Inhibition of Splicing in HIV-1 - A Novel Approach in the Fight Against the Virus

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Degree project in biology, 2006
Examensarbete i biologi 20p, 2006
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SUMMARY

According to ‘UNAIDS 2004 Report on the global AIDS epidemic’ 38 million people were living with Human Immunodeficiency Virus (HIV) in 2003. The greatest problem with HIV is the high mutation rate leading to resistance to drugs. Research has shown that indole derivatives can affect the production of HIV and I wanted to study the effect of 13 indole derivatives on RNA and protein levels. I have constructed a test system; the plasmid pDP containing the Human Immunodeficiency Virus type 1 (HIV-1) genome except for a deletion in the pol gene. Some of the 13 substances were shown to decrease the pDP RNA and Gag protein levels. These results suggest that some of these substances might be potential inhibitors of HIV-1 and need further examination.
INTRODUCTION

The acquired immunodeficiency syndrome (AIDS) was discovered in 1981 by health workers in United States and 1983 a retrovirus now known as Human Immunodeficiency Virus (HIV) was identified as the causative agent for AIDS. AIDS is a condition where the immune system, especially the T-lymphocytes, is strikingly reduced and gives susceptibility to cancers and opportunistic diseases. The AIDS mortality rate is close to 100 % which makes HIV one of the most lethal known pathogen ever (Flint 2004).

According to ‘UNAIDS 2004 Report on the global AIDS epidemic’, almost five million people became infected with Human Immunodeficiency Virus (HIV) in 2003 and 38 million people were believed to be living with the infection. Most of these people are living in Africa, around 25 millions in sub-Saharan Africa. In Asia and Eastern Europe the infection is expanding very fast mostly due to injections of drugs with contaminated needles (http://www.unaids.org/bangkok2004/report.html 051203).

Human immunodeficiency virus type 1, HIV-1, comes from Simian immunodeficiency virus, SIV, which infects primates. The closest relative to HIV-1 is SIV<sub>cpz</sub> which infects chimpanzees, indicating that SIV<sub>cpz</sub> is the origin of HIV-1. The virus appeared in humans sometime between 1915 and 1940 but was not discover until 1980s. HIV is consisted of two strains; HIV-1 and HIV-2 of which type 1 is a more common and a more virulent strain (Campbell and Reece 2002). HIV-1 viruses can be divided into three different groups depending on the nucleotide sequences and represents three different transmissions from chimpanzees to human; M, containing most of the isolates can be divided into subtypes, N, a new group nearest related to M, and O, the most divergent group. HIV-1 is transmitted via sexual contact, via blood and from mother to child. Except for blood transfusions and needles,
HIV enters through mucosal surfaces and more likely via infected cells rather than free virus particles (Flint 2004).

The HIV-1 genome

The Human Immunodeficiency Virus type 1 (HIV-1) genome consists of different genes that all are transcribed from a promoter in 5’-long terminal repeats (LTR) into a 9 kb pre-mRNA that is spliced into different mature mRNAs (fig. 1). The parts and the genes in the genome are:

![Diagram of HIV-1 genome](image)

**FIG. 1.** The HIV-1 genome adapted from Schwartz et al. 1990. The figure shows some of the different mRNAs that are produced from the genome by alternative splicing. *tat*, *rev* and *nef* are examples of fully spliced mRNAs, *env* is an example of singly spliced mRNA.
LTR

LTR, long terminal repeats, situated in the 5’- and the 3’-end of the HIV-1 genome consists of U3-, R- and U5-region (Schwartz 1991). The 5’-LTR functions as a transcriptional promoter by binding cellular transcriptional activators (Flint 2004). Upstream, 22 nt from the transcription initiation site there is a TATA box homologue essential for activation of transcription. There is also an enhancer sequence in the 5’-LTR where the cellular transcription enhancer NFkB binds and stimulates production of virus. The LTR also contains an RNA sequence called TAR in the R-region. The TAR RNA forms a stem-loop which is recognized by the viral Tat protein and affects the viral elongation (Steffy et al. 1991).

gag

The gag gene encodes the structural proteins that form the inner matrix of virus particles. The Gag precursor polyprotein, p55gag, is proteolytically cleaved by the viral protease in the immature virus particle into four different proteins; p24gag (capsid), p17gag (matrix), p7gag and p6gag (nucleocapsid) (Steffy et al. 1991).

pol

The products from pol gene are produced through ribosomal frame shifting during translation of the 9 kb mRNA, resulting 5 % of time in a Gag-Pol polyprotein which is cleaved into three important proteins: protease (PR), integrase (IN) and reverse transcriptase (RT) (Schwartz 1991).

eenv

The env gene encodes the envelope glycoproteins positioned on the surface of the virus particles. The translation product of env mRNA is a 160 kDa precursor protein that is
endoproteolytically cleaved into gp120 (major envelope glycoprotein) and gp41 (small trans-
membrane envelope protein). gp120 binds the cellular receptor CD4 and co-receptor CCR5 on T-cells and CCR5 on other immune cells, gp41 induces fusion of viral and cellular 
membranes prior to virus entry into the cell (Steffy et al. 1991).

