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INHIBITION OF AVIAN LEUKOSIS VIRUS REPLICATION BY ANTISENSE RNA

By

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ABSTRACT

INHIBITION OF AVIAN LEUKOSIS VIRUS REPLICATION BY ANTISENSE RNA

By

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Avian Leukosis Virus (ALV) is a class of retrovirus which causes lymphoid leukosis in chickens. Its presence in commercial chicken flocks affects productivity through disease and death. With the generation of ALV-resistant chickens as an eventual goal, the possibility of conferring such resistance using antisense RNA has been tested *in vitro*. The 5' end of the ALV genome was employed as a target for antisense inhibition, and four fragments of increasing size in this region were amplified by PCR. Antisense RNA generated from each PCR product was tested for its effect on replication of recombinant ALV vectors in stably transfected RP30 cell lines. One antisense transfectant showed a significant reduction of viral replication in repeated experiments. Other transfectants, however, did not show significant inhibition of viral replication even though a substantial amount of antisense RNA was detected in some of these cell lines.

Subsequently, an antisense oligodeoxynucleotide (ODN) approach was employed in hopes of locating the most effective target sequence in this region. Of the several ODN tested, one which was complementary to the retroviral primer binding site showed the most inhibition. However, when antisense RNA directly complementary to the primer binding site region was generated within transfected RP30 cells, this RNA again failed to substantially inhibit ALV replication. Finally, antisense RNA was generated complementary to the mRNA for the cellular receptor for subgroup A ALV in hopes of generating ALV resistance by blocking receptor expression. Again, although a substantial amount of antisense RNA was generated in several distinct cell lines, no significant reduction of viral infectivity was observed. It is concluded that, at least in our experiments, antisense strategies were not reliably effective in inhibiting the spread of ALV in culture, and that therefore this is not a particularly attractive strategy to generate ALV-resistant transgenic chickens at this time.

To my husband, my parents, and Eui-Suk's family for the

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INTRODUCTION

Avian Leukosis Virus (ALV) belongs to the Avian Leukosis-Sarcoma group of retroviruses. Some of the ALVs are pathogenic and cause lymphoid leukosis (LL) in chickens by transforming B cells in the bursa of Fabricius. These transformed B cells develop into bursal tumors which can metastasize to other organs resulting in eventual death. Although there has been significant progress in eradicating ALV from the breeder stock of commercial chicken flocks, no commercial vaccines are currently available. Pathogenic ALVs still exist in chickens and their effects are enhanced by nonpathogenic ALVs and other pathogenic avian viruses.

Antisense RNA has been implicated in the regulation of gene expression in various systems and has been employed to inhibit virus replication. It is thought to block gene expression by hybridizing to target RNA and rendering it functionally inactive, generally by an unknown mechanism(s). This thesis describes the effects of antisense RNA on ALV replication in tissue culture system.

Chapter 1.

Literature Review

Avian Leukosis Virus

Avian Leukosis Virus (ALV) is a class of retrovirus belonging to the avian leukosis-sarcoma group. ALVs lack cellular oncogenes but can still cause long latency neoplasms. DNA copies of the ALV genomes (provirus) integrate into the host cell genome and can activate adjacent host proto-oncogenes. Most chickens also contain one to several proviral remnants within their genomes. These DNA copies are called endogenous viral (*ev*) genes that in some cases can be expressed as complete endogenous viruses (EV) (reviewed in Crittenden, 1991).

A typical retrovirus contains two identical copies of genomic RNA that are packaged in a protein core together with reverse transcriptase (Figure 1-1). This core particle is encapsulated by a glycoprotein and cell envelope to make up the complete virion. The ALV genome is a dimer of two identical RNAs, each 7.5 kilobases (kb) in size, held together through the dimer linkage site . Retrovirus genomes are arranged so that almost all noncoding sequences that contain important recognition signals are located in terminal regions (long terminal repeat; LTR), with internal regions given over virtually entirely to protein coding functions. ALV is the simplest type of retrovirus, containing three genes (*gag*, *pol* and

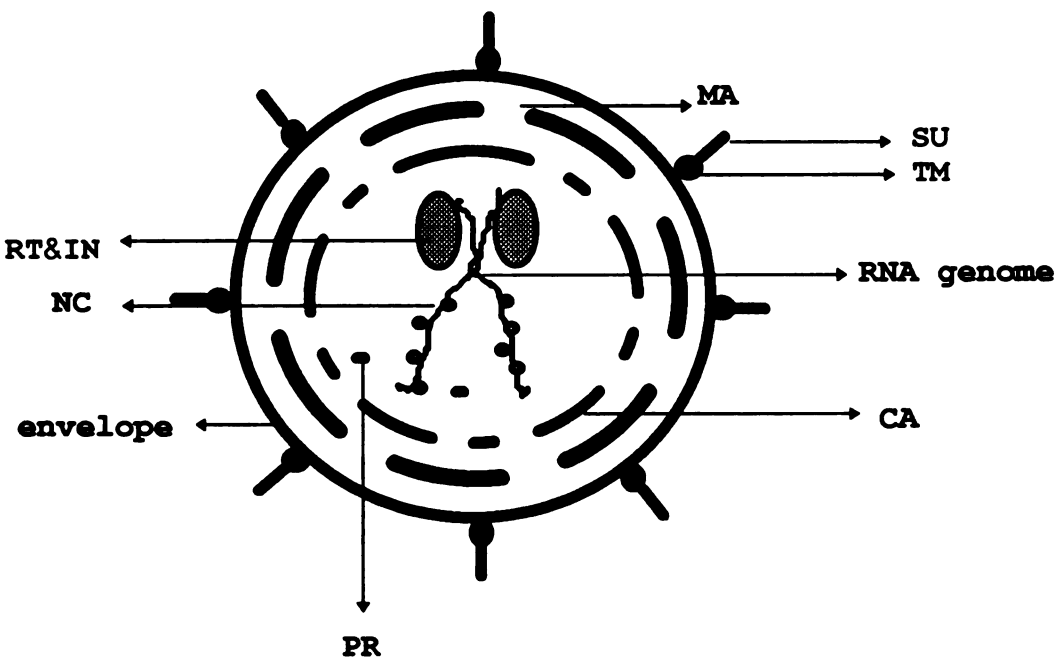


Figure 1-1. The retrovirus virion.

env), each of which, however, encodes more than one viral protein product or activity. The LTR is the primary cis-acting regulatory region, and it contains an enhancer, promoter and poly (A) signal. The LTR can be divided into three parts; U3, R, and U5 (Figure 1-2).

The U3 of ALV is 150-250 nucleotides (nt) long, depending on whether the virus is endogenous or exogenous. This region of exogenous ALVs, such as Rous Sarcoma Virus (RSV), contains a strong enhancer sequence that is required for high-level expression from viral promoters in different cell types (Crittenden, 1991; Fadly, 1986). The U3 region of avian endogenous viruses (*ev*) are distinct from those of exogenous viruses in that they lack a detectable LTR-associated enhancer. The absence of a strong enhancer in the *ev* LTR has been correlated with its low oncogenic potential relative to exogenous viruses (Fadly, 1986).

The R sequence is terminally redundant and present at the 5′ and 3′ ends of the viral genome. The R sequence is involved in the transfer of nascent DNA from one end of the genome to the other during the reverse transcription process.

