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masterthesis Infection and Immunity

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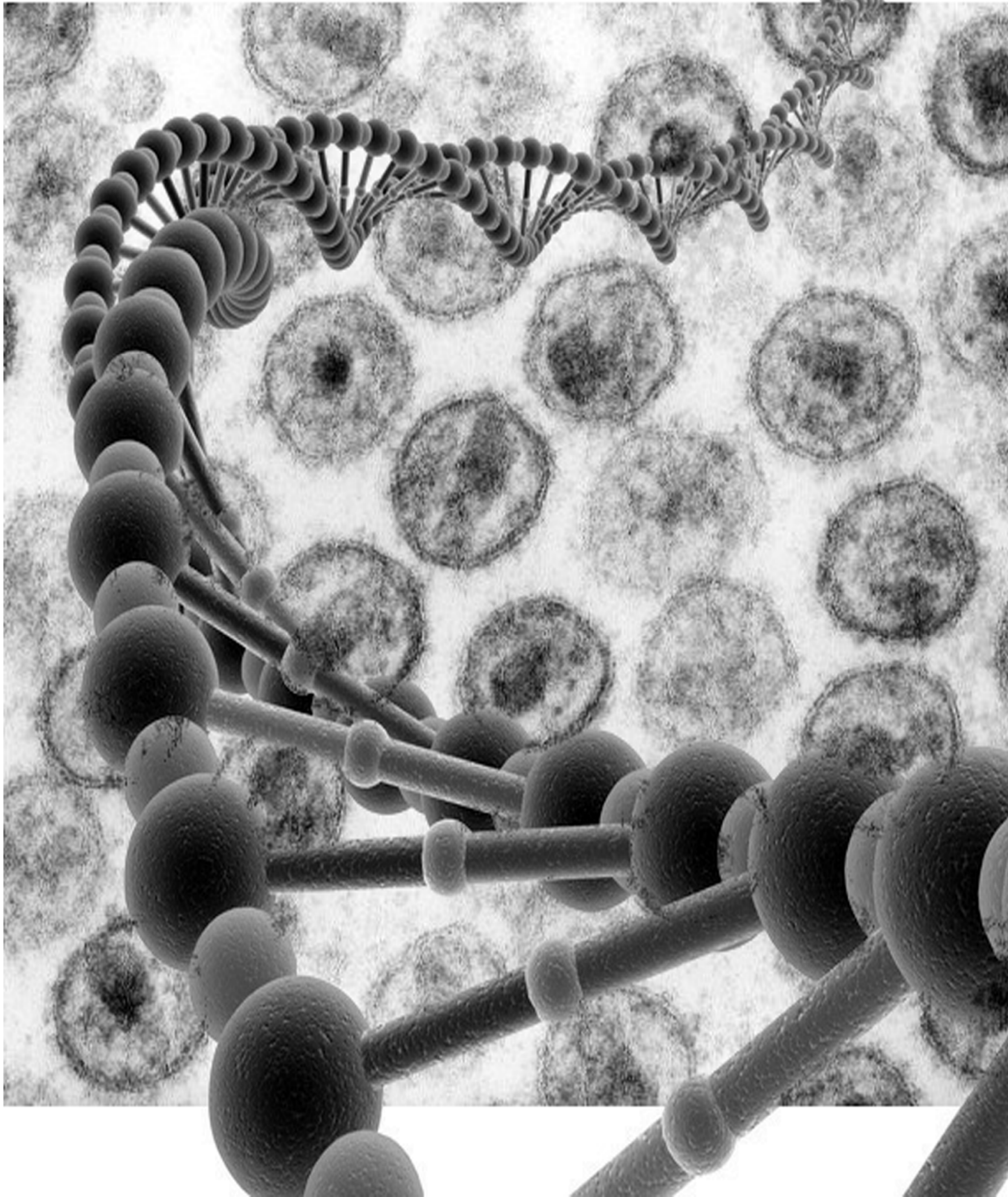

The molecular mechanisms of latency and reactivation in CD4$^+$ T cells during HIV infection

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Written under supervision of L.M van den Berg, MSc and Prof. dr. T.B. Geijtenbeek

List of abbreviations

AIDS - aquired immunodeficiency syndrome
HIV - human immunodeficiency virus
HAART - highly active antiretroviral therapy
LTR - long terminal repeat
NC - nucleocapsid protein
CA - capsid protein
MA - matrix protein
Env - envelope protein
RT - reverse transcriptase
IN - integrase
Pol - polymerase
CCR5 - C-C chemokine receptor 5
CXCR4 - C-X-C chemokine receptor 4
gp120 - glycoprotein 120
RTC - reverse transcription complex
PIC - preintegration complex
GCN5 - general control of amino acids 5
LEDGF/p75 - lens-derived-epithelium growth factor/p75
RNAPII - RNA polymerase II
TFII - general transcription factors
TBP - TATA-binding protein
TAR - transactivation reactivation element
P-TEFb - positive-transcription elongation factor b
Tc1 - cyclin T1
Cdk9 - cyclin dependent kinase 9
P/CAF - p300/CREB binding protein-associated factor
NELF - negative elongation factor complex
DSIF - DRB sensitive initiation factor
HAT - histone acetyl transferase
HDAC - histone deacetylation complex
TSA - trichostatin A
NFκB - nuclear factor κB
CBF-1 - C-promoter binding factor 1
LSF - late simian virus 40 transcription factor
YY1 - yin yang 1
Sp1 - specificity protein 1
AP-4 - adaptor protein 4
Suv39H1 - suppressor of variegation 3–9 homolog 1
HP1 - heterochromatin protein 1
TNFα - tumour necrosis factor α
SWI/SNF complex - switch/sucrose non-fermentable complex
Ini1 - Integrase Interactor 1
NFAT - nuclear factor in activated T cells
IκB - inhibitor of κB
CBP - CREB-binding protein

The molecular mechanisms of latency and reactivation in $CD4^+$ T cells during HIV infection

Aids has become the leading cause of death world-wide. Much research has been done on the causal agent of this disease, the human immunodeficiency virus (HIV). HIV infection in the western world no longer inevitably precedes the disease AIDS, due to the development of a highly active antiretroviral therapy (HAART). However, curing HIV-infected patients seems to be still impossible since several viral reservoirs are not affected by HAART. Replication competent HIV in latently-infected $CD4^+$ memory T cells forms currently the best–characterized long term reservoir of HIV in patients. For eradication of this viral reservoir reactivation of the viral life cycle is essential. Consequently, the current research goal is reactivation of the latent reservoir and thereby eradication of latent virus. In this thesis, recent literature on the molecular mechanisms of HIV latency and reactivation in $CD4^+$ memory T cells will be discussed. Profound understanding of the molecular mechanisms will hopefully lead to the development of more adequate therapeutics to cure HIV infection.

1 - Introduction

In 1981, the first patient with acquired immunodeficiency syndrome (AIDS) was described(1, 2). The disease was characterized by patients suffering from many opportunistic infections, caused by depletion of the $CD4^+$ T cell compartment. In 1983 the first isolations of the causal agent human immunodeficiency virus (HIV) were successfully performed(3). Subsequently, research on the causal agent could explain the clinical manifestations of the disease.

Pathogenesis

The human immune response upon encountering HIV includes generation of virus-specific $CD8^+$ T cells and neutralizing antibodies. A mild acute infection-caused disease period includes fever, malaise and swollen lymph nodes. After this short period of acute infection a transition towards an asymptomatic chronic phase occurs. How long this stage lasts depends on treatment, the phenotype of the virus and host response. When untreated, the constant production of high numbers of T cells during this phase will continue(4). The reason for the subsequent T cell depletion is still under debate at this moment. Currently, the hypothesis that depletion is caused by chronic T cell activation is favoured(5-7). Treatment will diminish T cell production, although major variations in treatment responses exist among individuals. Finally, the viral load increases and HIV starts to suppress the immune response towards itself and other pathogens. In this phase of the disease, the patient begins to suffer from opportunistic infections and has developed AIDS(4). The mean surviving time of untreated patients after the onset of AIDS is 9-13 months(8).

Research on HIV has led to the development of a highly active antiretroviral therapy (HAART)(7). HAART combines at least three antiviral drugs that each affect a different step in the viral life cycle, thereby reducing the change on drugs resistance(6). It is necessary to reduce this risk since HIV replicates at high rate and its reverse transcription has low fidelity. Furthermore, the ability to recombine during replication is very high, which in combination with the previous named characteristics of HIV leads to the presence of a highly heterogenic virus population in every infected individual. HAART decreases the mortality

rate due to AIDS(9, 10). Currently, HIV-infected individuals receiving HAART do not, or in a late stage, develop AIDS. This development has changed AIDS from a fatal disease into a chronic infection with HIV. Unfortunately, eradication of the virus from the patient seems impossible at this moment, due to viral reservoirs in the infected individual that are not sensitive to HAART.

Genome structure

HIV is a positive-stranded RNA membrane-coated retrovirus. Its virion contains two RNA molecules, stabilized by nucleocapsid proteins (NC) and surrounded by a typical cone-shaped core of capsid proteins (CA). Matrix proteins (MA) surround the core, associated to a host-derived membrane, containing both host proteins and viral envelope (Env) proteins, as is shown in Fig. 1. Within the virion, at least the viral proteins reverse transcriptase (RT), integrase (IN), protease and Vpr together with several cellular proteins are present(11). The genome of the virus consists of nine open reading frames which encode the Gag, Polymerase and Envelope (Env) structural polyproteins and regulatory proteins, as is shown in Fig. 1. Different mRNA products are generated by posttranscriptional mechanisms like several splicing steps. Moreover, translational mechanisms like read-through and frame-shifting lead to an even higher variability of protein products. Currently, two types of HIV are known, HIV-1 and HIV-2, of which HIV-1 is pandemic. HIV-1 is divided into three subgroups, which are again split into at least nine subtypes. The dominating subtype worldwide is HIV-1 C(7). Unless otherwise stated, the herein called HIV refers to this subtype.

