Construction of Adenovirus Vectors for Studies of Protein Function and RNA Interference

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Abstract
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During an adenovirus infection the accumulation of alternatively spliced mRNAs is subjected to a tight temporal regulation. The IIIa protein is a structural protein expressed exclusively late after infection. To study the significance of the restricted IIIa protein expression we used a Tet-ON regulated adenoviral vector to overexpress the IIIa protein during the early phase of infection. The results show that unregulated IIIa protein expression caused a reduction in late viral protein accumulation and a slight block of viral DNA replication. Further, the results indicate that IIIa splicing might be subjected to a regulation via a feed back loop stimulating its own expression.

To improve the efficacy of vectors for regulated transgene expression, we constructed binary adenoviral vectors based on the Tet-ON and Tet-OFF systems. These vectors encode both the transcriptional activator and the tetracycline-regulated expression cassette from the same viral unit, ensuring that each infected cell will express both the activator and the reporter gene. In model experiments this system was shown to result in a tight control of gene expression with no detectable background expression of the transgene and induction levels reaching 500-600 fold.

Introduction of dsRNA into a cell will induce a sequence specific degradation of the homologous mRNA via a mechanism named RNA interference (RNAi). The adenovirus VA RNAs are short highly structured RNAs that are expressed in large amounts late during an adenovirus infection. Here we showed that both VA RNAI and VA RNAII functions as virus-encoded suppressors of RNAi, by interfering with the activity of Dicer, the enzyme that cleaves the initial dsRNA to short-interfering RNAs (siRNAs) that mediate RNAi. Further, the VA RNAs themselves are substrates for Dicer and are cleaved into siRNAs in vivo that are incorporated into active RNA-induced silencing complexes.

There is a great interest in developing novel therapeutic strategies based on RNAi. We constructed adenoviral vectors that express short hairpin RNAs, which in vivo will be cleaved to siRNAs that induce sequence-specific RNAi. We compared the efficiency of RNAi induced by vectors based on the viral VA RNAI and the human U6 promoters. Our results suggest that under conditions where the recombinant virus does not replicate, the VA RNA promoter is more effective in down regulating target gene expression, whereas the U6 promoter was more effective under replicative conditions.

Keywords: Adenovirus, viral vectors, VA RNAI/II, RNAi, Tet-ON/OFF, RNA splicing

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To my family with love
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Abbreviations

CAR: Coxsackie and adenovirus receptor
CML: Chronic myeloid leukemia
CRAD: Conditionally replicating adenovirus
DBP: DNA binding protein
dsDNA: Double stranded DNA
FFU: Fluorescent forming unit
ITR: Inverted terminal repeat
MHC I: Major histocompatibility complex type I
miRNA: Micro-RNA
MLTU: Major late transcription unit
MOI: Multiplicity of infection
mRNA: Messenger ribonucleic acid
PKR: Double-stranded RNA-dependent protein kinase
Poly(A) site: poly adenylation site
pRb: Retinoblastoma protein
pTP: precursor protein for TP
RGD: Arginine-Glycine-Aspartic acid
RISC: RNA-induced silencing complex
RNAi: RNA interference
rtTA: Reverse tetracycline-responsive trans-activator
siRNA: Short interfering RNA
snRNA: Small nuclear RNA
Tet: Tetracycline
TP: Terminal protein
tRNA: Transfer RNA
tTA: Tetracycline-responsive trans-activator
VA RNA: Virus-associated RNA
Introduction

Viruses have played key roles in shaping the history of life on our planet by shuffling and redistributing genes in and among organisms and by causing diseases in animals and plants. They are unique from other life forms in that they can contain either RNA or DNA as their hereditary material. However, irrespective of their genetic material they are dependent on the host cell metabolic machinery for replication.

Properties that make viruses attractive as vectors is that during infection of a cell, viruses use specific entry mechanisms to bring in their own genetic material and subvert the biosynthetic machinery of the infected cell to ensure expression of their genes in order to allow viral replication and release. The development of gene delivery vectors suitable for \textit{in vivo} gene transfer has been actively pursued in recent years. Adenoviral vectors are widely used for efficient delivery of genes into a variety of cell types and organisms in basic research, in gene therapy applications, and in vaccine development.

Still there is a great need for further development of improved vector strategies that will enhance the specific gene expression, reduce vector dose toxicity and the induction of inflammation.

Introduction to adenovirus

Adenovirus continues to be an important model system for investigation of basic aspects of cell biology. It has been used to study several important cellular and molecular processes including cell cycle control, RNA splicing, and immune response regulation.

Taxonomy

Adenovirus was first discovered in 1953 as an agent causing upper respiratory tract infections in humans (Hilleman and Werner, 1954; Rowe et al., 1953). Adenoviruses are widespread in nature and the virus has been isolated from most types of animals. However, the best-characterized adenoviruses are the mastadenoviruses, which include the human, bovine, simian, equine, porcine etc adenoviruses, and the aviadenoviruses, which are viruses with a tropism exclusively for birds. The human adenoviruses comprise 51 distinct serotypes that cause acute respiratory, ocular, gastrointestinal and urinary
tract diseases and are classified into six subgroups (A through F), based on several different criteria like oncogenic potential, red blood cell agglutination and DNA homology (Davison et al., 2003; Wadell et al., 1980). Subgroup A are highly oncogenic while subgroup B adenoviruses are weakly oncogenic and the non-oncogenic viruses were originally classified as subgroup C. This group has subsequently been further subdivided into subgroups C, D, E and F based on additional criteria like DNA homology, restriction endonuclease cleavage pattern, polypeptide composition and immunological characteristics. Based on DNA sequence homology serotypes belonging to the same subgroup usually show a high degree of relatedness with more than 85% homology, whereas serotypes belonging to different subgroups show less than 20% sequence homology. Serotype 2 (Ad2) and serotype 5 (Ad5) belongs to subgroup C and are the most studied of the human adenoviruses. Despite the fact that several adenovirus serotypes can transform cells in vitro and in some cases also cause tumors in rodents (Trentin et al., 1962), the human serotypes have not been associated with tumour formation in humans.

**Structure**

Adenoviruses are non-enveloped viruses with icosahedral capsid symmetry of approximately 80 nm in diameter. The viral genome consists of a linear double stranded DNA (dsDNA) genome with a length of about 30-38 000 base pairs, and encodes for some 40-50 proteins, of which around 12 are components of the virion (reviewed in Russell, 2000; Rux and Burnett, 2004). The adenovirus genome is characterized by an inverted terminal repeat (ITR) ranging in size from 36 to over 200 bp, which functions as origins for viral DNA replication. The 5' ends of the viral DNA are covalently linked to a terminal protein (TP), which serves as a primer protein that brings the penultimate dCTP that serves as the start nucleotide for initiation of the first round of the DNA synthesis by the viral DNA polymerase (Liu et al., 2003). TP is made as a precursor protein, pTP, that is cleaved to mature TP by a virus-encoded cysteine protease during the final stages of virion maturation.