Mutational analyses have shown that U5 has multiple roles in the viral life cycle. Some U5 sequences are

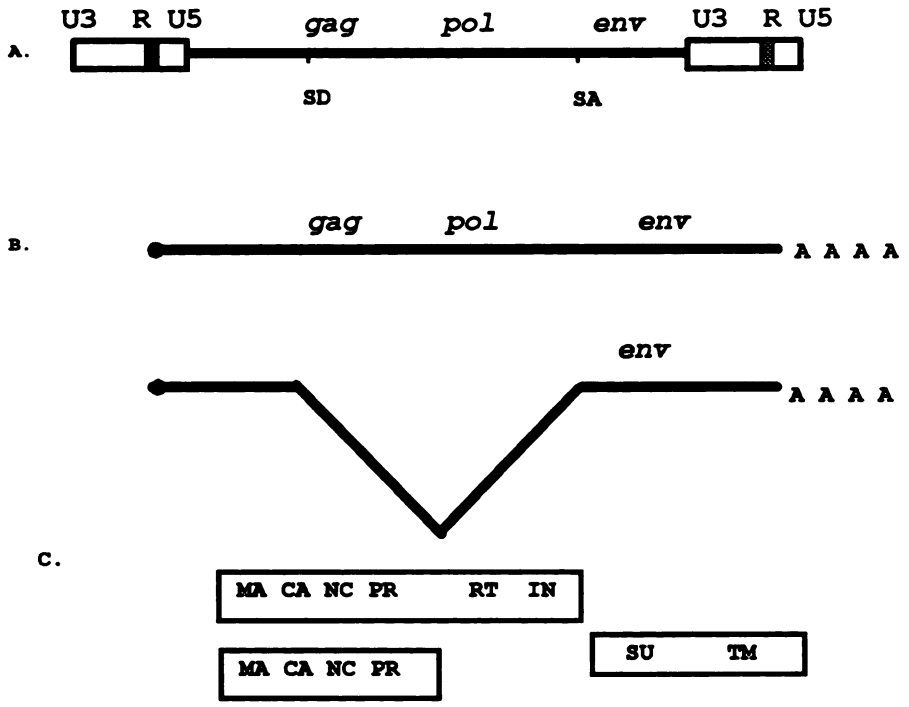


Figure 1-2. Diagram of ALV proviral DNA, genomic RNA and proteins. A: proviral DNA, B: Genomic and subgenomic forms of RNA, C: Virion proteins. The unprocessed proteins are indicated in the boxes. ALV sites shown include: SD, splice donor; SA, splice acceptor; MA, matrix protein; CA, capsid protein; NC, nucleocapsid protein; PR, protease; RT, reverse transcriptase; IN, integrase; SU, surface protein and TM, transmembrane protein (Crittenden, 1991; Coffin, 1991).

essential for reverse transcription (Cobrinik *et al.*, 1988). The 3'end of U5 contains the *att* sites (12-15 bp) necessary for integration , and there is evidence implicating this region in the packaging of viral RNA (Murphy and Goff, 1988). The primer binding site (PBS) binds the tRNA primer which is needed for the initiation of first strand DNA synthesis during reverse transcription. Different retrovirus groups use different tRNAs to prime. For example, ALV carries $tRNA^{trp}$ as its primer whereas HIV uses $tRNA^{lys}$ (Coffin, 1991; Crittenden, 1993).

The leader sequence, approximately 250 nt in length, follows the U5 at the 5'end of the viral genome just prior to the coding region. The important functions of this region are to specify incorporation of genome length RNA into virions (packaging sequence), RNA dimerization (Bieth *et al.*, 1990), and initiation of reverse transcription (Aiyar *et al.*, 1994; Cobrinik *et al.*, 1991). Knight *et al.* reported that a secondary structure of the packaging sequence in this region is required for efficient encapsidation of genomic RNA (Knight *et al.*, 1993).

The *gag* gene encodes a polyprotein precursor (Pr76) which is cleaved at the step of viral maturation by the *gag*-encoded protease (PR, p15). This cleavage gives rise

to the capsid protein (CA, p27), matrix protein (MA, p19) and nucleoprotein (NC, p12). The *gag* gene was reported to contain a regulatory sequence which confers stability to the RNA and also an enhancer sequence for viral gene expression (Federspiel and Hughes, 1994). CA forms the major internal structural feature of the virion. CA is also the major ALV-specific antigen that is detected in assays for the presence of ALV in chickens and cell cultures. There is a splice donor site 13 nt downstream of the ATG translation initiation signal in the *gag* gene and a splice acceptor site at the 5'end of *env*. The splicing step is indispensable in the virus life cycle because it allows expression of the *env* gene. The MA protein is in closest association with the viral membrane. Consistent with its membrane association, the N-terminus of MA is modified by the addition of a myristic acid group (Weiss et al, 1982). However, the MA of ALV has only an acetate group added at its N-terminus (Coffin, 1991). It has been suggested that the RSV MA plays a role in membrane binding during assembly of the virus (Parent *et al.*, 1996). The NC protein is a small basic protein found in the virion in association with genomic RNA.

The *pol* gene codes for the reverse transcriptase (p63 alpha, *RT*) that is used in transcription of the viral DNA

from the viral RNA genome. Retroviral reverse transcriptase has an RNase H activity which degrades the RNA strand of the RNA-DNA duplex during reverse transcription. It also encodes the integrase (p32,*IN*) that is specifically involved in the integration of proviral DNA into the host chromosome. Enzymatic assays for *RT* are often used for detection of ALV particles in chickens and cell cultures. Translational frameshifting fuses the *gag* and *pol* reading frames producing a 180 kd precursor protein which is processed into the mature *gag* proteins and *RT* and *IN*. These proteins are packaged together into the virion with viral genomic RNA.

env is expressed from spliced subgenomic RNA. Its protein products (envelope glycoproteins, gp85 and gp37) are important for attachment of the virus to the receptor on the cell membrane and penetration into cells. These proteins are used for classification of the virus into 5 major subgroups (Ishizaki and Vogt, 1966; Vogt and Ishizaki, 1965; Vogt and Ishizaki, 1966). gp85 and gp37 remain linked to each other by disulfide bonds on the virion (Leamson and Halpern, 1976). These glycoproteins confer three major subgroup-specific functions: induction of neutralizing antibodies; production of cell receptor interference against members of the same subgroup; and

control of host-range among avian and mammalian cell types. ALVs are divided primarily into the subgroups based on the host specificity conferred by the envelope glycoprotein (A through E). Endogenous ALVs belong to subgroup E. The remaining subgroups make up the exogenous ALVs. Subgroup F and G show host specificity to pheasants (Fujita et al., 1974). Recently, subgroup J has also been isolated (E. Smith, personal communication).

The susceptibility of chickens to infection by ALV is controlled by three genetic loci: *tva*, *tvb*, and *tvc* (Crittenden, 1991). The *tva* and *tvc* susceptibility alleles are thought to encode receptors or susceptibility factors for subgroup A and subgroup C viruses, whereas different alleles of *tvb* may encode receptors for subgroup B, D, and E.

The general features of replication include the following:

A. Binding the envelope glycoprotein to the receptor on the cell membrane. The initiation of a replication cycle begins with the specific binding and interaction of the receptor molecule on the cell with SU protein on the virion envelope. Bates *et al.* (1993) and Young *et al.* (1993) used gene transfer to clone the receptor specific for subgroup A in a quail and a chicken cell line,

respectively. This receptor was shown to have some homology to the light density lipoprotein receptor (Bates *et al.*, 1993; Young *et al.*, 1993). Neither its mRNA nor protein product in cells, however, was detected, indicating that the receptor gene is poorly expressed and/or expressed early in development followed by rapid mRNA decay. A recent study by Gilbert *et al.* (1995) showed conformational changes of subgroup A Env protein induced upon binding the receptor that is relevant to the activation of its fusion function.

B. Release of capsid into the cytoplasm. Following attachment, the virus envelope and the cell membrane fuse to release the virion core into the cytoplasm. This step is beginning to be understood and the TM protein of the virion seems to play a role. The internalization process seems to occur by receptor-mediated endocytosis followed by fusion of the viral envelope and the endosomal membrane, possibly provoked by the lower pH of the endosomal contents. However, neither HIV nor ALV requires an acidic pH for uncoating (Stein *et al.*, 1987).