The life cycle of HIV

HIV is a complex retrovirus, and each step in the viral life cycle consists of a fragile balance between evasion of the antiviral immune response and continuous progression to the next phase. The antiviral therapy HAART interferes with these different life cycle steps, thereby impairing progression of viral infection (Fig.2). Combining entry and fusion inhibitors, integrase inhibitors, reverse transcriptase inhibitors and protease inhibitors, it blocks the below described viral life

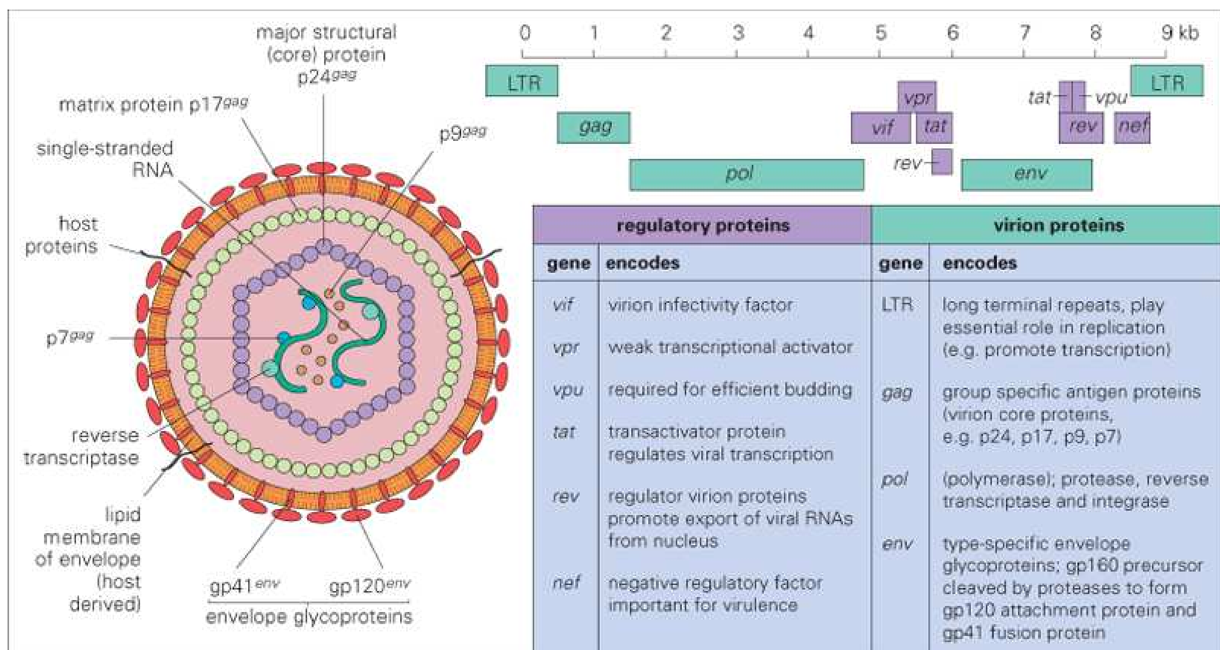

Figure 1 - Virion and genome structure of HIV particle

The virion structure contains the major structural (core) protein or capsid protein, the matrix proteins, RNA, host proteins, the nucleocapsid proteins, P7 gag, reverse transcriptase, envelope proteins and the p9 gag protein. On the right site the HIV genome is schematically represented. The three levels of depicted genes reflect the different splicing products. The proteins encoded by the presented genes are shortly described in the box.

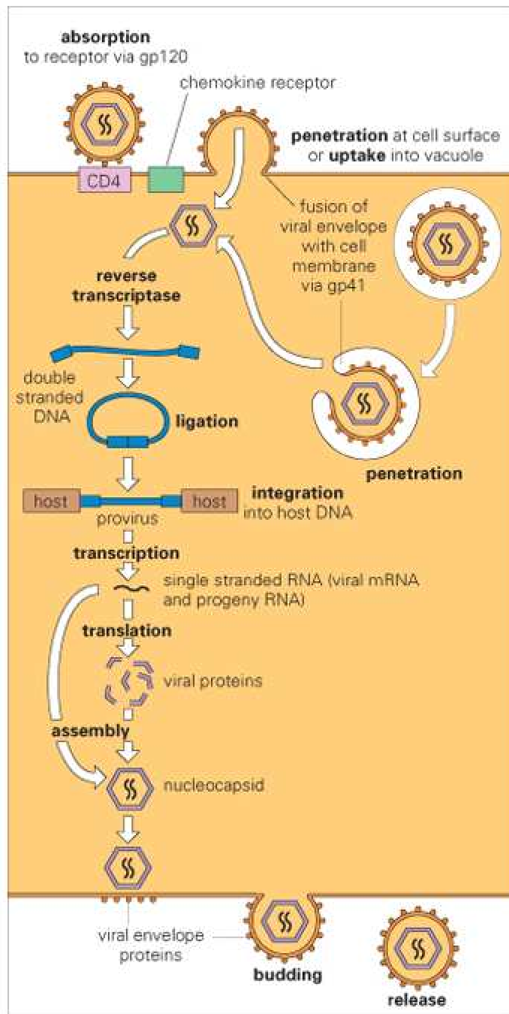

Figure 2 - The life cycle of HIV

The viral life cycle concerns the following steps: entry, reverse transcription, ligation, integration, transcription and translation and finally the assembly of new virions. New virions budd off from the host cell. Envelope protein gp120 binds to entry receptor CD4 and a chemokine co-receptor which is known as absorption. The alternative pathway is uptake via the endosomal route, which is depicted next to the plasma membrane fusion route. When the virus has entered the cell, reverse transcription by the viral enzyme reverse transcriptase takes place. Next, the virus is integrated into the host genome. Both processes are facilitated by the viral integrase protein. The transcription is mainly performed by the cellular transcription complex, although the presence of the viral transactivator catalyses the process. The final assembly of the viral proteins on the plasma membrane triggers the release of the virion. The infective particles released are then able to enter another cell and start a new viral life cycle.

Figure from Goering *et al.* (2008)(4)

stages and consequently eradicates viral infection. Currently, there are thirteen drugs available that block transcription and nine that target the viral protease and thus maturation. Viral entry, fusion and integration can be impaired by one available drug each(12, 13). The sensitivity of cells to HAART depends on the activity of the virus.

The viral protease

HAART consists often of a protease inhibitor in combination with two or more of the other classes of drugs. Protease inhibitors block activity of the viral protease, a key protein of HIV which acts in several phases of the viral life cycle. It is transcribed as polyprotein Gag-Pol, which also contains the reverse transcriptase and integrase domains. The protease is active as homodimer, and able to release itself from the Gag-Pol precursor via autocatalytic processing. The enzyme is indispensible within cytoplasm and virion, since the protein is required to activate the polyprotein domains via proteolytic processing, releasing the domains as separate proteins(14).

Entry

With HAART, entry can be blocked by the class of entry and fusion inhibitors. Entry is an important step in the viral life cycle of a virus, since preference for a certain receptor generally determines the tropism of the virus. For instance, cells that can be infected by HIV carry CD4 and one of the chemokine co-receptors C-C chemokine receptor 5 (CCR5) or C-X-C chemokine receptor 4 (CXCR4). These include T cells, macrophages and dendritic cells. The interaction of viral glycoprotein 120 (gp120) with its host receptor CD4 mediates not only attachment, but also primes the T cell for infection by the induction of signalling that activates the cell and probably further currently unknown processes(15, 16). Although CD4 is the main receptor for entry, the process of entry is suboptimal without the co-receptors CCR5 or CXCR4(17-21). During infection the tropism of HIV changes, but the first infection is always due to a CCR5-tropic strain. What the function is of the change of co-receptor preference is not clear, although a change in host cell type has been suggested(22). The viral receptor gp120 is transcribed as polyprotein Env. Env is divided into two glycoproteins through proteolytic processing by the viral protease. The glycoproteins gp120 and

gp41 form heterodimers, which are grouped as trimers on the membrane of the virus particle and can bind to the cellular receptors via its gp120 subunit. After binding to the cellular receptors, the proteins undergo conformational changes, which finally lead to membrane fusion. The exact conformation of this complex during the several stages of entry is still under debate(23, 24), but forms a putative important drugs target for more entry inhibitors.

HIV probably uses different mechanisms to enter susceptible CD4$^+$ T cells. It is generally believed that both plasma membrane fusion and endocytosis with subsequently endosomal membrane fusion can take place, although it is still controversial which mechanism the virus relies on *in vivo*(25). Conflicting results has been reported on the question whether fusion occurs at the plasma membrane or at the endosomal membrane. There have been several reports that showed reliance of HIV on direct fusion with the plasma membrane, supported by several different arguments. One of the major observations that argue for the reliance of HIV on plasma membrane fusion is that entry is not pH-dependent(26), suggesting that the endocytic pathway is not involved. Second, internalization of the receptor is not necessary for virus entry(27). This suggests that the virus does not rely on endosomal entry. On the other hand, endocytosis of the virus has never been convincingly excluded. pH-independency does not exclude the possibility of fusion with the endosomal membrane after endocytosis, since there might be another mechanism that triggers fusion of the virion with the endosomal membrane. The non-necessity of receptor internalization does not prove that it does not occur. Increasing the confusion, both endosomal and plasmamembrane localization of virus particles has been shown in microscopy experiments(28, 29). Recently, Miyauchi *et al*. showed via life-cell imaging that successful entry of HIV is mainly dependent on the endosomal route(30). These authors suggested that viral content of viral particles that are fused with the plasma membrane do not traffic through the cytoplasm. Although the life cell imaging experiments are quite sophisticated, Yu *et al.* questioned whether the biological confirmation of the microscopy data has been correctly interpreted(31). Miyauchi *et al.* treated the cells with dynamin, an inhibitor of endosomal fusion, to confirm that the block in cytoplasmic trafficking revealed by the microscopy data is induced by endosomal entry. They detected a complete absence of viral entry. Yu *et al.* could not repeat the experiment with WT-HIV, although entry of the VSV-G pseudotyped virus (of which it is confirmed that entry is dependent on the endosomal route) was completely blocked. These conflicting data show that the exact mechanism by which HIV is able to enter cells is still under investigation. As a third mechanism of entry, HIV mediates cell fusion of CD4$^+$ T cells with bystander cells, generating multinucleated syncytia of lymphocytes(32, 33). The CXCR4-tropic strain of HIV generates more syncytia and interaction of Env with CD4 seems required for this type of entry(34, 35). In conclusion, mechanisms of HIV entry are not completely revealed. A better understanding of these processes will probably lead to discovery of novel targets for inhibitors.