tat
The tat gene codes for the 16 kDa protein Tat that binds to RNA sequence TAR in LTR and 
stabilizes the viral mRNAs (Schwartz 1991). The binding of Tat to TAR enhances the 
elongation of viral mRNA and in the absence of Tat, viral transcription usually terminates 
(Flint 2004).

nef
The Nef protein is encoded by the nef gene and is incorporated into the virus particle but its 
role in replication is not known (Flint 2004). Nef seems to be important, however in 
pathogenesis, since nef-deficient viruses show decreased virulence. Nef down-regulates the 
expression of e.g. CD4- and MHC class I receptors, and enhances the infectivity of the HIV-1 
virion (Li et al. 2005).

rev
The rev gene encodes the protein Rev that acts by binding to the Rev-responsive element 
(RRE) present in the env region of mRNAs (Schwartz 1991). Rev contains a nuclear export 
signal (NES) and binds to nuclear pore complex (NPC) (Pollard and Malim, 1998). By 
binding to RRE on unspliced or partially spliced mRNA, Rev exports these mRNAs from the 
nucleus to the cytoplasm (Flint 2004).
\textit{vif}

The viral infectivity factor gene, \textit{vif}, encodes the RNA-binding protein Vif that seems to be important for virus assembly and maturation (Flint 2004). The infectivity ratio is decreased in the absence of \textit{vif} (Haseltine 1991). A recently discovered mechanism for Vif is the enhancement of proteasomal-mediated degradation of the cellular protein APOBEC. APOBEC causes hypermutation of DNA and RNA and is incorporated into the virion. Vif enhances the degradation and decreases the synthesis of this protein (Chiu et al. 2005).

\textit{vpr}

Vpr, the product from \textit{vpr} gene, causes a G$_2$ cell cycle arrest by a mechanism that is not really understood yet. There is an increased activity of LTR in the G$_2$ phase which is why Vpr indirectly enhances the activity of LTR. Vpr also promotes entry of viral DNA into the nucleus (Flint 2004). Vpr is packaged into the virions and thereafter released into the cell where it influences the correctness of RT and thereby the mutation rate (Le Rouzic et al. 2005).

\textit{vpu}

The Viral protein U gene, \textit{vpu}, facilitates the export of virus particles from the cell (Haseltine 1991). Vpu is involved in the release of viruses from cells and also degrades CD4 molecules caught in the ER by gp160 (Bour et al. 2003). For further information see the section on the virus capsid.

\section*{Splicing}

Transcription of DNA into RNA results in a pre-mRNA. This pre-mRNA is modified into mature mRNA by mechanisms such as capping, polyadenylation and splicing. During splicing
serine-arginine rich proteins (SR) bind a specific sequence in the exons known as exon splicing enhancer (ESE) that defines the exons (Sanford et al. 2005). Thereafter several splicing factors are recruited to the pre-mRNA starting with cellular splicing factor U1snRNP binding to the 5’-splice site and another cellular splicing factor, U2AF binding to the pyrimidine tract of pre-mRNA and 3’- splice site (fig. 2). Several other factors bind to the pre-mRNA and a splicing complex called a spliceosome is formed. The spliceosome is a catalytically active complex that removes the introns from pre-mRNAs, resulting in a mature mRNA (Graveley 2000; Sanford et al. 2004). Splicing can be used to produce many different mRNAs from the same pre-mRNA, a mechanism known as alternative splicing. All HIV-1 pre-mRNAs are polyadenylated at the 3’-LTR and transcribed from the 5’-LTR promoter into one full-length transcript ~9 kb long (Pollard et al. 1998). This pre-mRNA is alternatively spliced into > 40 different mRNAs (Soret et al. 2005). The unspliced full-length mRNA encodes Gag and Gag-Pol proteins. The singly spliced mRNAs are ~4 kb and encode Env, Vif, Vpr and Vpu and the fully spliced mRNAs encode Rev, Tat and Nef (Pollard et al. 1998). Incompletely spliced mRNAs cannot be transported from the nucleus to the cytoplasm, so cellular mRNAs are fully spliced. HIV-1 uses the Rev protein to transport its incompletely spliced mRNAs to the cytoplasm (Flint 2004).

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FIG. 2. Spliceosome. SR proteins recruit cellular splicing factors like U1snRNP to the 5’-end of intron and U2AF to the polypyrimidine tract and the 3’-end of intron.
Life cycle

HIV virions enter cells by gp120 binding to CD4, a surface protein present mostly on T-lymphocytes but also e.g. on monocytes and macrophages. CD4 is normally a receptor for the major histocompatibility complex (MHC) class II on antigen presenting cells (Steffy et al. 1991). gp120 binds to CD4 and goes through a conformational change leading to binding of the co-receptor α-chemokine receptor (CXCr4) in T-cells and β-chemokine receptor (CCR5) in macrophages. These bindings make gp41 dissociate from gp120 and induce fusion of the viral and cellular membranes (Krambovitis et al. 2005) (fig. 3). The HIV genome is stored in the virion as two copies of ssRNA (fig. 4) which are transcribed into dsDNA by viral reverse transcriptase (RT) (Pollard et al. 1998) within the first 6 h of infection (Haseltine 1991). Cellular tRNA\textsubscript{Lys} is used as a primer for RT during reverse transcription (Kleiman 2002). The transcribed DNA is incorporated into the host cell genome by viral enzyme integrase (IN). IN cleaves host DNA and viral DNA and joins the ends of the DNAs in the nucleus (Haseltine 1991).