C. Reverse transcription of a single-stranded RNA into a double-stranded DNA. This process takes place inside the virion core. Reverse transcriptase is carried within the virus in close contact with genomic RNA. tRNA, which is

attached at the PBS of the RNA genome, is used as the primer for initiation in this step. During reverse transcription, RNase H degrades the RNA strand of the RNA-DNA hybrid. This is the step during which a lot of mutations can be introduced into the genome due to the error-prone nature of RT. RT has higher error rates (ranging from 3×10^{-3} to 3×10^{-5} / replication) than that of the eukaryotic RNA polymerase II (less than 10^{-5} per animal generation; Gopinathan *et al.*,1979; Mizutani *et al.*, 1976). Template switches by RT upon confronting the strong-stop sequence during this step can generate recombinant forms of the virus (Coffin, 1979; Zhang and Temin, 1994).

D. Integration of viral DNA into the host chromosomal DNA to make the provirus. Linear and several circular DNA forms of the virus genome are found in the nucleus during integration. The results of *in vitro* experiments with murine leukemia virus favor the linear form as the structure that is integrated to form the provirus (Fujiwara *et al.*,1988). A small sequence at the site of integration in the host DNA, six base pairs long for ALV, is repeated at each end of the provirus. While it is clear that the integration machinery shows little sequence preference in the choice of integration, it has also been

observed that integration sites tend to map in or near transcriptionally active regions and nuclease-sensitive regions of host chromatin (Mooselehner and Harbers, 1990). RSV DNA has been observed to integrate at an unusually high frequency into certain preferred regions of the chicken genome, and, within those regions, insertions tend to occur into the exact same site (Shih and Coffin, 1988). Experiments done *in vitro* by Pryciak *et al.* (1992) were in accord with a model in which integration machinery has preferential access to the exposed face of the nucleosomal DNA helix.

E. Transcription of proviral DNA to viral genomic RNA and mRNA. Transcription of the provirus is initiated at the junction of U3-R, and it proceeds through the 3′ LTR into flanking cellular DNA, with the final 3′ end located at the end of R by cleavage and poly (A) addition. All retrovirus genomes are transcribed by host RNA polymerase II and host transcription factors. ALV contains CCAAT/enhancer motifs that cover most of the LTR enhancer regions (Ryden and Beemon, 1989). Interestingly, the CCAAT/enhancer motifs are absent in EV LTRs, which correlates with the very low transcriptional activity of those loci (Habel *et al.*,1993). The avian C/EBP-related factors designated a1/EBP and a3/EBP were shown to bind

these enhancer elements (Bowers and Ruddell, 1992; Smith *et al.*, 1994). cDNAs of both a1 and a3 encode leucine zipper transcription factors. It was recently found that an NF-kB/Rel-related protein is a component of the LTR CCAAT/enhancer binding complex through its interaction with a1/EBP (Bowers *et al.*, 1996).

The ALV genome has a splice donor site 13 nt downstream of the ATG in *gag* and a splice acceptor site near the 5' end of *env*. Splicing of the full-length mRNA is carried out by the host machinery and generates a mRNA of 2.0 kb which is essential for expression of *env* genes.

F. Translation of viral mRNA into proteins. This process is dependent on the cellular translation machinery, and it's identical to the translation of cellular mRNA (Lewin, 1994). The full-length RNA can have two different fates: some molecules become new genomes and others serve as message for the viral proteins. How these are selected to enter either the mRNA or the genome pool is not well understood. In most of the retroviruses, the *gag* reading frame ends in a translational terminator and is also in a different reading frame from that of *pol*. A shift of reading frame occurs at the junction between the *gag* and *pol* for translational readthrough. The first demonstration of this "frameshift suppression" was obtained from ALV

(Jacks *et al.*, 1988; Jacks and Varmus, 1985). In ALV, the probability of this frameshifting is about 5%. By regulating the frequency of this event, the virus balances the ratio of these two protein products.

G. Assembly of gag-pol protein-genomic RNA complex and budding. Assembly and budding of retroviruses depend on the product of a single viral gene, *gag*. For ALV, this process appears to occur at the plasma membrane (Weiss *et al.*, 1982). A conserved cis-acting packaging sequence (Ψ) in the 5′ leader of the avian sarcoma virus genome identifies the viral RNA and allows it to be incorporated into the virion (Linial and Miller, 1990). Upon budding, the virus particle undergoes maturation, in which the gag polyprotein precursor is cleaved into the several structural proteins (MA, CA, and NC, and in ALV, PR) by its own protease.

The disease induced by exogenous ALV is called lymphoid-leukosis (LL). When an exogenous ALV DNA copy is integrated by chance upstream in the host genome of a proto-oncogene, such as *c-myc,* its 3′LTR drives a high level of transcription of this gene and thereby causes the transformation of infected B-cells. The transformed B cell originates from the bursa of Fabricious and transformed cells metastasize to the liver, spleen and other visceral

organs, with eventual death (Crittenden, 1993; Fadly, 1992; Fadly, 1986). This underlying mechanism inducing cellular transformation is different from that of rapidly transforming retroviruses such as RSV. Most strains of RSV carry the *src* oncogene between the *env* gene and the 3' LTR. Acute transforming viruses such as RSV carry the oncogene sequences in their genomes and in most other cases, they are replication defective due to the deletion of essential virus gene(s)(Weiss *et al.*, 1982). These viruses arise from the transduction of a cellular oncogene, c-*src* or others. The readthrough transcripts, containing both viral and cellular sequences, are copackaged with viral genomic RNA. This is followed by illegitimate recombination events during reverse transcription to generate a rapidly transforming retrovirus (Hajjar *et al.*, 1993; Swain *et al.*, 1992).

The continued presence of ALV in commercial chicken flocks affects productivity through LL and death. Although there has been significant progress in eradicating ALV from the breeder stock of commercial flocks, no commercial vaccines are currently available. ALV still exists in commercial chicken flocks, and its effects are enhanced by endogenous viruses and interactions with other avian viruses (Crittenden *et al.*, 1984; Smith and Fadly, 1988).

Retroviral Vectors

Hughes *et al.* have developed a series of replication competent retroviral vectors derived from a cloned copy of the genome of the Schmidt-Ruppin strain RSV (Hughes and Kosik, 1984; Sorge and Hughes, 1982; Sorge *et al.*, 1983). They are called RCASBP (Replication Competent, SR-A LTR, Splice Acceptor, Bryan high titer polymerase) and RCOSBP (Replication competent, RAV-0 LTR, Splice Acceptor, Bryan high titer polymerase). RCASBP was made by removing the *src* gene and introducing a unique *Cla*I site that can be used to insert foreign DNA of up to 2 kb. RCOSBP was constructed by replacing the LTRs of RCAS with those of RAV-0 (Rous associated virus), which are lacking the enhancer elements. The RCOS and RCAS vectors express inserted sequences from the viral LTR. Therefore, insertion of a reporter gene and assay of its activity makes it easier to study tissue-specific or development-specific LTR regulation (Fekete and Cepko, 1993). In contrast, the RCON and RCAN vectors, which lack the splice acceptor present in RCOS and RCAS, can be used to express DNA inserts from internal promoters (Boerkoel et al., 1993). This makes it possible to separate the expression of the insert from viral gene expression. As an example, Petropoulos *et al.* (1992) somatically infected chickens

with an RCAN retroviral vector that contains the CAT gene linked to the chicken skeletal muscle α_{sk}-actin promoter and found high CAT activity only in striated muscle, whereas the chickens infected with the vector carrying the β-actin promoter/CAT cassette displayed low levels of the CAT activity in a wide range of tissues (Petropoulos *et al.*, 1992). This study suggested that gene expression can be targeted to a variety of other avian cell types by constructing similar vectors containing other tissue-specific promoters. The structures of these vectors are described in Figure 1-3.

These vectors are replication-competent and helper-independent. Therefore, rearrangements due to recombination with helper sequences are eliminated, and both vectors can be produced to a substantially higher titer. These vectors are widely used as vehicles of gene transfer into chickens and recently into transgenic mice carrying the subgroup A receptor (Hughes *et al.*, 1987; Federspiel et al., 1995).