Reverse transcription

When entering a host cell, the virus must be reverse transcribed and uncoated. Uncoating occurs by currently unknown mechanisms, after or during which reverse transcription takes place(11). Reverse transcription is a process in which the RNA genome of the virus is transcribed into a DNA equivalent, that is primed for integration into the host genome. The enzyme that transcribes the HIV RNA genome into DNA is a viral protein, reverse transcriptase (RT). This enzyme can be blocked by two different classes of reverse transcriptase inhibitors, nucleoside and non-nucleoside inhibitors. The nucleoside reverse transcriptase inhibitors are analogues of naturally occurring nucleosides, but terminate transcription when they are incorporated in the DNA. Non-nucleoside inhibitors inhibit the function of reverse transcriptase itself by binding to the enzyme and blocking structural rearrangements of the protein domains physically(12, 13). Reverse transcriptase is an asymmetric heterodimer of p51 and p66. The catalytic subunit p66 consists of palm, thumb, finger and RNase H structure, similar to the cellular and prototypical *E.Coli* DNA and RNA polymerases. The p55 subunit is believed to have a structural

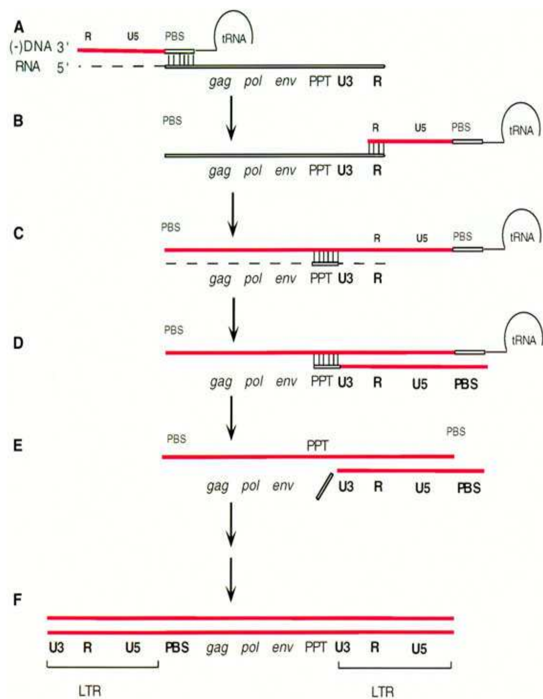

Figure 3 - Reverse transcription

Reverse transcription starts at the primerbinding site (PBS). A. First, the 5'- RNA end will be transcribed into DNA and the corresponding template RNA destroyed. B. The end of the newly produced DNA strand binds the 3'end of the viral RNA genome and a complete copy of the RNA strand is transcribed. C-E. One of the digested parts of the viral RNA genome functions as primer (PPT) for the positive strand synthesis. F. The double stranded DNA copy of the viral genome is ready for integration into the human genome.

Figure from Sarafianos *et al.* (2001)(40)

role only(36, 37).Within the virion, RT already displays enzymatic activity and is thus directly functional after delivery in the cytoplasm(38). RT shows, at least *in vitro*, poor processitivity and low fidelity, leading to high mutational rates and genetic diversity of HIV(39).

As described previously, RT and two single-stranded RNA molecules are present in the virion. The RNA molecules are paired by several regions and carry on the 5'side a specific lysine 3 tRNA primer molecule from cellular origin. This primer is necessary for the induction of RT activity. Reverse transcription starts by transcribing the last 5'part of the genome as is shown in Fig. 3. The RNase H domain of RT destructs the RNA genome that has been utilised as template. Transcription is often aborted at this point, producing only the small (-)5'end of viral DNA. When the process continues, the first template exchange take place. (-)5' DNA that has just been transcribed binds to the 3'end of the remaining RNA genome and facilitates transcription from this point until the moment that a minus DNA copy of the whole RNA genome is produced. The RNA genome is digested by the RNase H domain and a specific digested part serves as primer for the next step of positive DNA strand synthesis. All these processes have been described to occur simultaneously in infected cells, and a collection of different viral DNA products can be found(40, 41).

Although the process of reverse transcription is biochemically well-characterized, the reaction *in vivo* is located in a reverse transcription complex (RTC). The RTC is a protein complex that migrates from the plasma membrane to the nucleus. During migration reverse transcription occurs and the RTC changes its protein composition, maturating towards a pre-integration complex (PIC)(11, 42). Migration is targeted towards the nucleus by direct interactions of different RTC components with cellular proteins, for instance interaction of RT with actin filaments(11, 43, 44). The viral components RT, IN and NC are generally believed to be essential for function of the RTC. However, what the precise role of MA, CA, Nef, Tat, Vif and Vpr proteins during reverse transcription and maturation is, is still under debate(42). For instance, the role of CA proteins of the virion core in the processes of reverse transcription and nuclear import is controversial. According to one theory, the CA proteins are thought to deliver the virus into the cytoplasm and have no role in other processes(11). Another theory claims that the virus can be uncoated gradually, and be present and have a function during reverse transcription. A third option explains that uncoating occurs at the nuclear membrane, when the pre-integration complex (PIC) is generated. In this model, CA proteins stabilize the RTC during travelling to the nucleus(11). Another example of the conflicting data in this field are reports on the role of phosphorylation of the MA protein. On one hand is believed that phosphorylation of this protein forms switch for a chance in localisation, from membrane

attachment to a cytoplasmic localization(45). But on the other hand has been claimed that phosphorylation is not a localization signal(46). In contrast, the role of IN has been relatively well-defined. RT and IN interact directly with each other and presence of IN is indispensable for reverse transcription activity(47). Both initiation and elongation capacity of RT are enhanced in presence of IN and the overall processitivity increases(48). However, other groups have found that IN had no effect on RT processitivity, but is rather involved in strand transfer during reverse transcription(49). It has been suggested that the different results are due to different experimental conditions. However, both studies have reported an major effect of IN on the final outcome of RT activity and therefore, it is clear that the interaction between both proteins has an important function in the RTC. Cellular proteins that are contributing to the process of reverse transcription remain mainly unknown. Without cellular factors, the activity of the RTC is drastically diminished(50, 51). A recent overview of the cellular factors that are thought to be involved in RTC activity has been given in the review of Warrilow *et al.* (2009)(42). In conclusion, the complex process of reverse transcription *in vivo* remains to be elucidated.

Nuclear import and integration into the host genome

The unfinished RTC or PIC have to be imported into the nucleus. Nuclear transport is a regulated and energy-dependent process for large complexes like RTC and PIC and is therefore a major obstacle for the continuation of the viral life cycle. How import of HIV complexes takes place is still under debate. Early reports and some mutation studies found no role for IN(52, 53), but other studies did find a role for IN during import(54), and currently it is the favoured hypothesis that IN is involved in import. Furthermore, it is controversial whether cellular proteins like the below described LEDGF/p75 or nucleoporins, or viral proteins like MA do play a role(45, 52, 54, 55). The putative function of different players in the process of nuclear import and the discrepancies of the results of the experiments have been reviewed by Suzuki (2007)(55). Currently, nuclear import is no target of any drugs used in HAART.

Although it remains to be fully elucidated whether IN functions in the nuclear import of the PIC, it is generally accepted that the protein catalyses the integration of viral DNA in the host genome. The viral IN protein consists of three major domains: a N-terminal, a central and a C-terminal domain. The protein is generally believed to act in multimeric complexes(56, 57). The N-terminal domain is analogous to a cellular zinc finger domain, is involved in multimerization of IN and found to be indispensable for integration(58, 59). The highly conserved central domain is involved in the binding of IN to viral 3’end DNA(60, 61) and the unspecific DNA-binding properties of the C-terminal domain probably add stability to the PIC-chromatine complex. IN activity is regulated by the activity of two acetyltransferases: general control of amino acids 5 (GCN5) and p300. Acetylation stabilizes the interaction of IN with DNA, probably through recruitment of stabilizing host factors(62-64). Similarly to the reverse transcription reaction, the biochemical process of integration has been relatively well understood. Before integration happens, the viral DNA is processed at the 3’end by IN, where a few nucleotides are removed to create an overhanging 5’strand and an reactive 3’site. Subsequently, the reactive 3’strand interacts with the host DNA, thereby linking the viral 3’ strand to one of the strands of host DNA, a process called joining or strand transfer. Both actions are based on the same biochemically process. If joining is properly coordinated, each of both 3’ends of viral DNA act with a spacing of 5 nucleotides on the contemplated host DNA strand. Subsequent repair of the created gap is probably carried out by cellular gap-repair complexes(65). One of the developed integrase inhibitors has been approved by the FDA in 2007 for use in HAART(13). This approved novel drug inhibits the strand transfer capacity of IN via binding to the active site(66).

However, this process *in vivo* involves the action of two complex composites, the PIC and chromatin. The PIC is composed of remaining proteins of the RTC, like IN, and several cellular factors. Recently, there have been several attempts to identify the cellular proteins that are involved, but except for the Lens-derived-epithelium growth factor/p75 (LEDGF/p75), the results remained conflicting(62, 67). LEDGF/p75 was found to cooperate in the

integration process and its action is relatively well-understood. The cellular protein is an ubiquitously expressed transcription factor, implicated in apoptosis and growth processes(65). LEDGF/p75 consists of a DNA binding domain and a protein binding domain. It is generally accepted that IN has a direct interaction with the protein binding domain of LEDGF/p75(68). Interaction of LEDGF/p75 with IN seems to recruit cellular factors to the PIC, to affect the DNA binding capacity of IN and to target the virus to a specific site of integration site(69-71), that is preferably within transcriptional activated genes(72). Furthermore it has been reported that the interaction between LEDGF/p75 and IN was found to be essential for nuclear localization or import of the PIC(73). Interactions of viral proteins with IN has been suggested. For instance interaction of RT with IN, which was shown to increase the enzyme activity of IN(49). Recently, also the role of viral Rev(see Fig. 1) in the PIC has been questioned. Rev can be present before the viral genome is integrated into the host genome, since viral genes can be transcribed from not-integrated DNA(74). It has been shown that Rev interferes with the binding of IN and LEDGF/p75, and inhibited viral integration. Further research is needed to elucidate the precise function of Rev(75). An extensive description of the role of LEDGF/P75 in integration can be found in a review by Poeschla (2008)(65). Increased knowledge of the function of proteins that are involved in the integration complex will probably reveal novel drug targets that can be used in HAART.

Viral transcription and replication

HIV is for its transcription almost completely dependent on the host cellular transcription machinery. Upon HIV integration into the host genome, the LTR functions both as enhancer element and as promoter. Similar to human gene transcription, the RNA polymerase II (RNAPII), general transcription factors (TFII) and the TATA-binding protein (TBP) are involved in the transcription complex that functions in initiation of transcriptional activity. These factors are always present, but the transcription of HIV is prematurely aborted in absence of the viral transcriptional activator Tat. In absence of Tat, RNA transcripts of 55-59 nucleotides are produced, showing that presence of Tat primarily drives elongation of HIV transcription and that initiation of transcription does not require viral protein expression(76, 77). Currently, Tat is no target of any drugs used in HAART.

Tat is a viral transactivator that does not bind DNA, but contains an RNA-binding domain which associates to the RNA stem cell loop structure, a transactivation reactivation element (TAR) that is formed at the 5'side of all transcribed viral RNA. Tat interacts directly with the positive-transcription elongation factor b (P-TEFb), and recruits P-TEFB to the site where RNAPII is tethered. P-TEFb dissociates from its inhibitory element through direct interaction of Tat with cyclin T1 (Tc1), a subunit of P-TEFb(78, 79). P-TEFb increases the elongative activity of RNAPII via another subunit, the cyclin dependent serine kinase 9 (Cdk9), which hyperphosphorylates the c-terminal domain of RNAPII. Hyperphosphorylated RNAPII produces elongated transcripts, which is essential for successful HIV infection. Thus the key role of Tat is recruitment of host factors to stimulate transcriptional elongation. Further mechanisms and factors that are involved in this process has been described recently by Lenasi and Barboric (2010)(80) and by Bannwarth and Gatignol (2005)(81).