![Diagram of HIV attachment](image)

FIG. 3. Attachment of HIV-1 to CD4+ cell. gp120 binds CD4 and CXCR4, and gp41 induces membrane fusion.
The virus capsid

Gag and Gag-Pol are the precursor capsid proteins and replicative enzymes respectively. Gag binds viral RNA, buds through the cell membrane together with Gag-Pol and forms an immature virion. The viral protease (PR) cleaves out itself from Gag-Pol precursor and cleaves Gag into p17gag, p24gag, p7gag and p6gag to produce a mature virion (fig. 4). To make the budding possible the number of CD4 molecules on the cell surface has to be reduced. Nef increases the degradation of CD4 and gp160 blocks new CD4 molecules in the endoplasmatic reticulum (ER) (Sierra et al. 2005). Vpu activates the degradation of these molecules and allows gp160 to advance from ER to cell surface (Bour et al. 2003).

FIG. 4. The HIV-1 virion

Killing of infected cells

The mechanism of cell death in HIV-1 infection is much debated. One theory is that Env protein present on the cell surface in infected cells can bind to CD4 and CXCr4 on uninfected T-cells. This may lead to a semifusion of plasma membranes and cell death. Another theory is that normal fusion between these cells can make the nuclei fuse so that the cells die. Another
possibility for cell death is that a virus infected cell during apoptosis may fuse to another cell and transfer apoptosis signals (Perfettini et al. 2005).

**Therapy and resistance**

When HIV replicates widely during the asymptomatic phase many new mutations can arise because of the lack of proof-reading during reverse transcription and the high replication rate. In every new HIV genome there is at least one new mutation and these mutations may bring resistance to the immune system and antiviral drugs. If the replication is blocked no new mutations can arise and the virus will not become resistant. Unfortunately there are problems in reaching all infected cells with treatment, and it is difficult to distribute the correct amount of drugs in the patient. Insufficient treatment most probably leads to resistance to the drug. When trying to overcome the risk of resistance, combinations of drugs are used, bringing a lower probability of the virus getting resistant, at least to all drugs used in combination. One drawback with combination therapy is the number of pills the patient has to take every day. This requires the patient to keep track of all the pills because forgetting taking the pills may lead to resistance. Taking several pills instead of just one is also more expensive. Still the combination therapy is the most effective treatment today, particularly for long-term treatment of the infection.

A broad spectrum of drugs is used today to stop the progress of an HIV infection to AIDS. *Nucleoside analogs – inhibitors of RT* stop the chain elongation when incorporated into DNA. One example is Azidodeoxythymidine (AZT), the first drug to be licensed. AZT is activated when phosphorylated by cellular enzymes and stops the elongation because the 3’-OH group of adenosine is replaced by an azido (N₃) group. AZT is not a good substrate for cellular polymerases because it appears first and with highest concentration in the cytoplasm. That, in fact, makes it a good substrate for RT. The drawback is that AZT can damage the
bone marrow and gives side effects such as headaches, nausea and muscle wasting.

*Nonnucleoside inhibitors* of RT. These inhibitors are non-competitive and bind to RT where they induce an allosteric change and inhibit the enzyme (Tachedjian et al. 2001). *Protease inhibitors*, PR autocleaves from the Gag-Pol polyprotein and then cleaves at seven other sites to produce the enzymes integrase, reverse transcriptase and some other proteins. The seven cleavage sites are similar, which has been used to create powerful protease inhibitors, peptide mimics (peptidomimetics). *Integrase inhibitors*. There are no inhibitors of integrase available today but it is a field for research. A big field for research today is in the development of a *vaccine*, research that has been going on for more than 18 years. Some problems with vaccines as well as all the other drugs are the fast arising mutations and the fact that the virus infects the immune system. Several different vaccines have been tested but none of them have been successful (Flint 2004). Some newly discovered drugs interfere with viral attachment and entry into the cells. One of these is Enfuvirtide, the first *fusion inhibitor* approved by the American Food and Drug Administration and also approved by the European Commission for the Treatment of AIDS. Enfuvirtide is a synthetic peptide that interacts with sites in gp41 and gp120, preventing conformational changes and thereby fusion of virus and cell membranes. Even though approved, resistance to this drug has been reported in treated patients, and more studies are being done. There are more drugs in clinical trials targeting attachment and fusion, e.g. the entry inhibitor AMD-070 that inhibits binding of gp120 to CXCR4 (Krambovitis et al. 2005).

**Indole derivatives**

Soret et al. (2005) have shown that indole derivatives can inhibit or re-direct splicing in HIV-1 by interacting with serine-arginine-rich (SR) proteins. SR proteins are involved in splicing events and activate or inhibit splice sites in pre-mRNAs. The researchers screened several
indole derivatives and discovered that some of them completely prevented HIV-1 production while some re-directed splicing of virus mRNAs. Because alternative splicing is essential for HIV-1 13 different substances will be screened for influence on the splicing.