The host specificity of these retroviral vectors as well as ALV mainly lies within the gp85-coding domain of *env* (Bova *et al.*, 1986; Bova *et al.*, 1993). Replacement of the 1.1-kb region in a hypervariable region (*hr* 1) in this

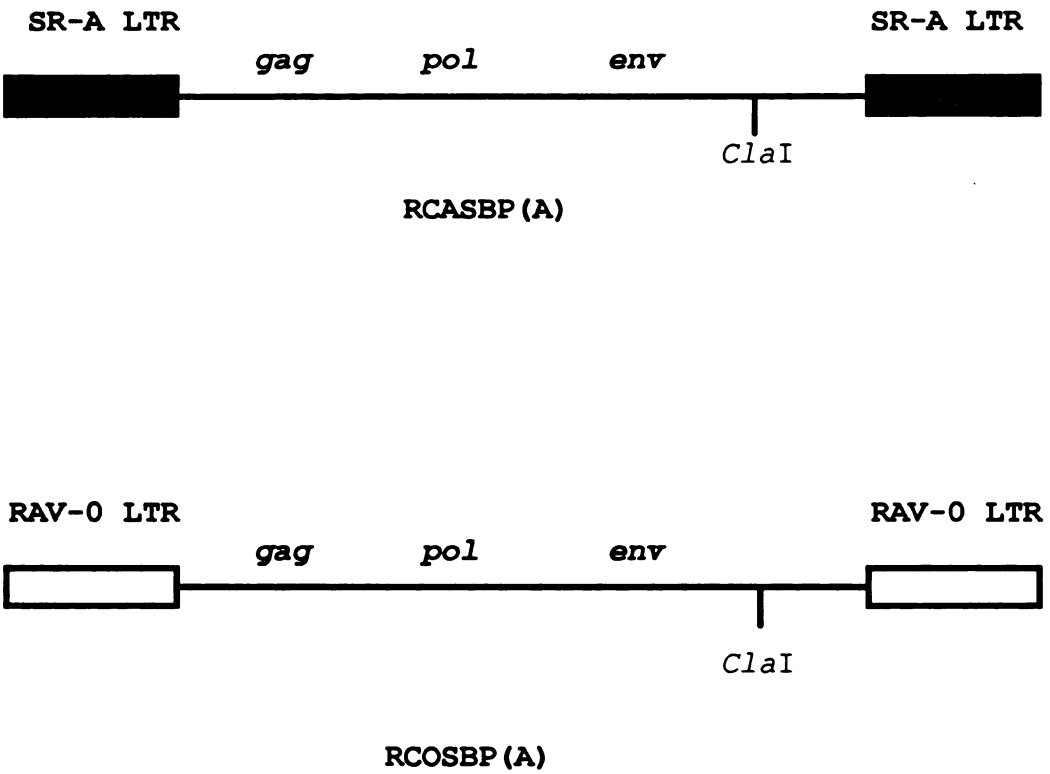


Figure 1-3. The genome organization of RCOSBP and RCASBP. SR-A LTR, Schmidt-Ruppin strain A long terminal repeat; RAV-0 LTR, Rous-associated virus type 0 long terminal repeat.

domain with that of another subgroup generates a virus of that subgroup.

Antisense RNA

1. Natural Antisense

Antisense RNA has a sequence complementary to its target messenger RNA. It has been thought to directly repress gene expression by hybridizing to target mRNA and rendering it functionally inactive. Although the mechanism of antisense RNA inhibition is not clear, antisense RNA has been receiving great attention and many trials are ongoing to test its specific inhibitory effect on target gene expression in several different systems.

The initial discoveries of natural antisense RNAs were in prokaryotes. For example, the initiation of ColE1 plasmid DNA replication in *E.coli* is negatively controlled at the level of primer formation by a small untranslated antisense RNA (Tomizawa, 1986). In addition, the regulation of the life cycles of bacteriophages P1 and P7, as well as plasmid incompatibility and copy number control has been shown to involve antisense RNA (Brantl and Wagner, 1994; Biere *et al.*, 1992; Siemering *et al.*, 1994). There are also many cases where the participation of

antisense transcripts in eukaryotic gene regulation is thought to occur (Simmons, 1993).

2. Artificial Antisense

Since natural antisense is effective and very specific, many artificial antisense RNAs have been designed to inhibit the expression of endogenous genes and the replication of pathogens (Biasolo et al., 1996; Ronemus et al., 1996; Scherczinger and Knecht, 1993). The antisense approach has been particularly effective in plants (Blockland et al., 1993). The well-known transgenic tomato, "*Flavr Savr*", has a longer shelf life by using antisense RNA against the message of the polygalacturonase gene, resulting in delaying of the softening. It is also interesting to note that, in a lot of studies with plants, antisense RNA against the entire coding sequence of the target gene was an effective inhibitor (Beffa *et al.*, 1996; Ronemus *et al.*, 1996).

There have been numerous studies on antisense RNAs which are stably expressed in either cell culture or in transgenic animals that inhibit the replication of (retro)viruses (Biasolo et al., 1996; Han et al., 1991). The aim of antisense techniques for the inhibition of viral infection is either to suppress the expression of the integrated provirus in chronically infected cells or

to prevent the virus from establishing itself in uninfected cells. To accomplish this goal, the obvious route is to disrupt the viral replication cycle. Antisense techniques can be especially effective in inhibition of retrovirus replication. Retroviruses have the advantage of introducing mutations into their genome during replication, especially by RT, which helps them escape the host immune system. Therefore, an antisense RNA which is designed to target a conserved viral sequence is expected to confer a strong and longer specific inhibitory effect on retrovirus replication. Additionally, the requirement of sequence complementarity increases the specific effect without interfering with any other cellular RNA. Retroviruses have many important signals at their 5′ ends, and those signals are relatively well conserved in a given virus due to the necessity of their interaction with either viral or cellular proteins. For example, PBS and Ψ are shown to directly interact with cellular tRNA and the viral capsid proteins, respectively. Therefore, the 5′ end of retroviral RNA seems to be the target that antisense approaches have to which most often been directed. Sczakiel and Pawlita (1991) have shown that stable expression of antisense RNA complementary to a 407-bp sequence of the 5′ leader-*gag* region of HIV inhibited

viral replication in human T cells. An antisense RNA against TAR has also been shown to inhibit viral replication by preventing its interaction with TAT as well as the other signals at the 5′ end (Chatterjee *et al.*, 1992; Vandendriessche *et al.*, 1995). The effect of antisense RNA was also demonstrated in a study in which MoMLV-induced leukemia was reduced by expressing an antisene RNA against Ψ in a transgenic mouse system (Han *et al.*, 1991).

Antisense Oligodeoxynucleotides

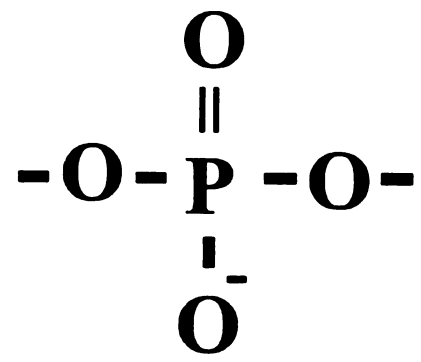
Antisense oligodeoxynucleotide (ODN), which are complementary to certain regions of gene messages or viral sequences, have been getting a lot of attention because of their ability to modulate gene expression. With its first success in inhibiting RSV replication in chicken embryo fibroblast (CEF) cells (Zamecnik and Stephenson 1978; Stephenson and Zamecnik, 1978), antisense ODNs have enjoyed considerable success as antiviral agents in various biological systems. There are even several antisense ODNs in clinical trials to regulate HIV replication in patients (Lisziewicz *et al.*, 1994).