Tat itself is regulated by several posttranslational modifications, including phosphorylation, acetylation and methylation. Phosphorylation by protein kinase R occurs on three residues, namely serine 62, threonine 64 and serine 68 and enhances the affinity of Tat for TAR. It is suggested that this increased affinity for TAR stabilizes the Tat-P-TEFb interaction, promoting thereby phosphorylation of RNAPII(82). Transcriptional elongation can thus be stimulated by regulation of Tat via phosphorylation. Furthermore, Tat activity is regulated by acetylation. Lysine residues can be acetylated by acetyltransferases, proteins that transfer acetylgroups to lysine residue of the target protein. Tat can be acetylated on two sites within its functional protein domains, lysine 28 and lysine 50/51. Lysine 28 modification by acetyltransferases p300/CREB binding protein-associated factor (P/CAF) and p300 enhances the binding affinity of Tat for P-TEFb, and promotes thereby the

processive capacity of RNAPII and the recruitment of other transcription stimulating proteins(83, 84). Acetylation of lysine 50 by acetyl transferases p300 and GCN-5 leads to release of Tat from the TAR element and dissociation of Tc1, while it stimulates the interaction of Tat with P/CAF(85, 86). Although the binding of Tat to the TAR element is initially required for elongation, dissociation of the TAR element is necessary for further elongation(86-90). There have been some conflicting reports on the mechanism of regulation of Tat activity via acetylation, but it is generally accepted that acetylation of both lysine residues is required for optimal transcriptional activation(83). Acetylation of Tat stimulates HIV transcription. Several different theories on how acetylation of Tat affects its functionality have been described by Bannwarth and Gatignol(81). Methylation of Tat disrupts the interaction of Tat with Tc1, thereby inhibiting formation of the transcription elongative complex with P-TEFb, and thus affecting transcription negatively(91). Tat can be methylated on the arginine residues 52 and 53 by peptidyl arginine methyltransferases PRMT6 and Set7/9-KMT-7(91-93). Although methylation decreases the transcriptional activity of Tat, the stability of methylated Tat is enhanced relatively to not-methylated Tat, due to inhibition of proteosomal degradation(93). In conclusion, methylation decreases the activity of Tat, but promotes its presence. The precise function of suboptimal activated Tat remains to be elucidated. In general, Tat is one of the key proteins in the viral life cycle. However, there are no drugs against Tat available An activated T cell phenotype and thus the activation of integrated HIV are essential for its eradication. Therefore, the inhibition of Tat would probably not add to faster eradication of the virus from the patient's body.

Virus production

When transcription occurs due to expression of Tat, the virus starts to make viral proteins of which several are utilised for generation of new virus particles. Viral proteins are translated from viral mRNA by the cellular machinery. Gag-proteins are thought to induce generation of new virus particles(94). Gag-Pol proteins are produced by a regulated frameshift during translation, and the ratio of Gag:Gag-Pol 20:1 has been found to be decisive in assembling the protein components and RNA into the virion(95).Gag and Gag-Pol proteins are targeted to the cell membrane when they become posttranslationally modified by addition of a fatty acid chain, a myristoyl group. Retaining of modified Gag molecules at the plasmamembrane recruits other non-modified Gag and Gag-Pol molecules and the proteins selfassemble into a virion-like structure. The presence of viral RNA catalyses the reaction, probably through an interaction with Gag proteins(96). The polyproteins Gag and Gag-Pol are budded off from the infected cell and are during maturation of the virion proteolytically processed. The matrix proteins form a layer associated with the membrane, the capsid proteins selfassemble into the typical core structure and the nucleocapsid proteins remain connected to the viral RNA, as is shown in Fig. 1(14, 97). The function of Gag proteins during virion assembly has been reviewed by Hill (2005)(14). Upon maturation, the virion can infect cells and is ready for a new infection cycle.

Since antiviral drugs interfere with the viral life cycle, eradication of all virus in a patient receiving HAART requires actively replicating virus. Cells containing non-replicating replication competent HIV are therefore not sensitive for drugs and cannot be eradicated by HAART. However, these cells harbour HIV that is still able to replicate upon reactivation of transcription. In theory, one latently cell might, upon reactivation, infect the patient again. Currently, the scientific community is struggling with the existence of a latent reservoir in memory T cells and attempts to find novel methods to reactivate the viral life cycle in these cells. However, to properly induce reactivation of a provirus in a certain cell type, thorough knowledge of the mechanisms of latency in this cell type is required. Herein, we will discuss the most recent knowledge on the molecular mechanisms of latency and reactivation in HIV-infected memory $CD4^+$ T cells.

2 - The molecular mechanisms of HIV latency in $CD4^+$ T cells

Latency

The term latency covers two ways in which virus can be present in a cell, namely as integrated or

non-integrated virus. Non-integrated virus is labile and is thought to be degraded with a half life of one day(98). Several hindrances for integration in for example reverse transcription, translocation or formation of the pre-integration complex are detected in cells that harbor only non-integrated provirus. However, integration stabilizes the viral genome and the virus will last within the infected cell until this cell dies. Latent reservoirs exist in several cell types, including macrophages, dendritic cells and T cells. However, the best-characterized HIV reservoir exists within the memory $CD4^+$ T cell pool. This reservoir is especially important since these cells have an exceptional long life-span compared to other potential reservoirs.

Upon maturation and selection in the thymus, resting, naïve T cells travel through the body to encounter antigens presented by cells. Co-stimulatory signals are necessary for further maturation and activation. The majority of these activated T cells dies after eradication of the pathogen and infected cells, but a minor subset returns to a resting state and are called memory T cells. These cells will be reactivated when they encounter their specific pathogen again. Both memory T cells and naïve T cells are called resting or quiescent T cells, since massive transcription and translation do not occur, in contrast to activated T cells. However, the viral reservoir within the naïve T cell pool is of less importance in pathogenesis. These cells die either because they are activated and will produce virus that is susceptible for treatment, or within a few days if they are not activated(98). Memory T cells are therefore the most important T cell reservoir of integrated, latently present HIV. Although this reservoir is very small (less than 10^7 cells per patient) in comparison to the total T cell amount, it is believed that this cell compartment harbors integrated replication competent virus with a half live of 44 months(99-101). Even when patients are good responders to HAART and have no detectable virus load in their plasma for a long period, the virus could be reactivated from their memory T cell compartment(102). This means that patients have to continue treatment for very long times. Eradication of these infected cells is one of the key steps towards total eradication of HIV in a patient.

Although it is generally believed that resting memory T cells form an important reservoir in patients, it is still controversial how these cells become infected. Since HIV has a tropism for cells high in CD4, CXCR4 and CCR5 expression, it prefers activated T cells for entry. One theory explaining the infection of resting cells claims that only activated cells are infected, but that some of these cells become memory cells before viral transcription is boosted by Tat expression(103, 104). The other theory claims direct infection of resting memory T cells. Although early reports have suggested that viral integration in a infected cell in quiescent state is not possible at all(105, 106), some later reports claim that this inability to detect active viral integration was due to insensitivity of the detection methods and insufficient incubation time in the experimental setting(107-109). However, it is still controversial whether resting cells can be infected. It is possible that minor activation of the T cell is sufficient for HIV to integrate, but not for conversion towards an activated phenotype. Signalling via viral envelope receptor subunit gp120 and co-receptor CXCR4 has been reported to change the intracellular environment(15, 16, 35). This effect could be the minor activation that is required for efficient HIV integration. However, further research is required to elucidate the precise mechanism of infection of resting memory T cells.

During latency, HIV transcription is inhibited. Putative transcriptional blocks as the site of integration, transcriptional interference, miRNA, absence of Tat and epigenetics are all generally believed to affect efficiency of HIV transcription. The effect of the site of integration, transcriptional interference and miRNA on HIV transcriptional latency have been discussed in reviews from Colin and Lint (2009) and Lassen (2004)(110, 111). Here, we will focus on the role of Tat and epigenetics.

Absence of Tat in latency

The viral transactivator Tat plays a very important role in activation of transcription and its absence is crucial for latency. Several models to investigate latency are based on a defect in Tat-function, like mutations in Tat or mutations in the Tat-binding sites on the LTR, which highlight the important role of absence of Tat in latency(112, 113). Elongation

is mainly Tat-dependent, in absence of Tat premature abortive transcription can occur. In a latent state, HIV transcription is fully dependent on host transcription complexes and regulation by the chromatin environment. Repression of transcription, which occurs in the latent state is maintained by host transcription complexes like the negative elongation factor complex (NELF). NELF interacts with RNAPII via DRB sensitive initiation factor (DSIF), which enhances pausing of the polymerase and thereby inhibits elongation of transcription(114). Furthermore, the specific knockdown of NELF changes the chromatin environment, including stimulation of H4 acetylation(115). This is an indication that NELF regulates transcription not only via direct interaction and inhibition of RNAPII but also affects chromatine modification enzymes. Strikingly, the inhibiting factors NELF and DSIF are not dissociated from the activated complex upon Tat expression. DSIF is, like RNAPII, phosphorylated upon Tat recruitment and affects transcriptional elongation positively in its modified form(116). This highlights the importance of absence of Tat for the maintenance of latency in infected T cells.

Epigenetics

Transcriptional inactivation of a reproduction competent virus in the latent state requires inhibition of the promoter. The LTR of HIV can act as a strong promoter. However, when HIV is integrated into the human genome, the LTR becomes a weak promoter and can even be completely silenced, which is the case during latency.