**Aim of project**

HIV-1 infection is persistent and today there are no drugs available to eliminate the infection. By constructing an HIV-1 plasmid and trying the effect of different substances I wanted to examine splicing in HIV-1 and to see if any of the substances could be a potential drug. The hope was to inhibit virus replication or re-direct splicing in HIV-1 so that immunogenic proteins such as Env can be over-expressed.
RESULTS

Construction of HIV-1 plasmid pDP

In order to investigate if HIV-1 splicing could be affected by a number of substances, previously synthesized to cure cancer, I had to start by developing a test system. The test system consisted of a plasmid containing an HIV-1 genome that could be transfected into human cells and express the HIV-1 mRNAs. In order to prevent production of infectious virions, I constructed an HIV-1 plasmid with a deletion in the pol gene that inactivated essential proteins. There already existed a plasmid with a deletion in the pol gene but also in the gag gene (pNL1.3AE). Because I wanted this plasmid to contain as much as possible of the HIV-1 genome and all the splice sites as well I constructed a new plasmid. A plasmid named pDP containing the HIV-1 genome with a 2 kb deletion in the pol gene was constructed by PCR and cloning (fig. 5). PCR from pNLΔenv (pNL4.3 with deletion in the env gene) with primers BSS/APAA resulted in a 1340 bp fragment called BA. PCR with MLUS/SALA from pNLΔenv should be 1845 bp but resulted in a >2 kb fragment. Product from PCR with the same primers using the primary plasmid pNL4.3 as template was of correct size and was used instead; called MS (fig. 6). The conclusion is that pNLΔenv sequence is incorrect, at least in this region.
FIG. 5. The construction of pDP from HIV-1 genome. The fragment BA was amplified from pNLΔenv with primers BSS and APAA (red) and MS was amplified from pNL4.3 with MLUS and SALA (blue). The two fragments were ligated into pNL1.3AE and resulted in a plasmid that contained all the splice sites and was deleted in the pol gene. Lines indicate the parts of the genome included in the plasmids.

FIG. 6. Agarose gel electrophoresis of the MS fragments obtained by PCR from pNLΔenv and pNL4.3.

The BA and MS fragments from PCR were ligated into a Topo vector and amplified in TOP10 cells. The fragment BA was cloned into pNL1.3AE when cleaved with BSHII and SalI, and transformed into HB101. Purification and cleaving of pNL1.3AE containing BA fragment showed a correct cloning and MS fragment was ligated into pNL1.3AEBA using MluI and SalI enzymes to produce plasmid pDP. After purification, the plasmid was cleaved
with restriction enzymes to verify that the sequences in the plasmid were correct and that the deletion was there. The pDP plasmid was 13 kb and cleavage with PvuII resulted in 7 bands. There should be 8, but the 3198 and 3480 bp fragments were probably too close to each other to give two distinguishing bands (fig. 7a). BSSHI and SalI cleaved out the inserted fragment which is totally 3.2 kb; this band showed that the deletion was there. SacI should give 5.8, 3.6 and 3.6 kb fragments. Two bands could be seen on the gel; 5.8 and 3.6. KpnI has three restriction sites in pDP and should give 8.1, 2.7 and 2.2 kb fragments, which it did (fig. 7b). The other enzymes cleaved pDP once and linearized the plasmid. The conclusion was that pDP had the correct sequences and could be used in further analysis.

**FIG. 7.** Agarose gel electrophoresis of pDP after cleavage with restriction enzymes. a pDP cleaved with PvuII and EcoRI b pDP cleaved with different enzymes.

**Test of pDP and analysis of substances possibly influencing splicing**

Cytoplasmic mRNA was extracted from HeLa-cells transfected with pDP, other HIV-1 plasmids and pDP together with substances A-M. The quality of RNA was checked by
running it on a 1 % agarose gel. The RNA was not degraded (fig. 8) and could be used for northern blot.

![FIG. 8. Extracted RNA, quality control on 1 % agarose gel. Each well represents one mRNA sample.](image)

To check pDP mRNA expression, mRNA from transfections with only pDP and pDP co-transfected with pL3tat, a \textit{tat} expressing plasmid, were run on northern blot gel together with HIV plasmids pNL1.4a7 and pNL1.5EU used as size markers. pNL1.4a7 and pNL1.5EU express \textit{rev} and \textit{env} mRNAs respectively. The bands from the pDP samples corresponded to ~7 kb full length mRNA, 4 kb partially spliced mRNAs and 2 kb fully spliced mRNAs. Samples co-transfected with pL3tat showed the same mRNAs together with \textit{tat} mRNA produced from pL3tat. No mRNA could be seen from samples with pNL1.4a7 and pNL1.5EU because these plasmids need Tat and Rev to express mRNA and should have been co-transfected with plasmids expressing those proteins (fig. 9). pDP was compared with pNL1.4a7 and pNL1.5EU in another transfection and that experiment showed correct sizes of mRNAs (fig. 10) thus, pDP could be used for analysis of the influence on splicing by various substances.
In order to test if substances A-M had any effect on splicing of transcripts from pDP, the
plasmid was transfected into HeLa cells together with the substances. Northern blots with the
extracted mRNA showed varying results. Analysis in Northern blot 1 (fig 10) was difficult to
do because the bands were very weak; this membrane had to be exposed for a long time.
Many substances showed varying results in different analysis.