Two critical factors to be considered in antisense ODN approaches are the efficiency of uptake and the stability

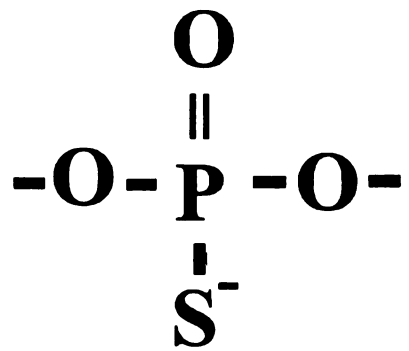
of the ODN. Due to the negative charge on the phosphate backbone of the ODN, direct penetration through the cell membrane is implausible. To increase its cellular uptake and intracellular stability , various modifications of the phosphate backbone have been applied. They are thiolation (Zhao *et al.*, 1993), methylation (i.e. alkylation) of the phophodiester bonds (Mckay *et al.*, 1996) as well as conjugation of ODN with peptides (Bongartz *et al.*, 1994). Figure 1-4 shows the structures of phosphodiester and phosphorothioate linkages. The suggested mechanism for cellular uptake of phosphodiester and phophorothioate ODNs is an endocytic process (Loke *et al.*, 1989) whereas methlyphosphonates enter cells by passive diffusion (Miller et al., 1981). Liposome-mediated delivery of ODNs has been developed to increase cellular uptake (Bennet *et al.*, 1992; Thierry and Dritschilo, 1992). Microinjection of oligonucleotides directly into the cell has shown to be an effective, though cumbersome, method for delivery (Graessmann *et al.*, 1991; Raviprakash *et al.*, 1995).

Mechanism of antisense RNA or ODN action

There have been many studies to elucidate the mechanism of antisense inhibition. However, relatively little is currently understood. Considering the process of gene



phosphodiester



phosphorothioate

Figure 1-4. Phosphorothioate and phosphodiester linkages.

expression, the following were suggested as possible steps where antisense inhibition might take place (Mirabelli and Crooke, 1993);

A. Transcriptional arrest. ODN may bind to DNA and prevent initiation of transcription by preventing effective binding of factors required for transcription, thus, producing transcriptional arrest. (Nielsen *et al.*, 1991; Svinarchuk *et al.*, 1996).

B. Inhibition of post-transcriptional processes. Antisense RNA or ODN that bind to sequences required for splicing may prevent binding of necessary factors or physically prevent the required cleavage reactions. This would result in inhibition of the production of the mature mRNA (Zamecnik *et al.*, 1986). Another possible mechanism includes inhibition of 5′ capping (Westermann *et al.*, 1989). Inhibition of 3′ polyadenylation has not been directly proven. However, antisense ODNs targeting the 3′ untranslated region (UTR) have shown inhibitory effects (Chiang *et al.*, 1991).

C. Translational arrest. The mechanism for which the majority of antisense RNA or ODN have been designed is translational arrest, in which recognition and binding of target mRNA by ribosome are prevented (Agrawal *et al.*, 1988; Lemaitre *et al.*, 1987; Sburlati *et al.*, 1991;

Sullenger *et al.*, 1990). It was demonstrated in HIV-1 that sequences essential for packaging the viral RNA are located around the *gag* initiation codon and can form a stable secondary structure. An ODN which is complementary to this region was found to be an effective inhibitor of HIV replication. This ODN might block the translation of *gag* mRNA and also disrupt the secondary structure of RNA (Agrawal and Tang, 1992). The positioning of the initiation codon within the area of complementarity and the length of antisense RNA or ODNs have varied considerably.

D. Disruption of RNA structure. RNA adopts a variety of three-dimensional structures induced by intramolecular hybridization, the most common of which is the stem-loop structure. These structures have been shown to play crucial roles in a variety of functions. As an example, antisense ODNs designed to target the transactivation response (TAR) element in HIV were shown to disrupt the structure of the stem-loop and inhibit TAR-mediated expression of a reporter gene (Vickers *et al.*,1991).

E. Activation of RNase H. RNase H is a ubiquitous enzyme that degrades the RNA strand of an RNA-DNA duplex. It has been identified in organisms as diverse as *E.coli* and human cells (Mirabelli and Crooke, 1993). It was

demonstrated that many ODNs may activate RNase H in cell lysates and purified assays (Gagnor *et al.*, 1987; Walder and Walder, 1988). ODN with a phophodiester bond seemed to be a better RNase H activity inducer than phophorothioate ODN (Boiziau et al., 1995). However, direct proof has not been found that RNase H activation is the true mechanism of antisense action in cells. It was also demonstrated that RNase L activity can be induced in infected cells by 2′, 5′ oligoadenylate (2-5A). This is formed by 2′, 5′ oligoadenlyate synthetase (2-5OAS), activity of which depends on the presence of viral or cellular double-stranded RNA (dsRNA). (Maitra *et al.*, 1995). RNase L is an endonucleolytic enzyme and it degrades both cellular and viral RNA, resulting in removal of the infected cells. Schroder *et al.* (1994) have developed new strategies which yield a selective avtiviral effect of 2-5A against HIV infection by application of the LTR-2-5OAS hybrid genes.

An antisense RNA molecule which can cleave the target RNA upon binding would further increase its efficiency. A ribozyme is a RNA molecule with a certain sequence motif which can recognize and cleave the target RNA molecule (Zaug *et al.*, 1986). There have been many applications incorporating a ribozyme motif into the antisense sequence, and this improved the antisense effect (Sun *et*

al., 1995). Sullenger and Cech incorporated a tethering ribozyme to a retroviral packaging signal for colocalization with the target RNA and showed cleavage of the target RNA which leads to the protection of uninfected cells (Sullenger and Cech, 1993).

There are several factors which may affect the efficiency of antisense inhibition. They are as follows:
A. The place of action. Many investigators have questioned whether different cellular sites are involved in antisense control of gene expression. A transgenic tobacco experiment showed a reduced level of translation efficiency of target mRNA and suggested a cytoplasmic interaction between the antisense RNA and the target message (Cornelissen and Vandewiele, 1989). Meanwhile, Liu and Carmichael (1994) have suggested from their study with polyoma virus that antisense RNAs that are retained in the nucleus bind to target transcripts and appear to lead to the degradation of their targets. This suggested that nuclear antisense RNAs were significantly more effective than were conventional antisense molecules, which were processed by polyadenylation (Liu and Carmichael, 1994).
B. The abundance of antisense RNA. Viral RNA may contribute up to 10 % of the total polyadenylated RNA in

infected cells (Coffin, 1991). Therefore, an efficient antisense system will have to introduce into the target cell a sufficient amount of antisense RNA in order to overcome this level of expression and do so without overwhelming the normal functions of the host cell by their sheer quantity (Izant and Weintraub, 1985). In several other cases, antisense RNA was not detected by typical techniques even though an antisense effect was obtained (Koschel *et al.*, 1995). It was suggested that this could be due to the instability of the antisense transcripts.

RNA Polymerase III (Pol III) is a ubiquitous enzyme with a transcription efficiency higher than that of RNA polymerase II (Gabrielsen and Sentenac, 1992). Sullenger *et al.* (1990) and Biasolo *et al.* (1996) showed a high level expression of antisense transcripts against the *gag* and *pol* genes of MoMLV and the first exon of *tat* of HIV-1, respectively, and significant inhibition of viral replication using Pol III-tRNA promoter systems.

C. The target regions. In most cases, including the repression of retrovirus replication, the 5′ end of the target RNA has proven most effective as a site for antisense expression. Typical retrovirus contains the PBS, Ψ, the leader sequence and the ATG translation

initiation signal, and, in some cases, the splice donor/acceptor sites. Secondary structures of the packaging signal have been implicated in efficient encapsidation of the virus particles. Also the leader sequence contains the PBS, onto which antisense RNA might compete with tRNA for binding. In the case of HIV-1, although the 5′ end of its RNA has been proven to be a good target in various experiments, antisense RNA against the first coding exon of *tat* showed a significant reduction of viral replication as well (Biasolo *et al.*, 1996). However, in other experiments in different systems, the inhibitory effect was achieved with the antisense transcript targeting the entire coding sequences or regions at the 3′ end (Scherczinger and Knecht, 1993; Sullenger *et al.*, 1990). Therefore, it is difficult to predict the optimal design of an antiviral antisense strategy.