DNA in a cell is stored in an efficient and organized way; DNA molecules are tightly wrapped around nucleosomes. A nucleosome is formed by two identical histon cores of each four different histone proteins, H2A, H2B, H3 and H4, from which the tails are subject to several modifications. These

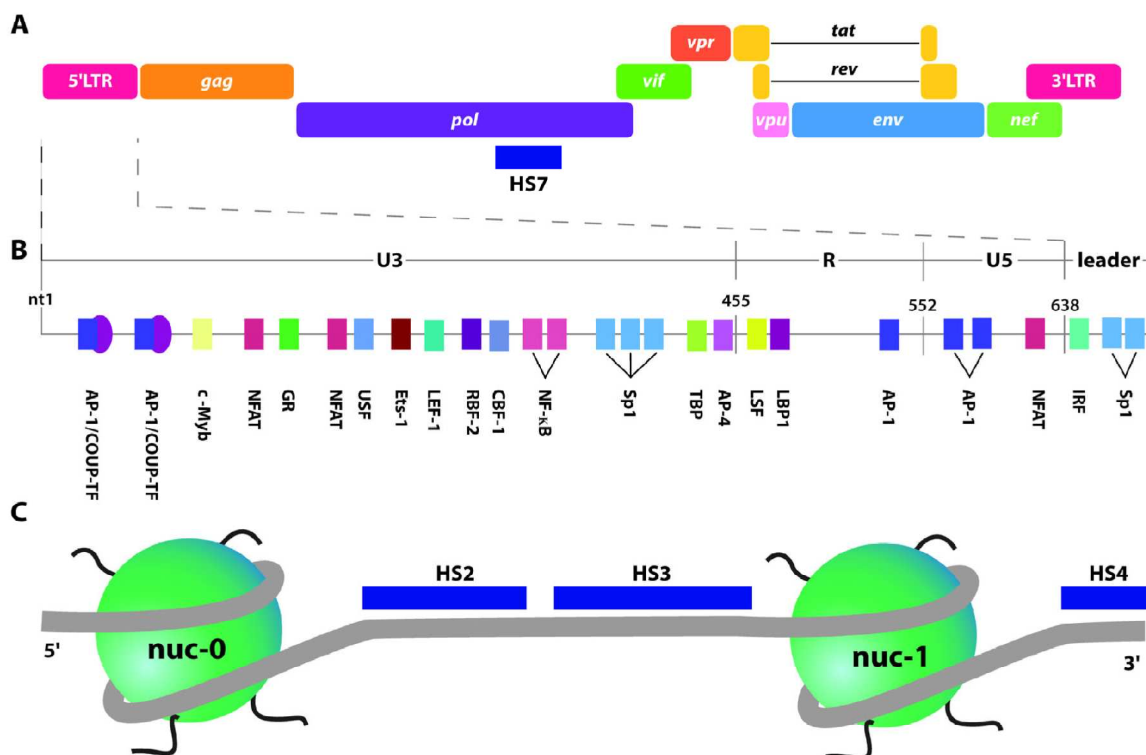

Figure 4 - The nucleosomal organization of the LTR

A. The genome of HIV consists of nine proviral genes, indicated by the collored boxes. The 5'LTR and 3'LTR are depicted at each final site. The hypersensitivity regions HS7 is depicted. B. The transcription factor binding sites in the 5'LTR are represented by the colored boxes. The site of transcriptional initiation is located at nucleotide 455 in this scheme. C. Schematic representation of the physical structure of the 5'LTR with nuc-0 and nuc-1. The hypersensitivity regions HS2, 3 and 4 are indicated.

Figure from Colin and van Lint (2009)(110)

modifications include phosphorylation, acetylation, ubiquitination and methylation. The effect of a specific modification is dependent on the position of the modified residue in the histone tail. Another major way of transcriptional regulation is ATP-dependent remodelling. HIV uses both in order to regulate its own transcription, since HIV is packaged into a host-like nucleosomal structure when integrated into the human genome. The five nucleosomes, called nuc-0, nuc-1, nuc-2, nuc-3, and nuc-4, have certain fixed positions on the integrated virus independent of the site of integration(117, 118). DNA hypersensitive regions (HS) in between nucleosomes are freely accessible for transcription factors(118). The most important nucleosomes considering transcriptional regulation of the HIV-genome are Nuc0 and Nuc1, localised at the enhancer region and the site of transcriptional initiation, respectively(117, 119). As is shown in Fig. 4, both nucleosomes are located at the site of several transcription factor binding sites. Nuc0 and Nu1 itself have been described to be target to modifications as acetylation and methylation(120). In conclusion, the nucleosomes play an important role in regulation of HIV transcription, including latency and reactivation.

Histone acetylation

In general, histone acetylation by so-called histone acetyl transferases (HATs) leads to transcriptional activation and deacetylation by histone deacetylation complexes (HDACs) to inhibition of transcriptional activity. Acetylation of positively charged lysine residues in the histone tail increases the negative charge of the lysine residue, thereby reducing affinity of the histone proteins for DNA and making the DNA more accessible for transcription factors. Deacetylation condenses DNA conformation, making promoters less accessible for transcriptional activators and hence silencing gene expression.

In a latent state, deacetylation of the subunits H3 and H4 plays an important role(121, 122). This was first established by the use of a potent HDAC inhibitor, trichostatin A (TSA), which leads to an increase in transcription by replacement of Nuc-1(119). Furthermore, the specific knockdown of HDACs also increased transcriptional activity, which highlights the importance of deacetylation in silencing HIV transcription(123). Since inhibition of HDACs leads to reactivation of latent provirus, the search to novel, non-toxic HDAC inhibitors is clinically relevant(124, 125).

Class I HDACs are recruited to the LTR by several cellular transcription factors. These transcription factors are themselves recruited to the LTR by the absence of stimulating signals. The inhibiting extracellular environment is thus required for the maintenance of a transcriptionally latent state of HIV(125). The inhibiting transcription factors interact with DNA and HDACs, consequently bringing HDACs in close proximity to their target histone proteins. Cellular processes in which these transcription factors are involved are for instance immunological activation, cell cycle, metabolic processes and apoptosis. Some of these factors are major players in signalling pathways like the MAP kinase and Notch pathway. Despite their different roles in the host cell, they all repress HIV transcription via HDAC recruitment. However, they use different mechanisms to bring HDACs to the LTR.

One of the best-characterized transcription factors involved in latency and reactivation of HIV transcription is the nuclear factor κB (NFκB). The subunits p65, p50 and p52 of the NFκB family can bind to the HIV κB binding sites as hetero/homo dimers in both an activating and inhibiting fashion. The proteins are involved in many processes, but are specifically known in T cells for their key role in immune activation(126, 127). The subunit p50 binds the activating p65 subunit when it is present, but forms homodimers in absence of p65. This p50 homodimer can bind to the two κB binding sites in the LTR. This leads to recruitment of HDAC1 and subsequent deacetylation of nuc-1, specifically lysine14 of histone3(121). Interestingly, not only p50 homodimers recruite HDACs to the κB binding sites in the LTR. Another protein that also recruits HDACs to the κB-bining sites is the C-promoter binding factor 1(CBF-1). CBF-1 is a key transcription factor in the Notch signalling pathway, a pathway that is involved in important developmental and proliferation processes. The protein can bind DNA in the HIV LTR and directly recruit HDACs(128). Both CBF-1 and the p50 homodimer are thus binding to the same κB site, thereby contributing

to a similar mechanism that promotes a latent state.

Not all HDAC-recruiting transcription factors interact directly with both DNA and HDACs. In case of HDAC recruitment by late simian virus 40 transcription factor (LSF) and yin yang 1 (YY1), both proteins are required for proper DNA and HDAC binding(129-131). LSF binds DNA and does not interact with HDACs. YY1 cannot bind DNA, but binds to LSF and is thus located on the DNA via LSF. YY1 in turn interacts directly with HDAC1(131). Interestingly, it has been shown that the binding affinity of LSF is regulated by phosphorylation, proposing a manner of regulation of latency. The protein is phosphorylated by ERKp38, a kinase from the mitogen-activated protein kinase (MAPK) family, of which the pathway is involved in processes of growth and cell cycle regulation(132).

Another factor that is associated with HDAC recruitment is c-Myc(133). c-Myc is a protein that is involved in crucial cellular processes like apoptosis and proliferation. The mechanism of HDAC recruitment by c-Myc is less well-defined than that of YY1 and LSF. However, similarly to these proteins the HDAC activity of the c-Myc containing complex seems to depend on a second factor, namely specificity protein 1 (Sp1). Sp1 is a transcription factor with three putative transcription factor binding sites in the LTR of HIV, as is shown in Fig. 4. The binding sites of Sp1 are required for c-Myc repressing activity, indicating at least a functional interaction between both proteins. The direct interactions in this complex have not been characterized. The cooperation of more factors in the HDAC recruiting activity of c-Myc can thus not be excluded(134). Interestingly, c-Myc is also found in complexes with HDAC activity on promoter sites of cellular genes, where the proteins YY1, LSF and c-Myc interact and seem to function cooperatively(133, 135). Although the existence of a supercomplex including YY1, LSF, c-myc and Sp1 has never been described for HIV transcription, it is likely that these factors are cooperating in their HDAC recruiting activity.

All the aforementioned transcription factors are described to mainly function via the recruitment of HDACs, and the subsequent deacetylation of Nuc-1. For the adaptor protein 4 (AP-4) an additional mechanism of gene silencing has been described, besides the general HDAC recruiting mechanism. AP4 is a basic helix-loop-helix transcription factor that is able to bind the E-box transcription factor binding sites in several promoters. In the LTR of HIV the E-box motif is located adjacent to the TATA-box, were both TBP and TFIIB could bind to initiate transcription(136). AP-4 is suggested to inhibit the TATA-box binding of TBP by competition for that binding site, and thereby blocking the recruitment of the transcription complex(137). All these factors are more or less required for maintenance of the latent state of HIV by altering the chromatin environment. However, different mechanisms are utilised to recruit the HDACs to the HIV LTR and repress transcriptional activity of the integrated HIV genome.

Histone methylation

Methylation of Nuc-1 is less characterized than acetylation, although it seems to play an important role in transcriptional regulation. Histone methylation is the reversible attachment of a methyl group to an arginine or lysine residue. Similar to acetylation, the modification of distinct residues can lead to different effects, although methylation does not change the DNA-nucleosome interaction but offers a specific docking site for transcription factors. Methylation of histones is performed by histone methyltransferases, of which suppressor of variegation 3–9 homolog 1 (Suv39H1) and G9a are described to be involved the transcriptional regulation of HIV, via lysine 9 methylation of Nuc-1 histone 3(123, 138, 139). This type of methylation causes repression of transcription and consequently contributes to a latent state, although the precise mechanism of silencing is unknown.