Because the substances are thought to inhibit splicing one could expect the 7 kb mRNAs to be
more abundant and 4 kb and 2 kb mRNAs to be less abundant when affected by the
substances. This should be because inhibition of splicing inhibits production of Rev protein
which is encoded by a fully spliced mRNA. Rev is needed to transport the incompletely
spliced mRNAs to the cytoplasm. Depletion of Rev leads to accumulation of mRNAs in the
nucleus. The effects of the substances that could be seen was just a decrease in amount of all

FIG. 9. Control of pDP mRNA
expression by northern analysis using 5’-
LTR probe.
the different mRNAs, spliced and non-spliced. This is because only cytoplasmic RNA was extracted and not nuclear.

In fig. 12 the first six lanes showed mRNA from one transfection occasion and the other seven lanes showed mRNAs from another transfection occasion. Substance F decreased mRNA levels in fig. 11 while there was no difference between control and F in fig. 12. That could have been due to the fact that in fig. 12 the control (pDP alone) might have been too weak which gave the wrong results when comparing the mRNA levels with the substances to that one. Unfortunately there was only one control, so it was difficult to say if this was the problem. The control might have been weak due to low efficiency during transfection or errors when measuring mRNA concentration before loading on the gel. This might also be the reason for the apparent increase in mRNA levels with substances D and E. Other substances that gave different results were H and J. This might also have been due to errors during measuring and low transfection efficiencies.

There were some substances that seemed to decrease the mRNA levels. Those were substances G, I, L and M (fig. 11-12) which seemed to decrease the overall level of mRNA. A decrease in the mRNA levels indicated that the substances inhibited splicing.
FIG. 10. Northern blot showing mRNAs from transfection with pDP or PDP + substances.
FIG. 11. Northern blot with mRNAs from transfections with pDP and pDP + different substances.

FIG. 12. Northern blot showing mRNAs from transfection with pDP and pDP + substances. All wells contain 4 μg of mRNA except from pDP + B, C and L which contain 2 μg.
**pDP 4 kb and 2 kb mRNAs identified by RT-PCR**

To further analyse pDP and the expression of mRNA, RT-PCR was used to see different spliced mRNAs. mRNAs from pDP were transcribed into cDNA by reverse transcriptase and amplified with two primers in PCR. The primers bind to 5'-LTR and 3'-LTR respectively and will detect all the different mRNAs and their different splice versions (fig.1). The DNA after amplification should have been about 220, 300, 400, 600 bp and bigger (fig.13a) because of the primers positions in the genome (fig. 5). The marker (1 kb) used was not accurate enough, a 100 bp marker should have been used. The gel did not give enough sharp bands, probably because it was not completely polymerized and because there might have been air bubbles in the gel. The data suggest a small decrease in mRNA levels with substance I compared to the other substances (fig. 13b). The negative controls with only pDP transfected were different in the two samples, probably because of different transfection efficiencies and because of the low quality of the gel. This analysis has to be repeated to see the true effect of the substances.

![FIG. 13. RT-PCR from pDP mRNA. a) mRNA from pDP. b) mRNA from HeLa cells transfected with pDP and different substances.](image-url)
Expression of Gag from pDP

To see if the Gag protein was expressed from pDP, proteins were extracted 24 h post transfection. A Western analysis showed that the intermediate p55gag was expressed from pDP (fig. 14). Substance C, I and J seemed to decrease the expression of p55gag while substance G did not (fig 14). The decrease in p55gag levels might have been due to inhibition of splicing and thereby inhibition of the production of Rev protein because rev mRNA was fully spliced. Rev is needed to transport unspliced mRNAs like gag-pol to the cytoplasm, otherwise gag and gag-pol mRNAs will accumulate in the nucleus and no Gag or Gag-Pol proteins be expressed.

FIG. 14. Western blot of proteins extracted from HeLa cells transfected with pDP and substances C, G, I and J. The p55gag protein was detected with a p24gag monoclonal antibody.
Summary of the effects on mRNA and protein levels by the 13 different substances

Some of the substances had repeated negative effects on HIV-1 mRNA and protein levels. Those were G, H, I, J, L and M while substances C and F showed different results in protein and mRNA levels respectively (Table 1). Substances D and E seemed to increase the levels on mRNA (Table 1). Because substances C, H, J and M showed different results in different experiments they are sorted into more than one type of effect in table 2.

Table 1. Effect on mRNA and protein levels of the 13 substances\(^1\)

<table>
<thead>
<tr>
<th>Substance</th>
<th>mRNA(^2)</th>
<th>Protein</th>
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<tbody>
<tr>
<td></td>
<td>Fig. 11</td>
<td>Fig. 12</td>
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<td>B</td>
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<td>E</td>
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<tr>
<td>M</td>
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\(^1\)- = large decrease; - = small decrease; / = no effect; + = small increase; ++ = large increase

\(^2\)The data in Fig 10 are not included since the bands were too weak to permit any conclusions.

Table 2. Substances sorted by their effect on mRNA and protein levels.