D. The accessibility to the target mRNA sequence. Since RNAs can fold into various secondary structures, there have been many investigations of how to improve the binding efficiency between the antisense RNA or ODN and its target sequence. Although secondary structures can be estimated by computer programs based on thermodynamic stability (Hackett *et al.*, 1991), they may not reflect the

structure of the RNA molecule in the cell or when it's bound by certain proteins.

Research Proposal

Avian lymphoid leukosis (LL) is a neoplastic disease of chickens caused by ALV. An ALV infection spreads congenitally from dams to progeny or by chicken-to-chicken contact in the same flock. ALV infection in commercial stock is controlled by virus-eradication schemes that prevent vertical transmission of ALV from one generation to the next. No efficient vaccines are available and the pathogenecity of ALV is augmented by the presence of *ev* (Smith and Fadly, 1988).

Antisense RNA is complementary to its target RNA. It inhibits gene expression by hybridizing to the target RNA and rendering it functionally inactive. It has been widely tested in a variety of systems including inhibition of replication of other retroviruses such as HIV and MoMLV.

The long-term goal of this project is to generate transgenic chickens resistant to ALV infection by using antisense RNA techniques. In this study, we have tried to test the efficacy of using antisense RNA in an *in vitro* cell culture system. We have focused at the 5' end of ALV genomic RNA as the target since this region is relatively

well conserved among different subgroups of ALV and has many important regulatory signals. To approach this goal, we have applied two different methods of expressing antisense sequences against the viral RNA: the transcription of antisense RNA in a stable expression system and the use of antisense oligodeoxynucleotides to locate the best target region.

As another approach to inhibit replication of ALV, we have expressed antisense RNA against the message for subgroup A ALV receptor in a quail cell line. The gene (*tva*) for subgroup A virus receptor has been recently cloned (Bates *et al.*, 1993). However, neither its transcript nor protein product are detectable, indicating that the receptor gene is poorly expressed. Therefore, it seemed likely that the expression of this gene could be repressed more efficiently by an antisense RNA than the viral RNA might be. In this study, we have focused at the 5′ end of *tva*, including its ATG translation initiation signal.

Chapter 2.

Antisense RNA generation as a strategy for the induction of cellular resistance to ALV

ABSTRACT

Avian leukosis virus (ALV) belongs to the avian leukosis-sarcoma group of retroviruses. Upon infection, the ALV provirus can integrate into the 5′ end of *c-myc* cellular oncogene, leading to over-expression of that oncogene and resulting in a disease called lymphoid leukosis. No vaccines are currently available to prevent the spread of ALV in commercial chicken flocks. In other systems, antisense RNA has been used to inhibit retroviral replication in infected cells and to protect uninfected cells. We have examined the use of antisense RNA to inhibit ALV replication in an avian cell line, RP30. In an expression system where an antisense RNA is transcribed constitutively, one cell line showed a significant inhibitory effect on replication of test ALV strains. A low level of the antisense transcript was detected in this cell line by RT-PCR. However, this inhibitory effect was not reproducibly observed in other transfected cell lines or in cells in which the antisense transcript was generated by a tetracycline-regulatable promoter, even though a substantial amount of antisense transcript was detected in such cells.

INTRODUCTION

Avian leukosis virus (ALV) is a class of retrovirus belonging to the avian leukosis-sarcoma group (Crittenden, 1991). The genome structure of ALV is simple compared to those of some other retrovirus groups, containing three genes (*gag*, *pol* and *env*) and a regulatory region or long terminal repeat (LTR). The LTR contains an enhancer, promoter and poly (A) signal which are recognized by the cellular transcription machinery.

The general features of ALV replication include the following: binding of the envelope glycoprotein to the receptor on the cell membrane, release of the capsid into the cytoplasm, reverse transcription of a single-stranded genomic RNA into a double-stranded DNA, integration of the viral DNA into the host chromosomal DNA to make a provirus, transcription of viral DNA to viral genomic RNA and mRNA, some of which is spliced to form a subgenomic viral message, translation of viral mRNA into proteins and assembly of the protein-genomic RNA complex at the cell membrane followed by budding (Coffin, 1991).

ALV can be transmitted either through close contact or congenitally through the egg. ALV lacks host oncogenes and is not therefore an acutely transforming virus. Exogenous ALV can induce a variety of neoplasms, but

principally lymphoid leukosis (LL), while endogenous ALV is rarely oncogenic. This difference is determined primarily by the enhancer element being present only in the exogenous viral LTR (Crittenden, 1991). When an exogenous ALV provirus integrates by chance upstream of the *c-myc* gene in the host genome, (usually) its 3′ LTR can enhance the transcription of this gene and cause transformation of infected B-cells in the Bursa of Fabricious. Transformed B-cells metastasize to the liver, spleen and other visceral organs leading eventually to death (Fadly, 1992). The continued presence of ALV in commercial chicken flocks affects productivity both through LL disease and death (Crittenden, 1993).

The use of antisense RNA to inhibit RNA function within cells and whole organisms has the potential to provide a versatile molecular tool against viral infection. There are many reports of the use of antisense RNA to inhibit retroviral replication. For example, various regions of the HIV genome have been tested as targets for antisense RNA, with a resultant reduction in virus replication (Sczakiel and Pawlita, 1991; Vandendriessche *et al.*, 1995). Antisense RNA can bind in a highly specific manner to complementary sequences in mRNA or viral genomic RNA, potentially blocking processing or

translation of the RNA or, possibly, its interaction with sequence-specific binding proteins.

To test the efficacy of antisense RNA in the inhibition of ALV replication, avian cell lines containing antisense RNA sequences against the conserved elements of the ALV genome were generated and challenged with the ALV-derived retroviral vectors: RCASBP and RCOSBP (Greenhouse *et al.*, 1988). The 5′ end of ALV contains a variety of important regulatory signals and is well conserved among different subgroups of ALV (Figure 2-1). Important signals in this region include promoter/enhancer elements in the LTR, the primer binding site (PBS) for $tRNA^{trp}$ which is essential for initiation of reverse transcription, the packaging sequence (Ψ), the ATG translation initiation codon for gag-pol and env proteins, and a splice donor site. Therefore, this region is likely to be an ideal target against which to direct antisense RNA to inhibit ALV replication.