One of the proteins that utilises lysine 9 methylation of Nuc-1 as docking site is the heterochromatin protein 1 (HP1). HP1 is an adaptor protein that is known to recruit different repressing or activating factors to the DNA. The family of HP1 proteins are grouped into three isoforms with different functions, HP1α, HP1β, and HP1γ. The important role of HP1γ in HIV transcriptional repression was first defined by the treatment with TSA, an HDAC inhibitor. Treatment

with TSA leads to an increase of transcription, which was thought to be dependent on the *deacetylation* of the histone proteins on the LTR. However, HDACs are not exclusively deacetylating histone proteins, but they deacetylate other proteins as well. Thereby, they regulate the activity of these proteins. HP1 is one of these proteins who's activity is regulated by acetylation, and treatment with TSA promotes its dissociation from the DNA(140). So the effect on transcriptional activity that was seen when cells were treated with TSA was not fully dependent on the acetylation of histones, but also on the activity of a protein that docks on a methylated residue. The role of HP1γ was better characterized in studies from du Chène (2007) and Marban (2007), where it was shown that specific knockdown of HP1γ inhibits the activating effect of TSA, suggesting that HP1γ functions by repression of HIV-transcription(123). In addition, the knockdown of HP1γ in non-TSA-treated cells could increase transcriptional activity of HIV in several cell lines and primary cells, even towards seventy-fold(123, 138). For this activity of HP1γ, the methylation of lysine 9 of H3 by Suv39H1 was required, highlighting the importance of this methylated residue as docking site. Since the reactivation of transcription after HP1γ knockdown depended on the presence of Sp1 and Tc1, it is suggested that HP1γ blocks the recruitment of P-TEFb by Sp1(123). The absence of P-TEFb would impair the elongative capacity of RNAPII, since the polymerase cannot be activated by phosphorylation. Thus, according to the results of these studies, HP1 is an adaptor protein that represses HIV transcription by interaction with other transcription factors on the LTR by using the methylated lysine 9 as a docking site. However, in contrast to these results, another group reported the inhibition of HIV transcription in case of HP1γ knockdown(141). So in this case, HPγ activated HIV transcription. Furthermore, the authors showed that HP1β is associated to the LTR when the HIV genome is silenced, and that this one is replaced by HP1γ when the LTR becomes activated. They claim that the different results were due to the activation of the MAPK pathway by TNFα treatment that was administered to the cells. Treatment would lead to H3 serine 10 phosphorylation, and thereby regulate HP1 isoform localization. This and the use of only one silencing RNA sequence to establish a specific knockdown would interfere with the results of the HP1γ knockdown(141). This group performed the same assays without TNFα treatment and with other RNA sequences to knockdown HP1γ. According to their data the phosphorylation of H3 serine 10 in activation led to a switch of isoform recruitment, in which the methylated residue still functioned as docking site, since the lysine 9 residue is not demethylated during activation. In conclusion, different isoforms of HP-1 play different roles in transcriptional regulation of the HIV gene. Unfortunately, there is currently no conclusive data available about the role of HP-1 isoforms in the regulation of HIV transcription, although the recruitive character of the methylated lysine 9 residue was shown by both groups. Thus, from this, it can be concluded that the methylated lysine 9 residue forms an important docking site for the HP1 family proteins during HIV infection, although the precise mechanisms remains to be unravelled.

ATP-dependent remodelling

For ATP-dependent remodelling energy in the form of ATP is required to remodulate the nucleosome-DNA interaction, thereby either increasing or decreasing the transcriptional activity. The ATP-dependent remodelling complex that is associated with HIV transcription is the switch /sucrose non-fermentable (SWI/SNF) complex. Although the role of SWI/SNF complexes in latency is poorly defined relative to the current knowledge of their function in activation, the core subunit Integrase Interactor 1 (Ini1) is suggested to play a role in latency. It was shown that Ini1 has a repressive effect on the transcriptional activity of the LTR, but only early after integration. This effect is suggested to be caused by the recruitment of several chromatine remodelling enzymes, since SWI/SNF complexes are known to function as a platform(142). Temporal distribution of different functions of similar complexes may be included in the switch towards either active transcription or silencing of transcription early after integration of HIV.

CpG methylation

DNA itself can be methylated on its cytosine ring, which provides heritable information in transcriptional control. However, in case of HIV

transcription, it is not clear whether CpG methylation plays a role in control of HIV transcription, since opposite results have been described, mainly received in cell line systems(120, 143). Two recent reports describe the involvement of CpG methylation in transcriptional regulation, and show also *in vivo* differences in CpG methylation of actively transcribed and latently expressed provirus(144, 145). However, the role of CpG methylation in HIV transcription remains to be fully elucidated.

In conclusion, latency in T cells is initiated and maintained by different cellular mechanisms and some properties intrinsic to the virus. The cellular mechanisms that promote latency mainly rely on the activation status of the infected cell. HIV does not actively control the state of latency, although several binding sites for transcription factors that favour the transcriptional inhibited environment might provide evolutionary advantages.

3 - Molecular mechanism of HIV reactivation in T cells

Reactivation of the latent reservoir of HIV is of clinical importance since reactivated virus replicates and consequently is sensitive for eradication via HAART. Tat is not present in the host cell during latency. To initiate reactivation of viral transcription, transcription must be induced *independent* of Tat. Once transcription of HIV is induced, Tat will be transcribed and from that moment on transcription is Tat-*dependent*.

In absence of Tat, HIV-transcription depends on the activity of host proteins only. The activity of these host proteins is regulated by the activation status of the T cell. The cell has to be immunologically activated to transcribe the HIV genome independent of Tat. The activation state of T cells is defined by the extracellular environment of the cell, for instance by cytokine composition and direct cell-cell contacts. The transcription factors NFκB and the nuclear factor in activated T cells (NFAT) are key molecules in different cellular signalling pathways that are triggered by a stimulating extracellular environment. During the Tat-independent induction of HIV transcriptional activity they play a major role and both proteins can bind to the κB sites of the LTR. The κB and Sp1 binding sites on the LTR are the only sites that have been reported to be indispensable for reactivation of transcription(146). Therefore, the focus of this chapter will be on NFκB and NFAT, since they both reactivate transcription in absence of Tat.

Tat-independent reactivation of HIV transcription

The role of NFκB in HIV reactivation

During latency, reactivation of transcription of proviral HIV is induced by immunological activation of memory T cells. This can be mimicked in culture by treating latently infected T cells with cytokines like TNFα, although it has been shown that the reactivation in primary cells does require more stimulatory factors(147). Stimulation with TNFα reactivates latently present HIV in cell lines via the NFκB pathway, of which the binding sites are indispensable for induction of HIV-transcrription both in presence or absence of Tat(121, 148, 149).

Without stimulation, the NFκB p65-p50 heterodimer is trapped in the cytoplasm via interaction with the inhibitor of κB (IκB). Phosphorylation of IκB is induced by various signals, including those via Toll-like receptors, tumour necrosis factor receptors, growth factor receptors, and the T cell receptor. Phosphorylated IκB is prone to ubiquitination, and subsequently target of proteosomal degradation. The NFκB p65-p50 heterodimer is released in the cytoplasm and translocates to the nucleus, where it competes with the inhibiting p50-p50 homodimer and binds to the κB transcription factor binding sites(150). The activating p65 subunit of NFκB itself is regulated by phosphorylation, acetylation and ubiquitination, of which the function of phosphorylation and acetylation have been best-characterized(151). Phosphorylation of p65 upon TNFα treatment increases transcriptional activity of the NFκB subunit. It regulates binding of p65 to either HDACs or p300/CREB-binding protein (CBP), wherein CBP is a homolog of p300(152, 153). The acetylation status of the chromatin environment and of p65 itself is a crucial factor in NFκB induced HIV-transcription(154). Phosphorylation of NFκB is thus enhanced by a stimulus that induces activating signal pathways, like TNFα, and regulates transcription by recruiting HATs that change the chromatin environment and modify

NFκB. Furthermore, DNA-binding of the phosphorylated protein to the κB sites leads to disruption of binding of CBF-1 and the p50 homodimer of these sites, consequently dissociating HDACs from the LTR(121, 153).CBP/p300 and P/CAF can acetylate NFκB. Acetylation of p65 generally increases transcriptional activity, with or without affecting the DNA or IκB binding capacity(155). In conclusion, phosphorylation of p65 and subsequent acetylation is crucial for activation of the transcriptional activity of NFκB, which is necessary for transcription of Tat.

Strikingly, Sp1 is present on the LTR in both latent and activated state(156) and seems to be essential for both latency and reactivation. Direct interaction of p65 with Sp1 is essential for recruitment and maintenance of p300 on the LTR, and consequently for induction of transcriptional activity via the above described mechanisms(146, 157-159). Although the interaction of Tc1 and Sp1 has been found to induce HIV transcription(160), it is unlikely that Sp1 is able to induce full Tat-independent transcription by itself, since it has been shown that presence of Sp1 stimulated initiation only(161). Conclusively, Sp1 seems to be indispensable for elongation, although NFκB activation is required.

Several conflicting observations have been described concerning the mechanism of NFκB induced Tat-independent transcription. Analysis of the LTR in the basal transcription state during latency and after reactivation revealed that the general transcription factor TFIID and a hypophosphorylated form of RNAPII were already present in the latent phase, but that TFIIH was recruited to the promoter by NFκB stimulation(162). TFIIH contains a kinase subunit, CDK7, which is able to phosphorylate RNAPII and thus stimulates elongation. Moreover, elongation induced by NFκB has been suggested to be CDK9-independent(154, 162). In contrast, other groups found a direct interaction of NFκB with, and recruitment of the P-TEFb complex to the promoter. They claimed that CDK9 phosphorylates RNAPII and promotes elongation upon NFκB activation(163, 164). In conclusion, presence of NFκB enables the production of elongated transcripts, which is stimulated via a mechanism independent of Tat activity.

It has been generally accepted that induction of transcription by NFκB is less robust than transcription evoked by Tat activity. Several differences in NFκB-induced transcriptional activity are probably contributing to the suboptimal elongation capacity of the transcription complex in comparison to Tat-induced transcription.

There are several hypotheses explaining the suboptimal activation by NFκB, but none of them clarifies satisfactorily the low level of activation. According to one hypothesis the non-continuous presence of NFκB plays a role. It has been shown that the nuclear presence of NFκB follows an oscillating pattern, as expression of NFκB stimulates the transcription of IκB. Nuclear presence of NFκB recruits the elongation complex to the LTR. The elongation complex consists P-TEFb and leads to phophorylation of RNAPII. In periods of relative absence of NFκB the amount of phosphorylated RNAPII decreases, which has been suggested to be due to the activity of a currently unidentified phosphatase. The elongation complex dissociates from the DNA when RNAPII is dephosphorylated, and transcription is consequently prematurely aborted(164). Very recently, it has been described that the St. John's Wort DING phosphatase can dephosphorylate RNAPII and thereby is able to inhibit elongation. The expression of St. John's Wort DING phosphatase decreased elongation even in presence of Tat and p65(165). The regulation and function of this phosphatase protein family in eukaryotic cells has currently not been fully characterized, although the proteins seemed to be ubiquitously expressed(166). It would be interesting to know whether this protein is the phosphatase that diminishes the effect of NFκB in reactivation. In conclusion, release of the activating subunit of NFκB from the cytoplasm is due to a stimulating extracellular environment that induces signalling via several activating pathways. Nuclear localization of NFκB in turn leads to enhanced initiation and suboptimal increased elongation of viral transcription, via recruitment of HATs and components of transcription complexes. Subsequent production of a few elongated

transcripts will cause production of Tat and boost HIV reactivation.

The role of NFAT in HIV reactivation

More recently, an important role for NFAT in Tat-independent induction of transcriptional reactivation of HIV has been reported(167, 168). However, the mechanism behind reactivation by NFAT is still not clear. The NFAT-family is a major transcription factor family that consists of five NFAT members, from which NFAT1 and NFAT2 have been reported to be involved in HIV transcriptional activity and T cell activation(169). However, it has been suggested that NFAT1 will possibly have a positive effect on HIV transcription, while the by NFAT1-induced NFAT2 has a negative effect(170, 171).