<table>
<thead>
<tr>
<th>Effect</th>
<th>Substance</th>
</tr>
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<tbody>
<tr>
<td>Large decrease</td>
<td>H, I, J, L, M</td>
</tr>
<tr>
<td>Small decrease</td>
<td>C, G, H, J, L, F</td>
</tr>
<tr>
<td>No difference</td>
<td>B, C</td>
</tr>
<tr>
<td>Small increase</td>
<td>none</td>
</tr>
<tr>
<td>Large increase</td>
<td>D, E</td>
</tr>
</tbody>
</table>
DISCUSSION

In this project I have constructed a plasmid containing the HIV-1 genome which was called pDP. The plasmid was constructed to be non-infectious with a deletion in the pol gene. pDP was tested for mRNA expression and was shown to express 7, 4 and 2 kb mRNAs. Western analysis showed that pDP expresses Gag protein thus Western blots can be used to analyze expression of proteins from the plasmid. I used 13 different substances to see the effect on mRNA and protein levels expressed from pDP. Analysis showed that substances G, I, L and M seemed to decrease the levels. These substances can be thought to have an inhibiting effect on splicing.

Different analyses sometimes demonstrated opposite effects which might be due to different transfection efficiencies. Differences in the confluence of the cells during transfection can affect the efficiency of the plasmid and substance uptake. The methods for transfection might not be accurate enough for these experiments. I will therefore add the substances to the cells first, and then I will add the plasmid and fugene mix; I will make a cocktail of plasmid, fugene and SFM which I will use for all the different transfections to exclude small volumes and eliminate pipetting errors.

The analysis by RT-PCR has to be improved when it comes to the acrylamide gel, either through more practise or by using another type of gel e.g. denaturing acrylamide gel containing urea. Different dilutions of cDNA before PCR amplification can be used to detect an optimal concentration for analysis.

The effects of the substances have to be further examined by repeated tests using internal controls to eliminate the possibility of general influence on all, including cellular, mRNA and
protein levels. It would also be interesting to extract nuclear mRNA to see if there is an accumulation of 7 kb mRNA due to inhibition of splicing and thereby no Rev transport. The substances that have interesting effects G, I, L and M, will be tested on HIV-1 isolates for their effect on splicing.
MATERIALS AND METHODS

Strains, plasmids and growth conditions

Chemically competent *E. coli* cells (table 3) were used for cloning of the plasmid pDP. The different plasmids used during cloning are described in table 4. The bacteria were grown at 37°C in 1 x LB (10 % tryptone, 5 % yeast extract, 170 mM NaCl and 10 mM NaOH) containing 50 μg/ml ampicillin. The plates that were used for growth of bacteria contained 1 x LB and 1.5 g agar per 100 ml of LB. During transfection of pDP, cancer cells were used derived from the cervical of a patient suffering from cervical cancer. She was called Henrietta Lack which is why the cells are called HeLa-cells. The cells are infected with the cancer virus human papillomavirus type 18, HPV-18, that makes them divide as cancer cells. The HeLa-cells were grown in monolayer cultures at 37°C and 7 % CO₂ in Dulbecco’s modified Eagle’s medium, D-MEM (GIBCO, Invitrogen, Sweden), containing 100 μg/ml penicillin and streptomycin (PEST) and 10 % fetal bovine serum (GIBCO, Invitrogen, Sweden).

Table 3. Strains

<table>
<thead>
<tr>
<th>Strains</th>
<th>Properties</th>
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<tbody>
<tr>
<td>DH5α</td>
<td>Efficient for cloning</td>
</tr>
<tr>
<td>HB101</td>
<td>Ability to grow large plasmids</td>
</tr>
<tr>
<td>TOPO10</td>
<td>Very efficient for cloning</td>
</tr>
<tr>
<td>HeLa</td>
<td>Dividing cancer cells</td>
</tr>
</tbody>
</table>

Table 4. Plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Properties</th>
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<tbody>
<tr>
<td>pNL4.3</td>
<td>Contains the whole HIV-1 genome, derives from HXB2 isolate</td>
</tr>
<tr>
<td>pNL1.3AE</td>
<td>pNL4.3 deleted in the gag and pol genes</td>
</tr>
<tr>
<td>pNLΔenv</td>
<td>pNL4.3 deleted in the env gene</td>
</tr>
<tr>
<td>pCRII-Topo</td>
<td></td>
</tr>
<tr>
<td>pL3tat</td>
<td>Contains tat coding regions and expresses Tat</td>
</tr>
<tr>
<td>pNL1.4a7</td>
<td>Expresses rev and env mRNA</td>
</tr>
<tr>
<td>pNL1.5EU</td>
<td>Expresses nef mRNA</td>
</tr>
<tr>
<td>pNL1.3AEBA</td>
<td></td>
</tr>
<tr>
<td>pNL1.3AE containing BA fragment</td>
<td></td>
</tr>
</tbody>
</table>
**Polymerase chain reaction**

Polymerase chain reaction (PCR) was carried out from a plasmid called pNLΔenv, i.e. HIV-1 plasmid pNL4.3 with a deletion in the envelope gene. The amplification BA, was obtained by using primers BSS and APAA (table 4). The second amplification was called MS and was obtained by using primers MLUS and SALA (table 5).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ – 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSS</td>
<td>GGCTTGCTGAAGCGCGACGGCAAGGG</td>
</tr>
<tr>
<td>APAA</td>
<td>GGTCGACGGACGCGTGCCCTTTTTCTAGGGGCTGC</td>
</tr>
<tr>
<td>MLUS</td>
<td>GGGGCCCCGGACGCGTATCAAGAGACTGATATACAA</td>
</tr>
<tr>
<td>SALA</td>
<td>TATTCGCTATGTCGACACCC</td>
</tr>
<tr>
<td>BAMA</td>
<td>GCCAAGGATCCGTTCTACTAATCGAATGG</td>
</tr>
</tbody>
</table>