The capsid contains 240 hexon capsomers, which form the triangular facets of the virus particle and twelve vertex proteins, named pentons. The penton capsomer consists of the penton base, which anchors the penton to the capsid and the fiber protein, which builds up the antenna like structures that protrudes from the vertices of the virus. The fibers functions as the attachment organ when adenovirus infects a cell and differ in length, and receptor binding capacity between serotypes (Philipson and Pettersson, 2004).
Genome organization

The lytic replication cycle is divided into two distinct phases; the early phase preceding viral DNA replication and a late phase, which follows viral DNA replication and is characterized by the synthesis of the structural proteins that are necessary for virion assembly. The viral genome encodes for a total of ten transcription units: five early transcription units (E1A, E1B, E2, E3 and E4), which encodes for proteins that are required to reprogram the host cell metabolism to favour virus replication; two that become activated at intermediate times of infection (pIX and IVa2); and one that become activated at late times of infection, the major late transcription unit, which give rise to multiple mRNAs by alternative splicing and alternative poly(A) site usage (L1–L5) (Akusjärvi et al., 1986). Of specific interest for this thesis the subgroup C adenoviruses also contains two transcription units that encodes for two small highly structured non-coding RNAs, the virus-associated RNAs (see the VA RNAs).

Figure 1. Adenovirus genome: the genome organisation consists of five early units (E1A, E1B, E2, E3 and E4), two delayed early units (pIX and IVa2), the major late transcription unit, which encodes for multiple proteins at late time of infection, and virus associated RNAI/II (VA RNAs). Kindly provided by G. Akusjärvi.
The virus life cycle

The primary receptor for the subgroup C adenoviruses is the cellular coxsackie and adenovirus receptor (CAR) (Bergelson et al., 1997; Tomko et al., 1997). Adenovirus binds to CAR via an interaction mediated by the knob part of the fiber protein. After binding to CAR, the Arginine-Glycine-Aspartic acid (RGD) motif in the penton base makes a critical interaction with the cellular $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins, which functions as secondary receptors for subgroup C adenovirus (Nemerow, 2000). The interaction with the integrin leads to an activation of an intracellular signaling cascade that results in a clathrin-mediated endocytosis of the virus particle. The secondary receptor interaction is not essential for virus entry but has been shown to accelerate endocytosis by a factor of 60. After entry into the cell the virus escapes from the acidic milieu in the endosome by disrupting the endosomal membrane. Recent data suggest that protein VI is the viral protein mediating the endosomal release (Wiethoff et al., 2005). Once in the cytoplasm the partly disassembled virus particle is transported to the nuclear pore by microtubule and dynein/dynamin-dependent transport motors. After docking at the pore the viral DNA is imported to the nucleus through an interaction with the nuclear pore complex receptor CAN/Nup214 and histone H1 (Trotman et al., 2001). The journey of the virus from the cell surface to the nucleus is an efficient process. Thus, most virus particle that binds to CAR at the plasma membrane delivers the viral DNA to the nucleus within 60 minutes (Greber et al., 1997).

The E1A gene product is the first to be expressed and it activates the expression of other adenoviral transcription units by binding to viral promoters (Liu and Green, 1994) and also has the capacity to regulate the expression of a variety of cellular genes. E1A forces the cell to S phase by binding to pRb, a tumor suppressor protein that inhibits cell cycle progression and arrest cells at G1 phase (Weinberg, 1995). Binding to Rb releases transcription factor E2F, which is required for the activation of genes needed for DNA synthesis. The binding to and inhibition of p300/CBP by E1A also contributes to E1A-induced G1 to S phase progression of the cell cycle. p300/CBP and p300/CBP-associated factor (PCAF) have intrinsic histone acetylase activities (Bannister and Kouzarides, 1996; Ogryzko et al., 1996; Yang et al., 1996) and function as co-activators for a variety of transcriptional activators. Deregulation of the cell cycle by E1A, leads to activation of the tumor suppressor protein, p53, and triggers a cellular defense mechanism leading to apoptosis (Debbas and White, 1993).

The adenovirus E1B-55K protein, one of the two major proteins encoded in E1B region, is a potent inhibitor of p53-mediated transactivation and apoptosis (reviewed in Ben-Israel and Kleinberger, 2002). E1B-55K is also required for efficient nucleocytoplasmic transport and translation of late viral mRNAs, and for the shutoff of host mRNA nuclear export and of host pro-
tein synthesis during late phase of infection. The 19-kDa polypeptide, also encoded by E1B, suppresses apoptosis via a mechanism analogous to the cellular Bcl-2 protein (Chen et al., 1996; Han et al., 1996).

The E2 region encodes proteins involved in viral DNA replication, which begins at about 8 hours after infection. The single-stranded DNA binding protein (DBP), encoded by the E2A region, is required for DNA replication and decorates the single-stranded viral DNA produced during the displacement synthesis used to replicate the viral genomes. The E2B region encodes for the precursor to the terminal protein (pTP) that is cleaved during viral assembly to the 55 kDa TP, which is covalently bound to DNA and functions as primer for initiation of viral DNA replication. The E2B region also encodes the 140 kDa DNA polymerase. Efficient viral DNA replication requires three additional cellular proteins: transcription factor NF-I, NF-III and Oct-I (reviewed in de Jong and van der Vliet, 1999).

Genes encoded by the E3 multi-gene transcription unit are non-essential for virus growth in tissue culture cells. The majority of the proteins encoded from the E3 region function to block the inhibitory effect the host immune response would have on the infection (Lichtenstein et al., 2004). For example, E3-gp19K prevents the major histocompatibility complex type I (MHC-I) from presenting viral antigens on the surface of the infected cell. Other E3 proteins, such as the RID, which degrades the receptors for TNFα and Fas, and the E3-14.7K protein, which blocks IKK activation, neutralizes the effector phase of the cellular immune response. E3 proteins also appear to be crucial for the capacity of adenovirus to establish a persistent infection. The E3 death protein (ADP) is expressed at very late stages of infection and is believed to mediate efficient lysis of cells and release of adenovirus from the infected cell (Tollefson et al., 1996).

The E4 region encodes for a total of seven proteins that participate at multiple steps in the viral remodelling of the host cell. For example, proteins from the E4 genes functions as regulators of transcription, alternative RNA splicing, the cell cycle, cell signalling, DNA replication and DNA repair (Tauber and Dobner, 2001). E4 proteins are also necessary for the selective transport of late viral mRNA to the cytoplasm and the shut-off of host cell protein synthesis (Halbert et al., 1985). In addition, E4 proteins have been shown to affect transgene persistence, vector toxicity and immunogenicity and also under some conditions cause oncogenic transformation.

Transcription of the viral structural proteins begins after initiation of DNA replication. These proteins are encoded by the major late transcription unit (MLTU). Since all mRNAs spliced from MLTU contain the common 5’ tripartite leader, they can be selectively exported to cytoplasm by the help of E1B-55K and E4orf6 (Pilder et al., 1986) and translated during the late phase of infection and during cell stress by a ribosome shunting mechanism (Yueh and Schneider, 1996; Yueh and Schneider, 2000). After protein synthesis, they are transported to the nucleus where the virus assembly takes place.
Adenovirus VA RNAI is required for efficient translation of viral mRNAs at late times after infection (Thimmappaya et al., 1982) and is described in more detail below.

Adenoviral vectors

Because of the high stability of the virus particle and the easiness by which the virus can be grown and purified adenovirus became one of the most important viral model systems during the 1960s. The large interest in adenovirus resulted in a rapid increase in the knowledge of viral genome organization and viral control of gene expression. Further, it turned out that adenovirus infects a wide range of dividing and non-dividing tissue culture cells and does not integrate into the host genome. The virus is also relatively easy to manipulate in vitro and can be purified to high titers in large quantities. Taken together all these features made adenovirus very attractive as a vector system for various gene transfer applications like gene therapy, cancer treatment and vaccination (reviewed in Hitt et al., 1997; Hitt and Graham, 2000).