The protein NFAT consists of a regulatory domain and a DNA-binding domain. It can form homodimers and heterodimers with proteins like AP1 and HDACs(172). In non-stimulated cells, hyperphosphorylated NFAT is trapped in the cytosol. This has been specifically described for resting T cells(171). Dephosphorylation of NFAT leads to activation and its subsequent translocation to the nucleus. The dephosphorylation step is regulated by signalling induced by stimulation of various receptors. These include cytokine receptors and the T cell receptor, of which is known that activation will increase intracellular calcium levels and activate calcineurin, a calcium-dependent phosphatase. Calcineurin is able to dephosphorylate and consequently induce transcriptional activity of NFAT. Thus, similarly to NFκB activation, HIV reactivation via this Tat-independent pathway depends on a T cell stimulating extracellular environment. Transcriptional activity of nuclear localized NFAT has been described to be regulated by phosphorylation as well. Phosphorylation abolished transcriptional activity via the promotion of export to the cytoplasm. One of the kinases that was able to promote nuclear shuffling of NFAT was ERKp38, also known to promote latency by phosphorylation of LSF(173).

Expression of NFAT target genes has been associated with an activated T cell phenotype, however, consequences of NFAT activation are defined by the protein that dimerizes with NFAT. The best-studied mechanism of NFAT-mediated transcriptional activation is activation induced by the heterodimer NFAT-AP1. HIV-transcription has been shown to be enhanced in the presence of AP1, although this has only been observed in presence of Tat(174). The precise effects of NFAT-AP1 heterodimers on Tat-independent transcription have currently not been described. Structural data showed that NFAT binds the LTR κB sites as homodimer(175, 176). Some groups have reported a major role for NFAT in activation, where others described a negative effect of NFAT homodimers on HIV transcription(169). However, this seems to be dependent on immunological and transcriptional activation status of the experimental set ups. Current data strongly indicate that NFAT homodimers does play a role in Tat-independent reactivation. Importantly, inhibition of NFAT translocation to the nucleus with several different inhibitors in different cell types all showed decreased HIV transcription(177). Furthermore, research on the role of NFAT in primary cells pointed unanimously to NFAT as able to reactivate latent HIV. Inhibition of NFAT impaired reactivation, although these data do not exclude the possibility of NFAT heterodimer formation(167, 168). However, it has been generally accepted that NFAT binds the κB binding sites of the LTR as a homodimer. Furthermore, the κB sites are indispensable for activation by NFAT and the activation of transcription is strictly NFκB-independent(175-179). Therefore, the ability of NFAT to reactivate latent HIV has to be at least partially dependent on NFAT homodimers. The mechanism of transcriptional activity induced by NFAT homodimers has not been elucidated. Structural data showed a major conformational change in the LTR upon NFAT homodimer binding(175, 176). This conformational change could influence binding of other transcription factors, and thereby affect the transcriptional activity of the LTR. Furthermore, NFAT has been shown to recruit p300/CBP, HATs that change the chromatin structure and serve as a scaffold for transcription complexes(180). However, the precise mechanism by which NFAT promotes HIV reactivation remains to be elucidated.

Tat-dependent reactivation of HIV transcription

Tat is the most powerful transcriptional activator of HIV transcription. Tat is present during reactivation upon by NFκB or NFAT induced minor elongation. Presence of Tat robustly enhances transcriptional elongation, providing viral proteins to regulate cell processes. Tat itself affects both processes that have a direct or indirect influence on transcriptional activity of the LTR, in order to stimulate viral transcription. For instance, to release cyclin-dependent kinases that are essential for phosphorylation of RNAPII, Tat inhibits cell cycle processes that utilise these kinases(181). Additionally, interaction of Tat with the HAT K-Acetyltransferase 5 which is also called Tip60, causes repression of cellular gene transcriptional activity. Along these lines, Tat is enhancing the available pool of general transcription factors that can be utilised by the HIV transcription complex(120). These are two out of more examples of the extensive influence of Tat on the regular host environment. However, the focus herein will be on the interactions of Tat with host proteins that influence transcriptional activity directly. To affect transcription, Tat interacts with various proteins including major (general) transcription factors and factors with a chromatin remodelling function.

Interaction of Tat with NFκB

Upon expression, Tat will be recruited to the LTR by interaction with the TAR element, as has been described in the introduction section. Besides this fashion of Tat recruitment, also TAR-independent mechanisms of Tat function have been reported. However, activity of NFκB and Sp1 have been shown to be indispensable for both TAR-independent and TAR-dependent Tat-induced transcriptional activation(149, 182). This underscores the importance of the interaction between NFκB and Tat during viral transcription. NFκB itself is one of the transcription factors who's function is altered by Tat expression. Tat affects NFκB activity via several mechanisms. It has been shown that Tat could bind directly to the κB binding sites and thereby induce transcriptional activity(183, 184). Besides binding to the κB transcription site, Tat activates NFκB and promotes its nuclear localization. However, the mechanism by which activation of NFκB is enhanced is still controversial and the hypotheses that explain the enhanced activation of NFκB in presence of Tat diverse from altering the cytoplasmic redox-potential to inhibition or activation of antiviral kinases(185-187). In addition, Tat mediates acetylation of the NFκB subunit p50 by interaction with p300/CBP. This modification of p50 enhances the transcriptional activity of the p50-p65 NFκB heterodimer in addition to the acetylation of p65(188). In conclusion, NFκB-induced expression of Tat positively influences HIV transcription by enhancing NFκB activity, a positive feedback loop.

Besides directly affecting NFκB function, Tat also stimulates NFκB activity by regulation of the activity of NFκB interaction partners. For instance, Tat is suggested to affect the transcriptional activity of Sp1. Direct interaction of Sp1 with Tat is still controversial, although a linking partner between the two proteins could not be found and the interaction between the two proteins have to be direct(189, 190). The group that reported direct interaction of Tat with Sp1, showed that direct interaction with Tat was required for phosphorylation of Sp1, which subsequently enhanced recruitment of transcriptional complexes at the LTR(191). Although Sp1 is indispensable for reactivation, it seems that the interaction with other transcription factors like NFκB and Tat forms the main function of the protein. In conclusion, Tat interacts with NFκB and this interaction affects the activity of NFκB positively through a positive feedback loop and stimulatory effects on cofactors of NFκB.

Interaction of Tat with NFAT

Tat can interact directly with NFAT(174). This direct interaction has been reported to enhance the transcriptional activity of both the LTR and cytokine promoters that are regulated by NFAT. For instance, IL-2 production was increased upon expression of Tat, and this effect was abolished when the NFAT binding site in the IL-2 promoter was mutated(192). However, the mechanism of increased transcriptional activity of NFAT in presence of Tat is likely to be dependent on the interaction of NFAT with AP1. Interaction of Tat with NFAT increased the affinity of NFAT for AP1, thereby increasing the NFAT-AP1 induced transcriptional activity. Tat did not seem to have an

effect on affinity of NFAT for itself in NFAT-NFAT dimerization(174).

Direct effects of NFAT on HIV transcriptional activity has been thought to be exclusively dependent on binding of the NFAT-NFAT dimer to the κB binding sites(175-177, 179). However, the affinity of the NFAT-NFAT dimer for the κB binding sites is lower than the affinity of activated NFκB for these sites and Tat specifically increases activation of NFκB. It is therefore likely that NFAT-NFAT homodimers do not play a major role in direct transcriptional activation in presence of Tat. However, interference of Tat with NFAT-AP1 heterodimers indicates that NFAT is exploited by HIV to create an optimal cellular environment for HIV replication. The cellular activation is driven by cytokines, of which expression is stimulated by activity of the NFAT-AP1 heterodimer. Although NFAT is very likely to elongate Tat-independent HIV transcription, it is unclear if NFAT directly affects Tat-dependent transcriptional activity. The indirect effects of the interaction of Tat with NFAT are probably favouring HIV transcription, suggesting an important indirect role for NFAT in Tat-dependent HIV transcription.

Tat affects the chromatine structure

Remodelling of the chromatine structure by epigenetic signalling is in general required for transcriptional activity, like it is for transcriptional inhibition during latency. Release of the tight packaging of the DNA around nucleosomes will increase the possibility for transcription factors to bind. Acetylation of histone tails leads to release of transcriptional repression by diminishing the electrostatical affinity of histones to DNA. During HIV reactivation, general cellular HATs are recruited to the LTR by both Tat and NFκB. Lysine 9 of the H4 tail of Nuc-1 becomes acetylated by GCN5 and P/CAF and leads to replacement of Nuc-1. This replacement will increase the accessibility of the LTR for activating transcription factors(119, 122, 193). Furthermore, the modification of H3 has been reported to play a major role in reactivation. Acetylation of lysine 14 is suggested to enhance transcriptional transcription(121). Interestingly, HATs that are involved in histone acetylation are also able to acetylate proteins, including NFκB and Tat. Moreover, interaction of Tat with several HATs increases HIV transcription via various mechanisms. During reactivation HATs like p300 are both recruited and tethered to the LTR by NFκB and Tat(159). Since physical interaction of p300/CBP with Tat increases the affinity of Tat to the TAR element, it increases also tethering of the transcription complex to the LTR(194). Furthermore, binding of p300/CBP to Tat increases the affinity of p300/CBP for general transcription factors TBP and TFIIB, which probably stabilizes the transcription complex(85). However, it has been found that histone acetylation following upon Tat activation depends on P/CAF rather than on the activity of p300/CBP(122, 195). Tat enhances transcriptional activity of the LTR thus by enhancement of both histone and protein acetylation.

Another, relatively recently discovered interaction partner of Tat that influences the chromatin structure is the human nucleosome assembly protein 1 (hNAP-1). The NAP-family assembles nucleosomes, thereby playing an important, but poorly defined role in transcriptional regulation. Direct binding of Tat to hNAP-1 has been shown to increase the transcriptional activity of Tat. The effect of hNAP-1 on Tat activity has been suggested to be based either on increased stability of Tat or on decreased affinity of the histone proteins H2A and H2B to DNA(110, 196).