The PCR program for BA was 1) 94°C 10 min, 2) 94°C 1 min, 3) 64°C 1 min, 4) 72°C 2 min, 5) 72°C 10 min. Steps 2-4 were repeated 30 times. The PCR program for MS was the same as for BA but with annealing temperature 45°C and extension time 3 minutes. The PCR products were purified on 1 % low melting point (Lmp)-gel using 5xTBE buffer (270 g Tris, 137.5 g boric acid, 0.01 M EDTA, total volume 5 l). The DNA of the correct size was cut out, placed in an eppendorf tube and left at 65°C for 10 min. After vortexing 1 ml of Wizard® Minipreps DNA Purification Resin (Promega, USA) was added and the samples were briefly vortexed three times over a 1 min period. The samples were purified in a Wizard miniprep column (Promega, USA) and the column was washed with 80 % 2-propanol. DNA was eluted by adding 30 μl dH2O to the center of the columns and spinning for 1 min at 200 g.
Cloning

Purified PCR fragments were ligated separately into pCRII-Topo vector (Invitrogen, Sweden) according to the manufacturer, transformed into chemically competent TOP10 E. coli (Invitrogen, Sweden) and amplified over night at 37°C. The BA fragment was cleaved out with BSSHII and SalI (Fermentas, Germany) according to manufacturer and ligated into pNL1.3AE (fig.5), an HIV-1 plasmid deleted in the gag and pol genes. pNL1.3AE containing the BA fragment (pNL1.3AEBA) was transformed into DH5α cells or HB101 cells and amplified over night at 37°C. The MS fragment was cleaved out from pCRII-Topo vector with MluI and SalI (Fermentas, Germany) as suggested by manufacturer and ligated into pNL1.3AEBA. pNL1.3AE containing BA and MS fragments were grown in HB101 cells and purified with QIAGEN® Plasmid Maxi Kit (Qiagen, Sweden). The final plasmid was called pDP and was checked by cleaving with SacI, PvuII, KpnI, SpeI, EcoRV, BSSHII and SalI (Fermentas, Germany) according to recommendations from the manufacturer. The concentration of the purified plasmid was measured by a spectrophotometer at 260 nm.

Reverse Transcription Polymerase Chain Reaction

The transcription of mRNA into cDNA was carried out using Superscript™ II RNaseH - Reverse Transcriptase (Invitrogen, Carlsbad, USA) according to recommendations from the manufacturer. PCR of cDNA was carried out with primers that bound to the 5’- and 3’-LTR respectively; BSS and BAMA (table 1). The PCR program for amplification was 1) 94°C 10 min 2) 94°C 1 min 3) 55°C 1 min 4) 72°C 1 min 5) 72°C 10 min step 2-4 was repeated 35 times. The products from PCR were run on a 5 % acrylamide gel containing 5 % 29:1 polyacrylamide mix (VWR International, Sweden), 0.375 M Tris-Cl pH 8.8, 1 % SDS, 1 % ammonium persulfate (APS), 0.1 % tetramethylethlenediamine (TEMED) and dH2O up to 40 ml.
**Construction of a probe**

A 600 bp fragment in 5’-LTR was prepared for a probe template by cleaving pDP with BSSHII and EcoRV, running it on 1% low melting point gel and purify it. The probe was radio labeled with P\textsuperscript{32} and DECAprime\textsuperscript{TM} II (Ambion, USA) according to manufacturer.

**Transfection**

HeLa-cells were transfected with pDP and other HIV-plasmids called pL3tat, pNL1.4a7 and pNL1.5EU to use as size markers on northern blot. HeLa-cells were also transfected with pDP and different substances (A-M) to see if they had any effect on splicing of pDP mRNA. The substances were diluted in DMSO, as a negative control pDP was transfected together with the same amount DMSO as substances. For transfection 1 µg of each plasmid was used and 10 µM of substances. Transfection was done with 200 µl serum-free medium (SFM, D-MEM GIBCO, Invitrogen, Sweden) per transfection and 3 µl Fugene per µg of DNA (Roche, Basel, Switzerland). SFM and Fugene were incubated together at room temperature. After 5 min the SFM and fugene mix was added dropwise into the DNA and incubated for 15-30 min at room temperature. HeLa-cells were transfected when they were 50-70 % confluent by adding the DNA, SFM and fugene mix dropwise to the cells.