In spite of the above-mentioned advantages, there are some obstacles that hamper pre-clinical or clinical applications of recombinant adenovirus vectors. Thus, viral proteins produced in low amounts, also with non-replicating templates, may provoke anti-viral immunological reactions of the host against adenovirus-infected cells. The first generation of adenovirus vectors has been created by deleting the E1 gene cassette, which is essential for activation of viral gene transcription. By removing both the E1 and the E3 regions, the vectors are capable to harbor inserts up to 7.5 kb (Graham, 1987). The E3 gene encodes proteins that counteract the host immune response and is not necessary for virus growth in tissue culture cells. First generation adenovirus vectors are useful in settings, such as cancer therapy and vaccination. However, due to the risk of viral gene expression at high MOI, which promotes viral gene expression and DNA replication in certain cell lines and some level of toxicity in vivo (Gorziglia et al., 1996; Lieber et al., 1996), they are not suitable for long term expression of the transgene in immunocompetent individuals (Li et al., 2005). Thus, viral gene expression in E1-deleted viruses are greatly reduced but not completely turned off leading to a stimulation of the immune system. Also, since the first generation of viral vectors typically is propagated in 293 cells there is a low risk that homologous recombination between the vector and the integrated copy of the E1 region regenerates replication competent adenoviruses. The second generation of adenovirus vectors with additional deleted genes, for example in region E4, in addition to E1, have shown long term survival of transduced hepatocytes in mice for the transgene product (Dedieu et al., 1997). Also E1 and E2 deleted adenovirus vectors have been shown to have an increased
persistence (Hu et al., 1999). More recently, the whole adenovirus coding sequence has been removed to form minimal sequence or “gutless” vectors (Kochanek, 1999). These vectors have an enormous capacity to carry therapeutic genes (around 35 kbp). However, since they just contain the inverted terminal repeat (ITR) sequences and the viral packaging sequence, they have to be grown in the presence of a helper virus that supplies all the viral gene products necessary for viral replication and virus assembly. Consequently, homogenous preparations of gutless vectors are difficult to produce. Usually a small, but detectable contamination of helper virus can be detected in the final virus preparations (around 0.1%) (Palmer and Ng, 2003).

For the use of adenovirus vectors in tumour therapy, conditionally replicating adenoviruses (CRADs) have been developed. The best-studied CRAD is the ONYX-015 virus (Bischoff et al., 1996). This virus contains a deletion that inactivates the E1B-55K gene. Since the E1B-55K protein binds to the p53 tumour suppressor protein and inhibits p53 as a transcriptional activator protein this viral protein serves as an important function in lytically infected cells by blocking p53-mediated activation of apoptosis. The majority of human tumours contain a mutation in the p53 gene, hence, ONYX-015 should, in theory, be able to replicate in these cells causing cell lysis and release of progeny viruses that can spread and destroy neighbouring tumour cells. In contrast, in normal cells (p53 wild type), virus replication should, in theory, be seriously impaired by the active p53 protein, which will be capable of inducing apoptosis. The results from clinical studies with ONYX-015 have shown that replication of the virus in tumours are transient with only a limited clinical effect. However, in combination with chemotherapy or radiotherapy the anti tumour effect of ONYX-15 was considerably improved (Rogulski et al., 2000; You et al., 2000). Recently it was shown that the tumour selectivity of ONYX-015 was greatly improved if infections were done in combination with a heat-shock treatment (O'Shea et al., 2005).

**Retargeting adenoviral vectors**

Genetically engineered viral vectors theoretically retain the cellular tropism of the wild-type virus and deliver the transgene to the malignant cell population without being propagated as an ongoing infection. Transduction efficiency is believed to mainly depend on the surface receptor expression on the host cell. To re-target the vectors specifically to cancer cells, attempts have been made to modify the tropism of the wild-type virus by replacing the viral components that mediate cell binding and internalisation with re-engineered receptor binding modules that recognize cancer-associated antigens (Cosset and Russell, 1996; Kasono et al., 1999; Krasnykh et al., 1996; Wickham, 2000).
Adenovirus belonging to subgroup C binds to the cellular CAR receptor with high affinity. The initial interaction occurs between the knob part of the fiber and CAR. In a second step the penton base interacts with the cellular $\alpha_\text{v}\beta_3$ and $\alpha_\text{v}\beta_5$ integrin co-receptor. However, human leukemic cells express low levels of these receptors and infection of these cells with the traditional type of adenovirus vectors is therefore very inefficient (Mentel et al., 1997) (our own observation). Fiber modifications have the potential to increase the efficiency of adenovirus-mediated gene delivery to specific cell types. It has been reported that subgroup B adenoviruses use the CD46 as the receptor (Sirena et al., 2004). Reconstruction of recombinant viruses were the Ad5 fiber has been substituted with the Ad35 fiber has shown a dramatic increase in transduction efficiency of leukemic cells (Nilsson et al., 2004; Yotnda et al., 2001).

Tetracycline regulated system

Regulation of expression of toxic proteins is desirable for a safe and effective cancer gene therapy. To avoid an inflammatory response using viral vectors at high doses and yet reaching the transgene expression at the levels required to achieving therapeutic doses, regulation of gene expression by the bacterial tetracycline repressor system has been developed into a strong and efficient tool for transcriptional regulation in eukaryotic cells.

The tetracycline-responsive trans-activator (tTA) and the reverse tetracycline-responsive trans-activator (rtTA) proteins used in mammalian expression systems are fusion proteins between the *Escherichia Coli*-derived tet repressor protein and the herpes simplex virus VP16 activation domain (Gossen and Bujard, 1992). In the Tet-On system (rtTA), the activator protein requires doxycycline for binding to the tet operator and activation of the transcription. In contrary, with the Tet-Off system (tTA), transcription of the transgene is turned off in the presence of tetracycline. These systems have been widely used in combination with different RNA polymerase II promoters to express a transgene under regulatable conditions in different tissues (Russell et al., 2004; Srour et al., 2003; Xu et al., 2005).

The Tet-Off and Tet-On systems have been adapted and inserted into different viral vectors, like adenoviral, lentiviral and other retroviral vectors (Peng et al., 2004). The Tet system is useful even for solving the problem of rescuing adenoviral vectors expressing proteins that are either cytotoxic to the cell or interfere with virus replication. By combining the inducible promoter with a Lac repressor based block to transcription elongation, basal transgene expression was reduced significantly in a 293 cell-line expressing the Lac repressor protein (Edholm et al., 2001).

Beside the regulation of gene expression by the use of RNA polymerase II promoters, conditional regulation of gene expression using the mammalian
RNA polymerase III system has also been explored in combination with tetracycline regulated promoter cassettes. For example, insertion of a tet operator just upstream of the transcription start site in an U6 promoter cassette made it possible to control the expression of an antisense RNA against a chimeric epidermal growth factor receptor - green fluorescent protein gene (Ohkawa and Taira, 2000). Similarly, it has also been reported that U6 snRNA transcription can be regulated in an *E. coli* lac repressor system (Luukkonen and Seraphin, 1998). The Tet-ON system has also been applied to control gene expression using “Gutless” adenoviral vectors (Xiong et al., 2006) to reduce the stimulation of the immune system.