In addition, Tat recruits one of the essential chromatine remodelling complexes during transcriptional activation, the ATP-dependent remodelling complex SWI/SNF. SWI/SNF complexes in human cells have been grouped in two different classes, based on the differences in composition. The BRG1 or human brm-associated factor (BAF) complex and the polybromo-associated BAF (PBAF) complex are characterized by one of the unique subunits, BAF250a for BAF and BAF180 for PBAF(197). These two classes function at different times during HIV infection, affecting transcriptional activity by translocation of nucleosomes. According to the prevailing hypothesis, SWI/SNF complexes stimulate DNA release from histone cores but not from histone tails, increasing the accessibility of DNA for transcription factors and transcription complexes(198). During latency the BAF complex is thought to interact with the inhibited LTR,

promoting exclusively initiation of transcription. The BAF complex interacts with Tat if present, but only with deacetylated Tat which has a suboptimal elongation capacity(199). During reactivation, elongation is stimulated with the recruitment of PBAF to the LTR via acetylated, and thus optimal activated Tat(199, 200). Furthermore, recruitment of SWI/SNF complexes was found to be required for Tat-independent transcription and is thus essential for reactivation of HIV transcription(201). Interestingly, the acetylated p65 subunit of NFκB can recruit proteins from the bromodomain family(151, 155). The different function of the PBAF and BAF complexes can be explained by variation in recruitment of cofactors, since the complexes also function as platform for other activating or repressing transcription factors. Indeed, interactions of SWI/SNF complexes with several important regulators of HIV transcription have been reported, like c-Myc, IN and Tat(142, 202-204). Several subunits of SWI/SNF interact specifically with lysine 50 and 51 of acetylated Tat and can act in cooperation with p300 in nucleosome remodelling(203, 205-207). Interestingly, one of the subunits that directly bind to acetylated Tat is Ini, the subunit that also represses HIV transcription in the early phase after integration and interacts directly with IN(205). However, it has been shown that the interaction of Tat with Ini in a later stage of HIV infection affects transcription positively. The precise mechanism of this positive effect on HIV transcription is currently not understood. According to the currently available data, a SWI/SNF complex PBAF is recruited during reactivation by both acetylated NFκB and acetylated Tat, via direct interactions with several subunits of this complex. This direct interaction forces Tat to dissociate from the TAR element and to form an elongation complex, including p300. In conclusion, SWI/SNF complex PBAF stimulates the elongation of transcription by the direct interaction with Tat and promote HIV expression.

Reactivation in general

In conclusion, HIV transcription can be divided into processes of initiation and elongation, like cellular transcription processes. The switch from initiation to elongation is a deciding moment in HIV transcription. Initiation is mainly Tat-independent, although presence of Tat stimulates initiation by release of normal transcriptional repression. Furthermore, Tat immediately starts recruiting transcriptional activators and general transcription factors to enhance transcription of HIV, to favour viral transcription instead of transcription of host genes. In contrast, the elongation phase is strongly Tat-dependent, although NFκB and NFAT are able to induce Tat-independent elongation as well. The sequential interaction of Tat with various activating factors causes formation of an highly effective elongation complex, that is able to transcribe and replicate the HIV genome.

HIV transcription in latently infected memory cells is inhibited and no viral proteins are present. Reactivation of latently expressed HIV in resting memory $CD4^+$ T cells is mainly dependent on the immune status of the extracellular environment. However, when the activating NFAT or NFκB signalling pathways are triggered, the virus will quickly control its own expression via Tat. Reactivation of the latent reservoir and subsequent eradication of the virus from a patient's body by HAART is the goal of many clinical researchers. However, finding novel ways to safely reactivate a minor subset of T cells without infection of other cells requires a thorough knowledge of the molecular mechanisms of reactivation.

4 - Discussion

The major cause of failure to cure HIV-patients who receive HAART is the persistent existence of a replication-competent virus in a latent reservoir. Latency remains one of the most puzzling features of the virus after thirty years of research on HIV. The best-characterized reservoir of latent virus is the exceptionally long living memory T cell pool. Attempts to understand the mechanisms of latency are hampered by either the relatively few latently infected cells in this small T cell compartment in human patients and the lack of a reliable model system. However, increasing amounts of data have established the role of epigenetics and Tat in latency and reactivation of HIV transcription. Epigenetic mechanisms are involved in immunological activation of T cells and activation of memory T cells is tightly linked to reactivation of

HIV transcription. Reactivated virus will replicate and form infective particles, infecting the patient's CD4 effector T cells, thereby reactivating disease. Since transcriptional inhibition is maintained by host proteins, the state of latency seems to be mainly dependent on external factors from the viral point of view. Although a latent state is not favoured by the virus, the phase of latency has major advantages for viral existence. It functions as a non-immunogenic reservoir in the infected individual. For maintenance of this state, persistent absence of viral or cellular transcriptional activator proteins and presence of an inhibiting chromatin environment is essential. Subsequent reactivation requires activation of NFAT or NFκB, of which activation depends on immunologically activating stimuli received by the latently infected T cell. Upon stimuli leading to elongated transcription, Tat will be transcribed and translated and will robustly regulate transcription. The expression of Tat affects most host processes and the cell starts to effectively produce virus. It is thus clear that many processes that lead to the initial events of latency or reactivation are not regulated by the virus but are instead fully depended on the host environment. Posttranslational modifications are initially regulated via the recruitment of general cellular enzymes by transcription factors NFκB and NFAT. Upon expression, viral proteins cooperate with these existing cellular mechanisms to create an optimal situation for replication and generation of new infective virions.

The complex interplay between host and viral transcriptional activators and repressors makes understanding of transcriptional latency and reactivation difficult. The HIV LTR is one of the best characterized promoters and the available information on transcriptional regulation of the HIV genome is overwhelming. Nevertheless, we are in many ways no closer to understanding of the enigmatic regulation of cellular processes by HIV than thirty years ago, since new knowledge often disposes new questions on already old topics. The field of HIV research is characterized by conflicting data. Causes of these controversies lie in different experimental set-ups and complexity of the research topic. Difficulties in experimental set-up exist of unknown reliability of cell systems and an extremely small subset of primary cells. The reliability of viral activity on host environment increases the variability between experiments. Some discrepancies have been described in this thesis, for instance the role of HP1γ in latency and the recruitment of P-TEFb on the LTR during Tat-independent reactivation. However, when consensus about these topics will be established, the data could be used to find novel ways to interfere with latency.

Latency and reactivation are features that are not only interesting from the point of view of a molecular biologist, but are also a clinically relevant topic since maintenance of a latent reservoir within patients is one of the major obstacles to cure patients with HIV and is the reason for the use of life-long HAART. Reactivation of this reservoir would reactivate viral replication and sensitize the virus for therapy, consequently eradicating virus from the T cell reservoir. However, activation of the whole pool of $CD4^+$ T cells would lead to massive immune activation. Therefore, more subtle molecular mechanisms of reactivation have to be identified. This knowledge can be utilised to achieve suboptimal activation of memory T cells and optimal reactivation of HIV transcription.

It is likely that multiple targets are required for optimal transcriptional reactivation of HIV. The existence of various variants of HIV world-wide and in the patient reduces the change of success for a therapy that only targets one individual mechanism of latency. Moreover, one-target therapy will increase the chance of development of resistant viruses and probably requires a higher dose, enhancing possible side-effects. The ideal future therapy exists of several synergistically acting components, specifically targeting as many mechanisms of latency as possible.

Research on methylation and ATP-dependent chromatin remodelling during latency and reactivation have shown that these processes are involved in HIV transcription. However, insight in the mechanisms of their role in latency and reactivation has to be increased before a highly specific therapy based on their function could be developed. At this moment, the best-understood epigenetic mechanism that leads to latency is

deacetylation of histone proteins. There are many reports on the attempts to reactivate transcription via inhibition of HDACs. In cell culture, it has been shown to be possible to stimulate reactivation with HDAC inhibitor treatment probably due to non-physiological concentration of HDAC inhibitors and use of a cell line(124, 125). Although there have been many clinical trials with HDAC inhibitors that show reactivation of latent HIV was enhanced, most of them intensified the use of HAART. Consequently, the net effect of administering HDAC inhibitors as sole drug is still controversial(208). It might be that treatment with HDAC inhibitors in physiological relevant amounts primes reactivation of viral transcription, but is unable to efficiently boost elongation. The inhibitory transcription factors have not been replaced from the LTR by HDAC inhibitor administration and are thus still able to actively recruit new active HDACs. Without activation and recruitment of HAT recruiting transcription factors, the reactivation will probably remain insufficient to eradicate a viral reservoir. Consequently, to increase the effect of HDAC inhibitors, also the transcription factors that recruit HDACs must be replaced by activating ones. Therapy that combines an activating pathway stimulus and HDAC inhibitors is therefore most likely to be successful. Currently, one of the most promising HDAC inhibitors is the specific class I inhibitor *suberoylanilide hydroxamic acid*. This inhibitor is already used for cancer treatment, but is currently not used for HIV reactivation. However, experiments in primary cells show that this inhibitor is able to promote reactivation, to a rather high extent when combined with a NFκB activator(209).

Thus, activating transcription factor recruitment is important to induce some robust HIV transcription. The activating transcription factors that can elongate HIV transcription in absence of Tat are NFκB and NFAT. Both proteins can be activated by stimulatory cytokines. It has been shown that TNFα-induced NFκB activation leads to HIV transcription in a Tat-independent fashion(210). However, HIV reactivation via NFκB will also lead to T cell activation, since NFκB is one of the most potent transcriptional activators in T cells. Cytokine administration that activates NFκB, for instance IL-2, led to the reactivation of a certain part of the viral reservoir. However, massive immune activation caused extremely large side effects.

A promising cytokine to modulate the transcription factor composition on the LTR is IL-7. IL-7 has been shown to be able to reactivate HIV transcription *in vitro.* In patients, IL-7 could modulate IL-2 induced reactivation of memory T cells(211). In naïve T cells, reactivation of HIV by IL-7 was mediated exclusively by NFAT. NFκB was inactivated and cell proliferation was not stimulated(212). IL-7 could thus be used to modulate the overall response in a combination therapy, however, the precise mechanism of regulation of HIV transcription remains to be elucidated.

Several key players in HIV reactivation can be specifically targeted by activating compounds. Specific activation of NFκB with prostratin leads to reactivation, although no data of clinical trials has been reported at this moment(209). Furthermore, the *in vitro* results of some compounds that increase the available P-TEFb pool are promising. It has been shown that the compound *hexamethylene bisacetamide* could induce reactivation in primary cells without overall activation of T cells(213, 214). However, currently no clinical trials with these compounds have been performed.

Combination therapy of different drugs is likely to achieve the best results to eradicate the viral reservoir. The results in cell systems point towards a combination of NFκB activation and HDAC inhibition(209). However, the activating dimer NFAT might form a novel promising target. The structure of the homodimer provides a specific target for intervention, since it binds quite specifically the κB sites on the LTR(176). When formation of these homodimers can be stimulated, reactivation of HIV would probably be very specific. This would minimalize the chance on side effects.

In conclusion, there are several very promising developments towards a therapy that could be able to eradicate the latent reservoir in HIV infected patients. The most important lesson to be learned from these studies is that the regulation of immune responses requires a thorough knowledge

of the molecular mechanisms. The most promising future therapies will probably combine drugs targeting different mechanisms to modulate the immune response to the appropriate level. In the ideal world, treatment could be adapted to every individual's response. However, the eradication of this particular latent reservoir does not solve all problems of HIV treatment. Nevertheless one of the best-characterized reservoirs of HIV in patients receiving HAART, the memory T cell pool is not the only one. For instance, HIV that infect cells in the brain is less sensitive to HAART. To develop drugs that is able to cross the physical barrier to the reservoir in the brain is likely to be the next challenge in the development of a cure for HIV. However, data of continuously ongoing research on novel drugs to fight HIV shows that knowledge of molecular mechanisms leads to more specific targeting and is likely to be the way to find a successful cure for HIV.

5 - References

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