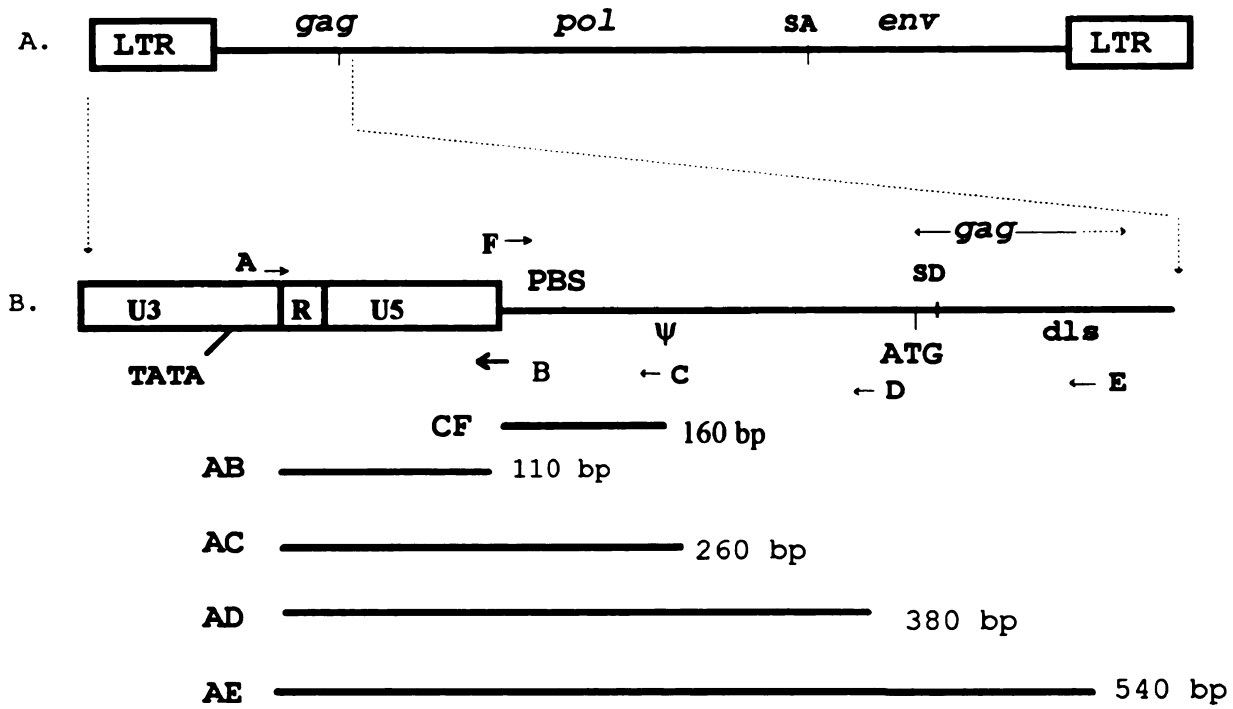


Figure 2-1. The regions targeted by antisense RNA.
A. The proviral structure of ALV.
B. The 5′ end of the ALV provirus is shown.
The primers are indicated by A, C, D, E, and F.
ALV sites shown include: TATA, promoter; Ψ, packaging signal; PBS, primer binding site; ATG, translation initiation signal; SD, splice donor; SA, splice acceptor and dls, dimer linkage sequence (Coffin,1991). Various target amplified fragment regions are shown by lines below the ALV diagram with their sizes indicated at the right.

MATERIALS AND METHODS

Cell culture

RP30 clone5 is a Marek's disease virus-transformed turkey lymphoid cell line and is free of endogenous virus. This cell line and all of its derivatives were maintained under 5% CO_2 in Leibovitz L-15/ McCoy's 5A medium (Life Technologies, Gaithersburg, MD 20877) containing 10% chicken serum, 5% fetal bovine serum, 2.5% tryptose phosphate broth supplemented with gentamycin (10 µg/ml) and amphotericin B (2.5 µg/ml).

Electroporation

Cells were washed twice with phosphate-buffered saline (PBS) and resuspended in 0.5 ml cold PBS in a 0.4 cm Gene Pulser cuvette (Bio-Rad, Hercules, CA 94547). Ten µg of each construct was added and the mixture was placed on ice for 5 min. The electroporation was performed at room temperature using the Gene Pulser set at 960 µF and 250 V. After electroporation, cells were incubated on ice for 5 min and then resuspended in 10 ml of growth media. After 24 h of incubation, cells were spun down, resuspended in 24 ml of selection media (0.4 mg/ml Geneticin [Life Technologies] ± 1 µg/ml of puromycin

[Sigma, St. Louis, MO 63178]) and plated onto a 24-well plate. Resistant colonies typically developed in 10 d.

Luciferase assay

Colonies to be tested for effectiveness of tetracycline (tet) regulation were transiently transfected with 10 µg of pUHC13-3, containing a luciferase (*luc*) gene expressed from the tet-regulatable promoter (Gossen and Bujard, 1992). After 24 h in growth media, cells were split into media ± tet (4 µg/ml)or doxycycline (dox, 1 µg/ml) and incubated overnight. Cells were counted, washed once with PBS and lysed in 1x cell culture lysis buffer (Promega, Madison, WI, 53706). Fifty µl of each cell lysate was mixed with 100 µl of luciferase substrate, prepared according to the manufacturer (Promega). The activity of luciferase was measured using a Turner TD-20e luminometer (Turner Designs, Inc., Sunnyvale, CA 94086) and normalized to the cell number.

Polymerase Chain Reaction (PCR) and plasmid construction

The sequences of the PCR primers used to amplify the 5′ end of the ALV proviral genome are listed in Table 2-1. Each primer was synthesized by the DNA Core Facility at Marshall University, Huntington, WV 25704. PCR reactions were performed with a common 5′ primer (pA or pF) and

Table 2-1. Primers used in PCR amplification of antisense target regions of ALV.

Primer	Sequence
A	GTG GAA TTC TAA ACG CCA TTT GAC CAT
B	TTG GAA TTC AAT GAA GCC TTC TGC TTC
C	TAT GAA TTC GAG CTC CCT CCG ACG
D	CTT GAA TTC CTT GAT CCG CAG GCC G
E	AAT GAA TTC CGC AGT GAT GGG ATC C
F	TGG TGA CCC CGA CGT GAT CG

various 3' primers (pB, pC, pD or pE; see Figure 2-1) using a RCOSBPCAT-3(A) plasmid (Greenhouse *et al.*, 1988) as template as described (Sambrook *et al.*, 1989). Each PCR product was digested with *Eco*RI and ligated into pBluescript II plasmid (Stratagene, La Jolla, CA 92037) and sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977). The CF fragment was directly subcloned into the TA vector (Invitrogen, San Diego, CA 92121) and sequenced. Each PCR fragment was then transferred into the pRC-CMV eukaryotic expression vector (Invitrogen) in both orientations by digestion with *Hind*III and *Xba*I followed by ligation. In the tet-responsive system, PCR products were transferred to pUHD10-3Neo or pUHD10-3puro using the *Eco*RI site. Plasmid DNA purification, restriction and ligation of DNA and isolation of subclones were as described (Sambrook *et al.*, 1989). pUHD10-3, pUHD15-1, pUHC13-3 and pUHD172-1Neo were provided by Herman Bujard (Gossen et al., 1995). pUHD10-3Neo was generated by inserting the neomycin-resistance cassette (with the chicken β-actin promoter and the SV40 poly A signal) from TFANeo (Federspiel *et al.*, 1989) into pUHD10-3 via *Hind*III digestion and ligation. pUHD10-3Puro was generated by inserting the puromycin-

resistance cassette from pβpuro (Li,1996) into pUHD10-3: pUHD10-3 was digested with *Hind*III followed by filling in the ends and ligated with the puromycin-resistance cassette from pβpuro by digestion with *Xho*I and *BamH*I followed by filling in the ends as described (Sambrook et al., 1989)

Reverse-transcription (RT) PCR

First-strand cDNA was synthesized using oligo$(dT)_{18}$ (5 mM) and 1-2 μg of total RNA as described by the manufacturer of reverse transcriptase (Life Technologies). A reaction without reverse-transcriptase (RT) served as a negative control. PCR was performed as described above with 1 μl of each RT reaction using pA and pE (Figure 2-1). Each PCR product was run on 1.2 % agarose gel in 1X Tris-acetate buffer (0.04 M Tris-acetate, 0.001M EDTA).

Virus preparation

RCOSBPCAT(A) virus stock was generated after electroporation of RP30-5 with the RCOSBPCAT-3(A) plasmid. When necessary, transfected cells were passaged to allow virus spread. Culture fluid was collected after centrifugation at 1600 rpm for 5 min at 4°C. The viral titer was determined by infecting RP30-5 and chicken embryo fibroblasts (CEF) from line 15B1 and Line 0 (Astrin

et al., 1979) using the limiting dilution method, followed by ELISA assay (Smith *et al.*, 1979) for p27. RAV-49 is a field isolate of ALV. 15B1 CEF infected with RCASBPCAT(A) was provided by Mr. Bill Payne (Department of Microbiology, Michigan State University, East Lansing, MI 48824) and RAV-49 was obtained from the USDA Avian Disease and Oncology Lab, East Lansing, MI, 48824.

Challenge with virus

The general scheme of a challenge experiment is described in Figure 2-2. Drug resistant cell clones were counted and 4 x 10^5 cells were seeded onto 60 mm plates in duplicates. For the tet-responsive expression system, each transfectant was split into -/+ tet media (4 μg/ml) 2 d prior to infection. Cells were infected with RCOSBPCAT(A) and/or RCASBPCAT(A) at various multiplicities of infection (MOI). Four d post-infection, the culture supernatant was collected by pelleting cells at 1500 rpm for 4 min at 4°C. The cell pellet was washed with PBS and lysed in 0.1% Tween 80/PBS by two cycles of freezing and thawing. Both were assayed by p27 ELISA (Smith *et al.*, 1979). Colonies which showed a reduction in p27 were further tested by varying the MOIs and by virus titer assays.