RNA polymerase III promoters

There are three basic types of RNA polymerase III promoters, type 1, type 2, and type 3 promoters. Type 1 promoters are found in the 5S genes and consist of a gene-internal element called the internal control region (ICR), that is subdivided into the A block, intermediate element, and the C block (Bogenhagen, 1985; Sakonju et al., 1980). Type 2 promoters are found in tRNA genes, the adenovirus 2 VA RNAI gene, and other genes (Galli et al., 1981; Sharp et al., 1981). These promoters consist of two gene-internal elements called the A and the B boxes. Type 3 promoters consist of a distal sequence element (DSE) that serves as an enhancer, a proximal sequence element (PSE), and a TATA box (Lobo and Hernandez, 1989) and were identified originally in the mammalian U6 snRNA gene, which encode the U6 snRNA component of the spliceosome (Das et al., 1988; Krol et al., 1987; Kunkel and Pederson, 1988). They are also found in, for example, the H1 RNA gene, which encodes the RNA component of human RNase P (Baer et al., 1990).

The control of RNA polymerase III transcription involve RNA polymerase III transcription factors, in particular TFIIIC2 and TFIIIB. The activity of TFIIIB is regulated by a number of factors including p53 and the retinoblastoma protein (Brown et al., 2000). RNA polymerase III promoters with different structures respond to different DNA-protein and protein-protein contacts in order to recruit the same RNA polymerase to the basal promoter. It has been shown that there are two forms of hTFIIIB; hTFIIIB-α that does not contain the TATA binding protein (TBP) and shows strong preference for transcription of the U6 over the VA RNAI gene and the TFIIIB-β which is predominantly active on intragenic pol III promoters (Teichmann and Seifart, 1995).
RNA interference

RNA interference (RNAi) is a recently discovered mechanism that leads to posttranscriptional gene silencing by acting as a sequence-specific RNA degradation process. This mechanism was first reported in *C. elegans* (Fire et al., 1998) and has later been described in almost all eukaryotic organisms. The mechanism of RNAi involves the cleavage of large dsRNAs into 21-23 nucleotide short interfering RNA fragments (siRNAs) by an enzyme called Dicer. Subsequently, one strand of the siRNA assembles with protein components into an activated RNA-induced silencing complex (RISC). The activated RISC binds to the complementary transcript by a base-pairing interaction between the siRNA anti-sense strand and the mRNA. If the complementarity is perfect, Slicer (the Ago-2 component of RISC) induces a specific cut in the mRNA, which results in mRNA degradation and gene silencing. Dicer is a member of the RNase III family of nucleases that cleaves dsRNA into siRNAs (Bernstein et al., 2001) with a 3′ overhang of 2-3 nucleotides and a 5′-phosphate and a 3′-hydroxyl termini (Elbashir et al., 2001).

In addition to cleaving dsRNA into siRNA, Dicer also is involved in the processing of 21-23 nucleotide single-stranded RNAs, so called microRNAs (miRNAs), that are processed from larger imperfectly base-paired dsRNAs in a two-step process. In the initiation step, a primary nuclear transcript (pri-miRNAs) is cleaved into approximately 70 nucleotide pre-miRNAs by a nuclear RNase III-like enzyme, named Drosha, and then exported to the cytoplasm where Dicer cleaves these large dsRNAs into the 21-23 nucleotide miRNAs, which are incorporated into RISC. However, the miRNA-containing RISC inhibits gene-expression by a posttranscriptional process (probably at the level of initiation of translation) by binding to its target mRNA with a duplex containing mismatches near its center (Olsen and Ambros, 1999). Therefore, a miRNA induces mRNA degradation when the target mRNA is engineered to perfectly match the miRNA (Hutvagner and Zamore, 2002). Conversely, a siRNA when modified to include centered non-matching nucleotides acts like a miRNA, causing translational inhibition rather than RNA degradation (Doench et al., 2003). The natural function of RNAi and its related processes seem to be protection of the genome against invasion by mobile genetic elements such as viruses and transposons, as well as orchestrated functioning of the developmental programs of eukaryotic organisms (reviewed in Agrawal et al., 2003).
Figure 2. The RNAi pathway. The large dsRNA is processed by Dicer, a member of RNase III nucleases, into many \( \approx 22 \) nt siRNAs, which subsequently are incorporated into RISC. The RISC identifies target messages based on complementarity between the siRNA and the mRNA leading to post transcriptional gene silencing.

The adenovirus VA RNAs

The two adenovirus-associated RNA (VA RNA) genes, VA RNAI and VA RNAII, are synthesized by RNA polymerase III and contain intragenic transcriptional control regions. The highly structured 166 nucleotide VA RNAI, which accumulates to about \( 10^8 \) copies per cell late in infection, is a potent inhibitor of the double-stranded RNA-dependent protein kinase (PKR). It binds the dsRNA-binding region of PKR and blocks it from becoming activated. PKR is an interferon-induced enzyme that when activated causes a general repression of translation in virus-infected cells. PKR binds to and is activated by dsRNA longer than 50 bp. Activated PKR inhibits translation by phosphorylating the key cellular translation initiation factor eIF-2. Since long dsRNA is the hallmark of virus-infected cells activation of PKR functions as a powerful mechanism to silence gene expression in virus-infected
cells. In fact, in the absence of VA RNAI, the failure of adenovirus to inactive PKR results in a more than 100-fold reduction in viral mRNA translation and virion production (Thimmappaya et al., 1982).

**Delivery of siRNA to target cells**

siRNA-induced gene silencing in mammalian cells has emerged as a promising tool to knockdown the expression of specific genes. During the last couple of years this method has been used extensively to study gene function in mammals. It has also been tested as a method to target many genes associated with human diseases, such as Leukemia (Scherr et al., 2003), and infections by HIV-1 (Boden et al., 2004; Lee et al., 2002), influenza infection (Ge et al., 2003), hepatitis B virus (McCaffrey et al., 2003) and hepatitis C virus (Wilson et al., 2003). In many studies, gene silencing was induced by introduction of synthetic siRNA by electroporation (Scherr et al., 2003; Wohlbold et al., 2003; Wohlbold et al., 2005) or siRNA transfection (Elbashir et al., 2002). However, the method for delivery of these small dsRNAs affects the efficiency of transfection (Walters and Jelinek, 2002) and in contrast to plants, siRNA effects are transient and restricted by the rate of cell division.

As an alternative method to deliver siRNA to cells, plasmids or viral vectors have been tested. In principle two approaches have been used. Either the sense and the antisense strands of the siRNA duplex are transcribed from separate promoters (Donze and Picard, 2002; Uchida et al., 2004) or siRNAs are expressed as fold-back stem-loop structures, so called short hairpin RNA (shRNA), that can be cleaved by Dicer into an siRNA within the cell (Kuninger et al., 2004). In both approaches the siRNAs are usually cloned downstream of a strong RNA polymerase III promoter. The most commonly used promoters are the mouse or human U6 small nuclear RNA promoter and the human H1 promoter. The advantage with RNA polymerase III promoters is that they usually are very strong and require a simple transcription termination signal consisting of four to five thymidines. A specific advantage with the U6 and H1 promoters is that the all the transcription regulatory elements are positioned upstream of the transcription initiation site making it possible to replace the entire U6 or H1 genes with the siRNA or shRNA gene cassettes of interest. For tissue-specific siRNA expression, RNA polymerase II promoters have been used to produce a long hairpin RNA that are processed in the nucleus by Drosha, transported to cytoplasm and cleaved by Dicer into a functional siRNA that is incorporated into RISC (Shinagawa and Ishii, 2003). The use of RNA polymerase II promoters for siRNA production mimics the way miRNAs are produced in cells (Lee et al., 2004).