**Cytoplasmic mRNA extraction**

Cytoplasmic mRNA was extracted after incubation for 20 h at 37°C. The cells were lyzed with ISOB/NP40 (10 mM Tris-Cl pH 7.9; 0.15 M NaCl; 1.5 mM MgCl\textsubscript{2}) and spun for 2 min at 200 g. Cells were lyzed further with 5 x RPS buffer (0.5 M Tris-Cl pH 9; 50 mM EDTA pH 8; 2.5 % SDS). The RNA was extracted three times with phenol/chloroform (1:1) and precipitated with 0.25 M NaCl and 2-propanol in a 1:1 ratio. The RNA pellets were resuspended in 10-20 µl of RNase-free dH\textsubscript{2}O, depending on the size of the pellets.
Concentrations of mRNA were calculated from the absorbance at 260 nm according to the equation \((A_{260} \times 40 \times \text{dilution factor})/1000\).

**Northern blot**

mRNAs were separated on a gel containing 1 g agarose, 1 x 3-(N-Morpholino)-propanesulfonic acid (MOPS) running buffer, 1.23 M formaldehyde and dH\(_2\)O up to 100 ml. For running buffer 1 x MOPS was used (0.2 mol MOPS; 0.01 M Na-acetate; 1 mM EDTA pH 8). The gel was pre-run for 5 min at 5-6 V/cm before the samples were loaded. For each sample 2-5 \(\mu\)g of mRNA was loaded and the gels were run for 3.5 h at 80 V. Thereafter the gels were put on transfer to nitrocellulose membranes over night using 20 x SSC (3 M NaCl, 0.3 M sodium citrate) as transfer buffer. The membranes were baked in a vacuum oven at 80°C for 1-2 h to fix the RNA to the membranes. Pre-hybridization-hybridization solution was prepared by mixing 5 x SSC, 5 x Denhart’s solution (0.05 g Ficoll, 0.05 g polyvinylpyrrolidone, 0.05 g bovine serum albumine, total volume 50 ml), 10 % dextrane sulphate, 50 % formamide, 0.1 % Na-pyrophosphate, 100 \(\mu\)g/ml ssDNA and 1 % sodium dodecyl sulfate. The solution was used to block unspecific binding of the probe.

**Western blot**

Transfected HaLa cells were washed twice with 1xPBS (140 mM NaCl; 3 mM KCl; 1.7 mM KH\(_2\)PO\(_4\); 10 mM Na\(_2\)HPO\(_4\) x 2 H\(_2\)O) and lysed with 1xRIPA (50 mM Tris-Cl pH 7.5; 0.15 M NaCl; 1 % Triton X-100; 1 % Na-deoxycholate, 0.1 % SDS and dH\(_2\)O up to 50 ml). The cell suspensions were transferred to eppendorf tubes and spun at 200 g for 15 min. The supernatants were transferred to new tubes and put on dry ice to freeze. After freezing, samples were thawed at 37°C and this procedure was repeated three times. To each sample was added 1 x loading dye (625 mM Tris adjusted to pH 6.8 by HCl; 6.25 % SDS, 50 %
glycerol and 6.25 mg bromophenol blue) and the samples were boiled for 3 min. The proteins were separated by electrophoresis at 100 V on polyacrylamide gels. A 12 % resolving gel was used containing 12 % 37:1 polyacrylamide mix (VWR International, Sweden), 0.375 M Tris-Cl pH 8.8, 1 % SDS, 1 % APS, 0.1 % TEMED and dH2O to 40 ml. A 5 % stacking gel was used containing, 5 % 37:1 polyacrylamide mix (VWR International, Sweden), 0.125 M Tris-Cl pH 6.8, 1 % SDS, 1 % APS, 0.1 % TEMED and dH2O to 10 ml. For electrophoresis 1 x running buffer (25 mM Tris-Cl pH 8.3, 250 mM glycine and 0.1 % SDS) was used. The proteins were transferred to nitrocellulose filter (NC) by making a sandwich (fig. 15) and using 1 x transfer buffer (25 mM Tris; 190 mM glycine, 20 % methanol) and constant current 250 mA for 3h at 4°C. The NC filter was blocked over night in blocking solution 5 % non-fat dried milk in 1 x PBS-T (1.4 M NaCl, 27 mM KCl, 17 mM KH2PO4, 100 mM Na2HPO4 x 2 H2O and 0.3 % Tween-20) at 4°C. The filter was washed with PBS-T and incubated at 37°C for 1 h with primary rabbit anti-p24gag antibody, diluted in PBS-T to 1 µg/ml. The filter was washed with PBS-T and incubated with secondary antibody anti-rabbit IgG conjugated with horse-radish peroxidase (HRP) diluted 1:5000, at 37°C for 1h and washed with PBS-T. Enhanced chemiluminescence (ECL) detection was performed by mixing the two ECL reagents (Amersham Biosciences, Sweden) in a ratio 1:1. The NC filter was soaked in the mixture for 1 min, air dried, covered with plastic foil and exposed to X-ray film that was developed after for 1 min, 5 min and 10 min.

FIG. 15. Assembly of sandwich for transfer of proteins from the gel to the NC filter.
ACKNOWLEDGEMENTS

I would like to thank my supervisor professor Stefan Schwartz for the encouragement and patience, and for answering all my questions. I am so glad that I came to your lab.

Thanks to my lab partner Monika for a great support and for making the times in the lab full of joy.

Thank you Xiaomin and Margaret for helping me and for your valuable advice.

I would also like to thank all the other people in the Virology department. You have helped me with so many things.

Finally, thank you Jonas for all the advice and help and for always being there when I needed you!
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