic activity and seem to act together. Interestingly, deletion of PTOV-1 inhibits cell proliferation, whereas overexpression leads to strongly induced cell proliferation [Santamaria A., 2005].

As mentioned, PTOV-1 consists of two repeated ACID domains, that we characterized as an activator binding domain [Mittler et al., 2003]. Overexpression of PTOV-1 brings additional ACID domains into a cell that could compete with MED25 for activators to bind. This competition could impair cellcycle regulation and lead to the increased cell proliferation that is observed.

Figure 30: Sequence alignment of human ACID domains from MED25 (ACID1) or PTOV-1 (ACID2.1 and ACID2.2). Amino acids 20 to 29 of the MED25 ACID domain are partially deleted in the PTOV-1 ACID domains. Conserved regions are boxed in gray.

It seems possible that the ACID domain is not only a novel protein-protein interac-

tion domain but is also responsible for the cell-cycle dependent nuclear translocation of PTOV-1. To test whether MED25 also changes its cellular distribution, nuclear extracts of synchronized cells were monitored in Western blots for MED25 levels. However no change in MED25 protein levels were observed during the cell cycle (Fig.24B). A predominant nuclear localization was observed using confocal immunofluorescence microscopy where the localization of overexpressed flag-MED25 was analyzed (Fig.23). Despite the homology of the ACID domains, MED25 does not seem to have a cytoplasmic function like PTOV-1 and it localizes to the nucleus at all times. The reason for this might be in the ACID domain itself. There is a stretch of 10 amino acids in the ACID domain of MED25 that is partially deleted in the ACID domains of PTOV-1 (Fig.30). This region is not predicted to form strand or helical secondary structure but might nevertheless account for a specific function. Even though preliminary interaction studies indicate binding of VP16 to all three ACID domains, the binding to PTOV-1 derived domains seems to be less specific (data not shown). An other reason for the different behavior of MED25 and PTOV-1 might be the duplication of the ACID domain. This hypothesis could be tested by generating a MED25 mutant carrying a duplicated ACID domain or the PTOV-1 derived ACID domains. In such an experiment it would be interesting to see whether the localization or the specificity of the mutant MED25 is changed.

To conclude, the ACID domain of MED25 is not able to confer cell-cycle dependent translocation of MED25 from the cytoplasm to the nucleus. However the localization of MED25 is nuclear which might be due to the ACID domain. Whether the cycling of the PTOV-1 protein depends on slight differences within the ACID domain or in the duplication of the ACID domain remains unclear. An intriguing model would be that the ACID domain is responsible for the transport to the nucleus, where MED25, in contrast to PTOV-1, gets retained by its ability to bind to Mediator.

4.9 Conclusion

To summarize the emerging picture of VP16 activation through A-Med function the following working model can be drawn. VP16-dependent activation is mediated by A-Med, a complex lacking the Cdk8 module as well as the MED1 subunit. However A-Med contains the MED16/MED23/MED24 tail module. From *in vitro* data we can draw the conclusion that a large Mediator, probably TRAP or a TRAP-like complex gets recruited by VP16. The question remains whether the MED16/MED23/MED24 module is present in the recruited complex or if they get integrated separately. During open complex formation MED1 and the Cdk8 module are lost from the complex in a phosphorylation dependent manner to form an active A-Med complex. In this, Mediator shows not only a conformational flexibility but also a dynamic nature of subunit composition. In which probably its capacity as a processor of diverse signals that act on him in the form of activators, repressors and coactivators lies.

In addition A-Med can act as a central control panel and integrate several additional cofactors into the transcription complex before Pol II transcription finally starts. CBP can be directly recruited by Mediator through the ACID domain of MED25. Additionally, A-Med can target Dot1L the histone methyltransferase responsible for H3K79 methylation that is correlated to all actively transcribed gens. Finally, A-Med binds to the polyadenylation factors CPSF and CstF and targets these factors to the promoter where they get loaded onto the CTD of Pol II. Taken together, A-Med seems to integrate activities used for histone modification, transcription, polyadenylation and termination.

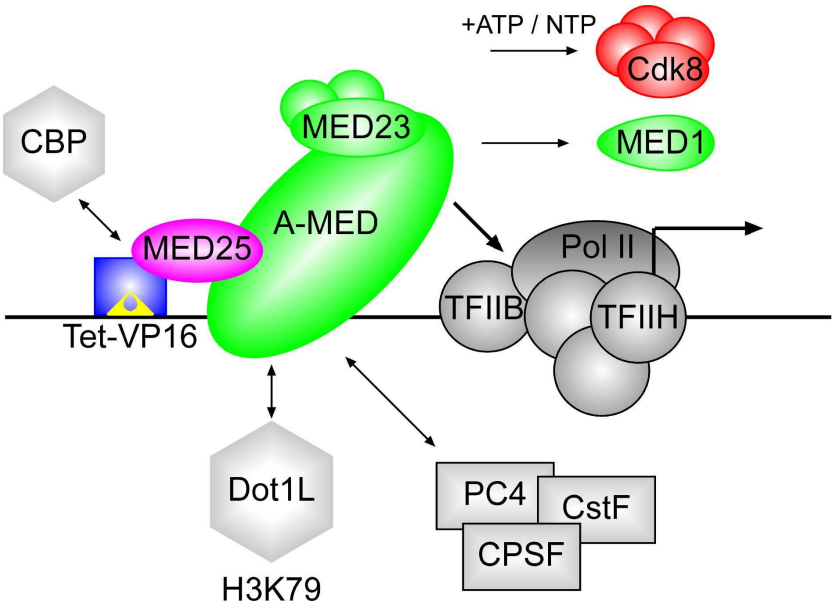

Figure 31: Model of VP16 activation and A-Med function. VP16 recruits A-Med through the ACID domain of MED25. A-Med contains the activating tail module formed by MED16/MED23/MED24 but looses MED1 and the Cdk8 module during open complex formation. The loss of the Cdk8 module phosphorylation dependent. In addition, A-Med can interact with further transcription coactivators like CBP, Dot1L, CPSF and CstF. Together A-Med forms a scaffold that combines activities required for histone modification, transcription initiation and RNA polyadenylation.

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