Due to difficulties in transfection of some cell types viral vector-based expression system have been used to increase the efficiency of RNAi. In most of these studies retroviral vectors have been used (Devroe and Silver,
although adenovirus-mediated RNAi have also been reported (Carette et al., 2004; Kasahara and Aoki, 2005; Zhang et al., 2004). The advantage with retrovirus-mediated RNAi is that the virus will integrate and produce cells that stably express the shRNA.

Chronic myeloid leukemia (CML)

CML is caused by a translocation between two unrelated genes, the Abl tyrosine kinase (145 kDa) located at chromosome 9, which is involved in cell cycle regulation (Deininger et al., 2000), and the Bcr (160 kDa) gene located at chromosome 22. This translocation, which is referred to as the Philadelphia chromosome, forms the hybrid Bcr-Abl gene characteristic of CML. The breakpoint in Abl is variable over a region of 200 kbp, often between the two alternative exons 1b and 1a, sometimes 5′ of 1b, or 3′ of 1a, but always 5′ of exon 2. The breakpoint in Bcr is in the M-bcr (major breakpoint cluster region), a cluster of 5.8 kbp, between exon 12 and 16, also called b1 to b5 of M-Bcr. Most breakpoints being either between b2 and b3, or between b3 and b4 resulting in a 8.5 kb mRNA that is translated into an approximately 200 kDa chimeric protein. The three most common Bcr-Abl fusion proteins in CML are e1a2, b2a2 and b3a2. The b3a2 form of the Bcr-Abl protein is a 210 kDa protein and is detected in 95% of CML patients. Bcr-Abl encodes a constitutively active tyrosine kinase that is both necessary and sufficient to induce and maintain leukemic transformation. The increase in kinase activity of the bcr-abl fusion proteins leads to pathological phosphorylation of several downstream targets and oncogenic growth and inhibition of apoptosis.

One of the drugs that are used for treatment of CML is imatinib mesylate, a signal transduction inhibitor, which targets the Bcr-Abl kinase as well as a few structurally related kinases. Imatinib binds to the conserved ATP-binding pocket of Abl and forces the activation loop into an inactive, non-phosphorylated conformation (Mentel et al., 1997). The SH3 domain in Abl appears to play a critical role in inhibition of the kinase activity (Mayer and Baltimore, 1994; McWhirter and Wang, 1993). Normally 3BP1 (binding protein) binds to the SH3 domain in Abl and prevents SH1 activation. In Bcr-Abl fusion proteins the breakpoint fusing Bcr to Abl results in a deletion of the SH3 domain, therefore creating a constitutively active kinase.

It has been reported that leukemic cells have evolved mechanisms to become resistant to imatinib (Hochhaus, 2003; Sacha et al., 2003; Tipping and Melo, 2003), a finding that makes it interesting to develop alternative forms of therapy. Several reports have described the successful application of siRNA methods to knockdown bcr-abl expression in CML (Jenke et al., 2005; Li et al., 2003; Scherr et al., 2003; Wohlbold et al., 2005). Interestingly, Bcr-Abl siRNA treatment has also been shown to sensitise CML cells
to imatinib treatment and also kill an imatinib-resistant Bcr-Abl kinase domain mutant cell line (Wohlbold et al., 2003) Plasmid based expression of shRNA has shown that there is a 1 of 20 case risk for integration of the vector due to the generation of linearized or nicked form of the DNA (Jenke et al., 2005).
Present investigation and discussion

**Paper I**

Unscheduled expression of capsid protein IIIa results in defects in adenovirus major late mRNA and protein expression

Human adenoviruses, especially group C viruses such as serotype 5 (Ad5), have been studied extensively. Despite the knowledge about their tropism, gene expression, host cell interactions, and transforming capabilities that is well-characterized, important aspects of adenovirus biology remain to be understood. One of these is the control of mRNA and protein expression from the major late transcription unit (MLTU) during the infectious cycle. The MLTU, which encodes the majority of the virus structural proteins, generates an approximately 28 000 nucleotide pre-mRNA that is processed into more than 20 cytoplasmic mRNAs, which are grouped into five families (L1-L5). Each family consist of multiple alternatively spliced mRNAs with a common position of the poly(A) site (reviewed in Imperiale et al., 1995). During the early stage of infection the majority of mRNAs expressed from MLTU are the mRNAs from region L1 (Akusjärvi and Persson, 1981; Chow et al., 1979; Larsson et al., 1992; Nevins and Wilson, 1981). After the onset of the late phase, the level of mRNAs from all five 3’ coterminal families (L1-L5) increases dramatically, probably because viral DNA replication generates a dramatic increase in template concentration. All mRNAs produced from the MLTU receive a common 201-nucleotide long 5’ leader sequence, the tripartite leader (TPL), which serves important functions for nuclear to cytoplasmic export, translation, and stability of the late mRNAs (Huang and Flint, 1998; Logan and Shenk, 1984; Yueh and Schneider, 2000). MLTU encodes three characterized nonstructural viral proteins; the L1-52,55K, the L4-100K, and the L4-33K proteins (reviewed in Akusjärvi et al., 1986). The two L4 proteins have recently been shown to play an important function for progression from the early to the late pattern of MLTU protein expression (Farley et al., 2004).

Alternative splicing is an important mechanism regulating gene expression in eukaryotic cells. The accumulation of adenovirus mRNA is subjected to a temporal regulation during the infectious cycle (reviewed in Akusjärvi and Stevenin, 2003). Adenovirus L1 is an alternatively spliced gene where one 5’ splice site is joined to two different 3’ splice sites giving rise to two
mRNAs, the 52,55K mRNA which is expressed during both the early and late phase of infection and the IIIa mRNA which only can be detected at the late phase of infection. The 52,55K protein binds to the viral DNA packaging sequence (Ostapchuk et al., 2005) and is required for viral DNA encapsidation, and also stimulates viral DNA replication, late protein synthesis and virion assembly (Gustin and Imperiale, 1998; Hasson et al., 1992). The IIIa mRNA encodes for a phosphoprotein, with an estimated molecular weight of 66 kDa and is characterized as a structural component of the viral capsid, located as elongated monomers between the hexons and are believed to interact with protein VII (Boudin et al., 1980; Rux and Burnett, 2004).

The accumulation of the L1-52,55K and the IIIa mRNAs is tightly regulated during the virus infection. *In vitro* studies have shown that the IIIa 3′splice site has a much weaker sequence context compared to the 52,55K 3′ splice site and this difference in strength correlates with the reduced efficiency of IIIa splicing (Kreivi et al., 1991). The adenovirus IIIa branch site/polyprimidinoid tract has been shown to function as a virus infection-dependent splicing enhancer, the 3VDE, and is essential for regulated IIIa pre-mRNA splicing (Mühlemann et al., 2000). In combination with the IIIa repressor element (3RE) the 3VDE controls the splicing phenotype of a pre-mRNA in nuclear extract prepared from adenovirus-infected cells (Ad-NE) (Mühlemann et al., 2000). Although the activity of the SR family of splicing factors is severely reduced by a virus induced dephosphorylation in late adenovirus infected cells (Kanopka et al., 1998) the 3VDE may provide a mechanism by which adenovirus can sustain an efficient splicing of the IIIa pre-mRNA, even under conditions of limiting concentrations of functional SR proteins (reviewed in Akusjärvi and Stevenin, 2003).

Since IIIa splicing is subjected to a tight control during virus infection, it was of interest to test if the expression of the IIIa protein during the early phase of infection would have negative effects on MLTU gene expression and virus growth.

In this paper we constructed a recombinant adenovirus expressing the IIIa mRNA from a Tet-ON-regulated gene cassette substituted for the E1 region. The IIIa gene was expressed by a double infection strategy in HeLa cells with an activator virus, AdCMVrtTA (Molin et al., 1998) encoding the Tet-ON transactivator protein (Gossen et al., 1995) and the AdTetTrip-IIIa reporter virus. By adding doxycycline to the cell culture medium we were able to achieve a regulated expression of the IIIa protein beginning immediately after infection. Since these recombinant viruses lack the E1 region (Molin et al., 1998), the infected cells could not progress into the late phase of infection. By superinfection with a wild type adenovirus the E1 proteins were supplied in trans, allowing all the adenovirus genomes in the cells to progress through a normal course of infection. Our results showed that IIIa protein expression is tightly regulated in HeLa cells, with no detectable background and high induced expression when doxycycline was added (Figure
2). Superinfection with the wild type virus resulted in an activation of viral DNA replication and a further increase in the induced level of IIIa protein expression. By using an immunofluorescence assay 22 hours after double-infection, we could also show that the overexpressed IIIa protein is transported to the nucleus in the absence of other viral proteins (Figure 3). Since the temporal control of the alternative splicing is an important mechanism controlling lytic virus growth (reviewed in Imperiale et al., 1995) we tested whether overexpression of the IIIa protein affected L1 mRNA accumulation. Using a northern blot assay (Figure 4), we could show that IIIa protein expression during the early phase of infection reduces 52,55K mRNA accumulation, a result that suggests that L1 alternative splicing might be regulated by an auto-regulatory feedback mechanism. Thus, the IIIa protein may stimulate its own expression through a selective inhibition of 52,55K mRNA expression. Further, we examined the role of the overexpressed IIIa protein on viral DNA replication and late protein synthesis. The result showed that unregulated IIIa protein expression before entry into the late phase of infection has a slight inhibitory effect on viral DNA replication (Figure 5) and viral late structural protein synthesis (Figure 6).

**Paper II**

**Binary AdEasy vector systems designed for Tet-ON or Tet-OFF regulated control of transgene expression**

Control over the timing and the level of gene expression is under certain experimental conditions an important parameter to consider in the analysis of gene function or application in gene therapy. Several inducible gene expression systems have been developed for this purpose (reviewed in Zhu et al., 2002). An ideal inducible gene expression system for clinical applications requires a low basal expression in the absence of inducers combined with a high induced expression (i.e. a high induction ratio), low non-specific effects on endogenous cellular process, low immunogenicity of system components and confirmed safety of available biological inducers.

Adenoviruses are generally associated with mild infections in humans and are characterized by a number of features, which makes them particularly attractive as gene transfer vectors for gene therapy or immunisation (for reviews see Hitt et al., 1997; Hitt and Graham, 2000). Adenoviruses are stable, can infect a broad range of human cells, including those of the lung, liver, blood vessels and brain, and deliver its genetic payload to the nucleus without integrating into the resident chromosomes except under rare circumstances, and allows for inducible transgene expression. Several reports have shown that adenovirus causes humoral and cellular immune responses in a vector dose-dependent manner (reviewed in Russell, 2000). Thus, a lower
viral load is desirable for gene therapy applications. Inducible adenovirus vectors not only permit a regulated protein expression but also produce higher levels of protein compared to constitutive promoters. Adenovirus expression systems containing regulatory promoters have been used to produce toxic proteins that interfere with adenovirus replication (Massie et al., 1998), and to study the significance of defined proteins on adenovirus multiplication (this study and Molin and Akusjärvi, 2000). There are several regulatory systems that fulfil the requirements of an appropriate inducible system: the Tet-ON (Gossen et al., 1995), the Tet-OFF (Gossen and Bujard, 1992), lac operator-repressor (Cronin et al., 2001), ecdysone (Saez et al., 2000), anti-progesterone (Wang et al., 1994) and dimer-based (Rivera et al., 1996) systems.

We previously used a double-infection strategy to control reporter gene expression in a Tet-ON adenovirus based vector system (Molin et al., 1998). In this protocol cells are infected with equal FFUs of activator and reporter virus. The system works efficiently in tissue culture experiments were unlimited amounts of virus can be used. However, in experiments using animal model systems the disadvantage with this strategy is the reduced possibility that both the vector containing the activator and the transgene infect the same cell. In this paper we aimed to construct AdEasy vector systems (He et al., 1998) based on the tetracycline-regulated Tet-ON and Tet-OFF gene cassettes (Gossen and Bujard, 1992; Gossen et al., 1995) expressing the activator proteins and the reporter genes from a single viral genome. To increase the efficiency of delivery of both the tetracycline-responsive transcriptional activator (tTA or reverse tTA) and the reporter gene cassette we constructed four binary AdEasy vectors encoding the activator gene cassette inserted into the non-essential E3 region of Ad5 (reviewed in Imperiale et al., 1995), in the rightward and leftward orientation. As a reporter gene we cloned the rabies glycoprotein gene downstream of the tetracycline regulatable promoter cassettes, resulting in vectors AdTet-ON/Rab, AdTet-ONrev/Rab, AdTet-OFF/Rab and AdTet-OFFrev/Rab. To further restrict transgene expression we included a Lac repressor-based block to transcription elongation (Edholm et al., 2001; Fieck et al., 1992; Matthews et al., 1999) downstream of the reporter sequence. The idea behind this was to reduce the background expression of the reporter gene, which might be toxic to the virus growth leading to inhibition of recombinant virus production or reducing virus yields by interfering with virus replication (see Edholm et al., 2001). The rabies glycoprotein has been reported to be toxic to 293 cells (Matthews et al., 1999).
Figure 3. Genome organization of the Tet-regulated adenoviral vectors, expressing the activator protein encoded in E3 region and the reporter gene expressed from E1 region, from a single viral genome.

To compare the efficiency of glycoprotein expression 293-LacI cells were infected with the different AdEasy vectors expressing the glycoprotein and mRNA production was measured by Northern blot (Figure 4) and protein production by Western blot (Figure 3). The results suggested that both the Tet-ON and the Tet-OFF reporter viruses functioned with a very similar efficiency. By combining the two regulatory systems (Tet and LacI) we further showed that gene expression was tightly regulated with essentially undetectable levels of the rabies-glycoprotein expressed in the absence of inducer and several hundred fold induced expression.

By using 293-LacI cells (Edholm et al., 2001) we could further demonstrate that the Lac repressor-based roadblock to transcription elongation was effective and could be regulated by addition of IPTG to the culture medium (Figure 6).

It has been reported that it can be technically difficult, or impossible, to reconstruct recombinant viruses expressing genes that are cytotoxic from constitutively active promoters (Hu et al., 1997; Matthews et al., 1999; Rubinchik et al., 2000; Yoshida and Hamada, 1997). One such protein that has been reported to be problematic is the rabies virus glycoprotein (Matthews et al., 1999). Since the gene of interest can be induced to high levels in our Tet-regulatable system (Edholm et al., 2001; Molin et al., 1998) we tested the toxicity of the rabies glycoprotein to virus growth. In a comparison between the infection of 293-LacI- and 293 cells with our vectors, we could show that overexpression of the rabies glycoprotein, under our experimental conditions, has only a moderate inhibitory effect on adenovirus growth (Figure 5).
Suppression of RNA interference by adenovirus VA RNA

Introducing dsRNAs into cells result in a specific degradation of homologues mRNA (Fire et al., 1998) through a mechanism that is called RNA interference or RNA silencing (for a recent review see Tomari and Zamore, 2005). Small interfering RNAs (siRNA) are produced in vivo by cleavage of the dsRNA introduced directly or via a transgene or virus. RNA silencing was first recognized as an antiviral mechanism that protect organisms from RNA viruses (Lecellier and Voinnet, 2004; Li and Ding, 2001; Waterhouse et al., 2001), or which prevent the random integration of transposable elements. Later on its role as a mechanism regulating gene-expression became apparent when it was realized that specific genes in plants and animals encode short forms of folded dsRNA (reviewed in Bartel, 2004). Vertebrates have a large number of mechanisms to combat viral infections. One of the best characterized is the so-called interferon system, which is activated by the presence of dsRNA (Goodbourn et al., 2000; Samuel, 2001). The two best-characterized interferon-induced enzymes are the dsRNA-activated protein kinase (PKR) and the 2′-5′-oligo (A) synthetase. Activated PKR phosphorylates the alpha subunit of the eIF2 translation initiation factor (eIF2α), which results in an inhibition of protein synthesis in the infected cell. Similarly, the 2′-5′-oligo (A) synthetase is activated by dsRNA and induces a general RNA degradation via activation of RNase L, leading to cell death via apoptosis.

Many viruses produce dsRNA as a replication intermediate or as a side product during transcription. To avoid the negative effects of the interferon response pathway on viral replication, many viruses have evolved mechanisms to block the action of PKR (Weber et al., 2004). For example, adenovirus VA RNAI, Influenza virus NS1, reovirus sigma 3, vaccinia virus E3L are examples of virus-encoded anti-interferon gene products. The interferon response pathway is activated by dsRNA longer than approximately 50 bp (Tian and Mathews, 2001). Thus, dsRNA shorter than 30 bp does not activate these mechanisms but trigger instead RNAi, suggesting that RNAi also may play a role in the cellular defence against infection by human viruses.

Since the structure of the adenovirus VA RNAs resembles that of microRNA precursors (Akusjärvi et al., 1980; Andersson et al., 2005; Lu and Cullen, 2004), it was of interest to investigate whether the VA RNAs also function as suppressors of RNAi. Although no human virus had previously been shown to suppress RNAi there was precedence from the plant viral systems that also human viruses may have evolved mechanisms to suppress RNAi (Lecellier and Voinnet, 2004; Li and Ding, 2001). Thus, in plants, RNAi is believed to function as an early form of the innate immune system and many
plant RNA and DNA viruses have evolved proteins that acts as suppressors of RNAi.

To determine whether human adenoviruses suppress RNAi, the CML fusion protein b3a2 was used as a target. 293 cells were co-transfected with a reporter plasmid expressing the fusion gene b3a2-EGFP (Scherr et al., 2003) and a vector expressing a 29 bp short hairpin RNA (sh-RNA) (Paddison et al., 2002) homologue to b3a2. The result showed that the b3a2 sh-RNA reduced the number of GFP-expressing cells compared to a control non-homologous sh-RNA vector. By infecting the transfected cells with wild type adenovirus, this effect was inhibited, indicating that adenovirus infection results in a suppression of RNAi (Figure 1).

Since VA RNAs are highly structured similar to miRNAs and their capacity to interact with a number of cellular dsRNA binding proteins (Liao et al., 1998), we wanted to test whether they function as suppressors of RNAi. Co-transfection of a firefly luciferase reporter plasmid and a vector expressing an shRNA against luciferase resulted in a specific reduction in luciferase expression (Figure 2). Including plasmids encoding for VA RNAI (pVAI) or VA RNAII (pVAII) (Svensson and Akusjärv, 1984) showed that VA RNAI was more effective than VA RNAII in rescuing shRNA mediated suppression of luciferase expression. This difference was expected since VA RNAII has a much weaker promoter compared to VA RNAI (Bhat and Thimmappaya, 1984) and during a lytic infection more VA RNAI is produced (Söderlund et al., 1976). By placing the VA RNAs under the transcriptional control of the strong H1 promoter (Brummelkamp et al., 2002) both VA RNAs were able to recover the activity of luciferase in a dose-dependent manner (Figure 2b). We conclude that VA RNAI and VA RNAII have the capacity to function as suppressors of RNAi.

To answer the question how VA RNAs suppress the RNAi pathway, we wanted to examine the activity of the two key enzymes involved in the RNAi. It has been reported that VA RNAs suppress RNAi by competing for the exportin-5 receptor (Lu and Cullen, 2004). We prepared cytoplasmic extracts from HeLa- and 293 cells at different time points after infection and measured the activity of Dicer using a radiolabeled dsRNA template and the activity of RISC by the cleavage of a radiolabeled mRNA incubated with a complementary synthetic siRNA. The result indicated that adenovirus almost completely inhibits both enzymes at 16 h post-infection (Figure 3). To test the effect of the individual VA RNAs on Dicer, we used a panel of mutant adenoviruses defective in VA RNA expression (Bhat and Thimmappaya, 1984) to infect 293 cells. Cytoplasmic extracts were prepared after 22 hpi and the activity of Dicer was measured (Figure 5). The result showed that the wild-type virus efficiently suppressed Dicer activity whereas the double mutant failed to block. The mutant virus defective in VA RNAII expression showed an almost identical inhibition of Dicer as the wild-type virus. In contrast, the VA RNAI mutant virus showed a drastically reduced capacity
to suppress the activity of Dicer. This result suggests that VA RNAI is primarily responsible for the inhibition of Dicer in infected cells. Since addition of an excess of target dsRNA to infected extracts restored the activity of Dicer the VA RNAs appears to act as competitive substrates. This can explain the inhibitory effect of the VA RNAs on RNAi since, specifically, the copy number of VA RNAI increase up to 10^8 per cell at late times of an adenovirus infection (Mathews, 1995; Söderlund et al., 1976).

Since our result indicates that the VA RNAs functions as suppressors of RNAi (Figure 2) and act as competitive substrates sequestering Dicer (Figure 4B), we tested whether they are cleaved by Dicer themselves. Incubation of in vitro transcribed 32P-labeled VA RNAs with cytoplasmic extracts prepared from uninfected cells resulted in efficiently cleaved VA RNAs to small RNAs with the same length as a synthetic 21-nucleotide siRNA or an siRNA cleaved from a 29-base-pair shRNA (Figure 6). Compared to VA RNAI, VA RNAII was cleaved to siRNA, with much reduced efficiency (compare lanes 6 and 8). The result also indicate that cleavage of the VA RNAs to siRNA appears to be structure-dependent since hybrid VA RNAs were not cleaved into siRNAs.

We also show that the terminal stem of both VA RNAs are cleaved to siRNA late during a lytic infection and that both strands of the siRNA from VA RNAI is incorporated into functional RISC (Figure 8). For VA RNAII only one strand of the siRNA is incorporated into RISC in vivo. The observation that the VA RNA-specific siRNAs are incorporated into RISC in vivo and that the activity of RISC is inhibited in virus-infected cells indicate that potentially VA RNA-specific siRNAs may saturate RISC in vivo. Since the VA RNAs are encoded by intronic sequences (reviewed in Akusjärvi et al., 1986), incorporation of such VA RNA-specific siRNA into RISC would not induce cleavage of cytoplasmic mRNA and have negative effect on virus multiplication.

It is well known that VA RNAI serves an important function during adenovirus multiplication by antagonizing the cellular interferon response pathway (reviewed in Mathews, 1995). Here our data point to the possibility that VA RNAI may also have a function to facilitate virus growth by suppressing the RNAi pathway.

The progress in this area of research has been dramatic. Thus, during the last couple of months it has been reported that several viruses encode inhibitors that block RNAi. For example, the B2 protein of Nodamura virus, which is a small RNA virus that infects both insect and mammalian hosts, binds to pre-Dicer substrate RNA and RISC-processed RNAs and inhibits the Dicer cleavage reaction and, potentially, one or more post-Dicer activities (Sullivan and Ganem, 2005). The vaccinia virus E3L protein and the influenza virus NS1 proteins functions as suppressors of RNAi in Drosophila cells (Li et al., 2004). Also, sequences within HIV-1 is processed to siRNA and further, the HIV-1 Tat protein functions as a suppressor of RNAi by
inhibiting the activity of Dicer (Bennasser et al., 2005). In an independent study it was shown that adenovirus VA RNAI in transient transfections inhibits biogenesis of siRNA and miRNA by binding Dicer and competing for binding to the exportin 5 nuclear transport factor (Lu and Cullen, 2004).

Paper IV. Manuscript

Construction of adenovirus vector systems based on the viral VA RNAI and the human U6 promoters for short hairpin RNA expression in mammalian cells

Most adenoviral vectors that have been described are replication deficient viruses that have deletions affecting the E1- and the E3-regions of the virus (for reviews see Hitt et al., 1997; Hitt and Graham, 2000). The E1 region is subdivided into the E1A and the E1B transcription units, both that are essential for efficient virus replication. The E1A region encodes for a virus-specific transcription factor that is essential for efficient activation of transcription from all the other viral transcription units (Akusjarvi, 1993). The E3 region encodes for multiple viral proteins that have their primary function to suppress the host immune response against the virus (Lichtenstein et al., 2004). Therefore, this region is not essential for viral replication in cultured cells. Removal of the 3.5 kbp E3 region provides more space for cloning DNA for expression of large proteins or multiple proteins. Expression cassettes are typically inserted into the E1-region (for examples see Kuninger et al., 2004; Uchida et al., 2004; Zhang et al., 2004) or E3 (Matthews et al., 1999; Yarosh et al., 1996) or both regions (Danthinne, 1999) and contain an appropriate exogenous promoter driving the expression of the gene(s) of interest.

Since VA RNAI requires distinct cellular transcription factors compared to the human U6 promoter (Schramm and Hernandez, 2002; Teichmann and Seifart, 1995), it was of interest to examine the efficiency of RNAi induced by expression cassettes driving shRNA synthesis using both promoter constructs. As a model system to test the efficacy of adenovirus-mediated delivery of siRNA and promoter strength in cancer gene therapy we selected the b3a2 variant of Chronic myeloid leukemia (CML) as our model system (reviewed in Deininger et al., 2000). Since the Bcr-Abl protein expressed in these cells are necessary for maintenance of the transformed phenotype a gene targeting strategy aimed at destroying this chimeric oncoprotein would be an ideal way to kill tumor cells.

We constructed plasmids and non-replicating adenoviral vectors expressing a 29 bp shRNA homologous to the b3a2 variant of CML in order to test the efficiency of adenovirus-mediated gene knock. As a control we used another vector expressing an unrelated shRNA. These shRNAs are expressed
either from the human U6 promoter or the adenovirus VA RNAI promoter. As a reporter gene, we used a plasmid expressing a part of the breakpoint region of the b3a2 fused to Enhanced Green Fluorescence Protein (EGFP) (Scherr et al., 2003) and constructed the viral vector expressing the same reporter gene. In these experiments C33A cells were transfected/infected with the reporter and the shRNA-expressing plasmid/virus.

Figure 4. The shRNAs are produced from either U6 or VA RNAI promoter and after cleavage to siRNA bind to the homologous sequence in the reporter gene mRNA leading to degradation.

The results showed that shRNA delivery via a viral vector system was more effective compared to plasmid DNA transfection, and further produced a silencing effect that was more rapid compared to transfection. Northern blot analysis of the production of shRNA and the processed siRNA revealed that there are very little shRNA/siRNA accumulating in plasmid transfected cells compared to viral infected cells. Further, the plasmid based shRNA delivery system was not dose-dependent. However, increasing amounts of plasmid cotransfection resulted in an increase in shRNA expression. One possibility might be that the efficiency of shRNA processing might be the limiting factor. An accurate measurement of the ratio between shRNA and siRNA produced by these vectors remains to be done. However, a visual inspection indicates that the increase in shRNA expression was followed by a similar increase in siRNA levels. The result also showed that in cells not supporting virus replication (i.e. cells other than 293 cells) the virus expressing the shRNA cassettes from the VA RNAI promoter produced higher level of
shRNA and siRNA and was more effective at inducing RNAi compared to an identical expression cassette based on the U6 promoter. The obvious next step to be taken would be to construct recombinant viruses lacking the natural VA RNAI and VA RNAII genes. Such viruses would be predicted to be much more effective at inducing RNAi.

During the last year reports have started to emerge where adenovirus vectors have been used to transfer siRNA for gene silencing (for examples see Bain et al., 2004; Kasahara and Aoki, 2005; Kuninger et al., 2004; Uchida et al., 2004). The reports are still few and generally based on replication deficient adenovirus systems. However, one interesting report describes the use of a conditionally replicating adenovirus (CRAD) for gene silencing (Carette et al., 2004). Surprisingly, a comparison of plasmid based and CRAD-based RNA silencing showed that the plasmid transfection strategy worked much better. The authors speculated that this might be due to suboptimal transcription from the U6 promoter in the replicating adenoviral genome. Alternatively synthesis of large amount of the VA RNAs during virus replication may reduce the silencing efficiency by competing for cellular factors involved in RNAi (Carette et al., 2004).
Conclusions

Paper I
Overexpression of the IIIa protein during the early phase of infection inhibited 52,55K mRNA accumulation suggesting that L1 alternative splicing might be regulated by an auto-regulatory feedback mechanism. Further, unregulated IIIa protein expression negatively affected viral DNA replication and viral late structural protein synthesis.

Paper II
Regulation of gene expression from both Tet-ON and Tet-OFF reporter viruses functioned with a very similar efficiency.

Further, gene expression was tightly regulated with almost undetectable levels of the rabies glycoprotein expressed in the absence of inducer and several hundred fold induced expression. The Lac repressor-based roadblock to transcription elongation was effective and could be regulated by addition of IPTG to the culture medium. Overexpression of the rabies glycoprotein had only a moderate inhibitory effect on adenovirus growth.

Paper III
RNAi is suppressed in adenovirus-infected cells via a mechanism that blocks the activity of both Dicer and RISC. Adenovirus VA RNA expression blocks RNAi and is necessary for inhibition of Dicer during lytic virus growth. Further, the suppression of the activity of Dicer in infected cell extracts could be overcome by increasing the substrate concentration, suggesting that VA RNAs act as competitive substrates. The VA RNAs are processed by Dicer into functional siRNAs that are incorporated into active RISC during lytic infection.
Paper IV

In our experimental system the adenovirus-based gene silencing technique have several advantages. First, viral infection works with 100% efficiency in a wide range of cells, making the strategy easier to apply. Second, RNAi induced by the viral system was more rapid compared to RNA silencing induced by plasmid transfection. A third advantage is that we could see a dose-dependent increase in the silencing effect with the viral vector system. Further, the virus expressing the shRNA from the VA RNAI promoter produced higher level of shRNA and siRNA in cells that did not support virus replication, leading to more effective RNAi induction compared to an identical expression cassette based on the U6 promoter.
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