Enzyme Linked Immunosorbent Assay (ELISA)

Samples were frozen and thawed twice in 0.1% Tween80 in PBS prior to the assay. 96-well immulon plates (Dynatech Laboratories, Inc., Chantilly, VA 22021) were coated with rabbit anti-p27 antibody (1 μg/ml, SPAFAS, Inc., Storrs, CT 06268) in coating buffer (0.01 M Na_2CO_3, 0.03 M $NaHCO_3$, pH 9.5) at 4 °C overnight. The ELISA assay was performed as described (Smith *et al.*, 1979) using 1 mg/ml of 5-aminosalicylic acid (Sigma) in the phosphate buffer (0.02 M, pH 6.0) as the substrate. Optical density was measured at 490nm using a EIA autoreader model EL 310 (Bio-tek Instruments, Inc., Winooski, VT 05404). The OD was normalized to cell number and/or the total cell protein, as measured by the BCA Protein Assay Kit (Pierce Chemical Co., Rockford, IL 61105).

Southern hybridization

Genomic DNA was extracted by digestion with proteinase K and extraction with phenol-chloroform as described (Sambrook *et al.*, 1989). DNA samples were digested with *Hind*III and subjected to 0.7% agarose gel electrophoresis, followed by transfer to a nylon membrane (Zeta probe, Bio Rad) and hybridization as described (Sambrook *et al.*, 1989). The hybridization probe was a

^{32}P-labeled AE DNA fragment made by *Eco*RI digestion of AE-pBS and labeled by random primer extension (Stratagene).

RNA isolation and northern hybridization

Total RNA was isolated from cells by lysis in 4 M guanidine thiocyanate, 42 mM sodium citrate, 0.83% N-lauryl sarcosine and 0.2 mM 2-mercaptoethanol followed by phenol/chloroform/isoamylalcohol extraction and isopropanol precipitation (Promega). In other cases, total RNA was isolated by lysis using Trizol (Sigma) as described by the manufacturer. 30 µg/lane of RNA was run on a 1.2% agarose gel containing 1x MOPS (morphilinepropanesulfonic acid), 0.66 M formaldehyde, and 1 µg of ethidium bromide per ml, and blotted to Magna charge membrane (Micron Separation Inc., Westborough, MA 01581) in 10x SSC. The blots were hybridized as described for Southern blot analysis.

RESULTS

Effect of constitutive expression of antisense RNA on ALV susceptibility

The target region against which antisense RNA was generated is shown in Figure 2-1. This region was chosen because of its high density of conserved, functional retroviral sequence elements. Various portions of the sequence of this region were amplified by PCR for subsequent cloning into appropriate antisense RNA-expressing vectors. Initially, a PCR reaction was performed using RCOSBPCAT(A) plasmid as a template and the primer pair of pA and pE as shown (Figure 2-1). The 540 base pair (bp) product, AE, was inserted into the pRC-CMV expression vector in both sense and antisense orientations. This plasmid uses the strong cytomegalovirus (CMV) promoter to drive transcription of the inserted DNA fragment, in this case, the AE sequence . Fourteen G418-resistant RP30 colonies transfected with the AE-pRC-CMV (antisense) construct were obtained. Those transfectants were screened for any inhibitory effect on the replication of ALV by infecting them with RCASBPCAT(A) or RCOSBPCAT(A). After 4 d of infection, p27 viral protein production was assayed by ELISA in both the

culture supernatant and cell lysate (Figure 2-2A). Initial results of the viral susceptibility assay for the 14 cell lines, along with vector alone controls and transfected cells with the AE fragment cloned in the sense direction are shown in Table 2-2. I, II, III, and IV represent each independent experiment and each transfectant containing AE-antisense construct is indicated as 1 to 13.

Further analysis of the reduced ALV susceptibility of 3B3

Only one out of the 14 transfectants showed a significant reduction of RCOSBPCAT(A) replication when compared to that of control cells (Table 2-2), and it repeatedly demonstrated a reduced susceptibility to RCOSBPCAT(A) in more than five repeated experiments (Table 2-3). To confirm the presence of the correct AE construct in the genome of this transfectant, named 3B3, genomic DNA was isolated and probed with the AE sequence (Figure 2-3A, lane 3). The 6 kilobase pair (kb) band observed corresponds to the full-length (linear) plasmid, generated by digestion with *Hind*III, which cuts the plasmid at one site. The pattern observed is consistent with the transfecting plasmid integrating into the genome in one site as a tandem multimer (Figure 2-3B). By comparing the intensity of the full length 6 kb band with that of the

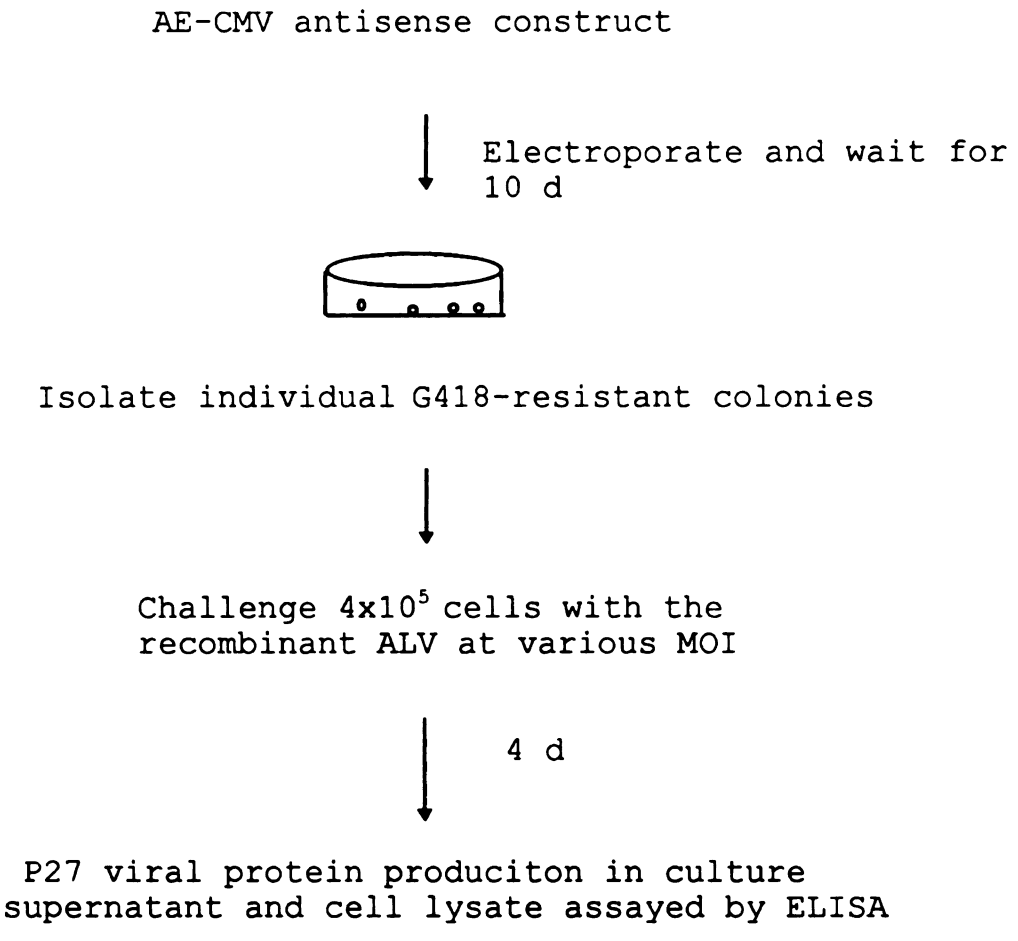


Figure 2-2A. Diagram illustrating the procedure emplyed to screen constitutive antisense-expressing colonies.

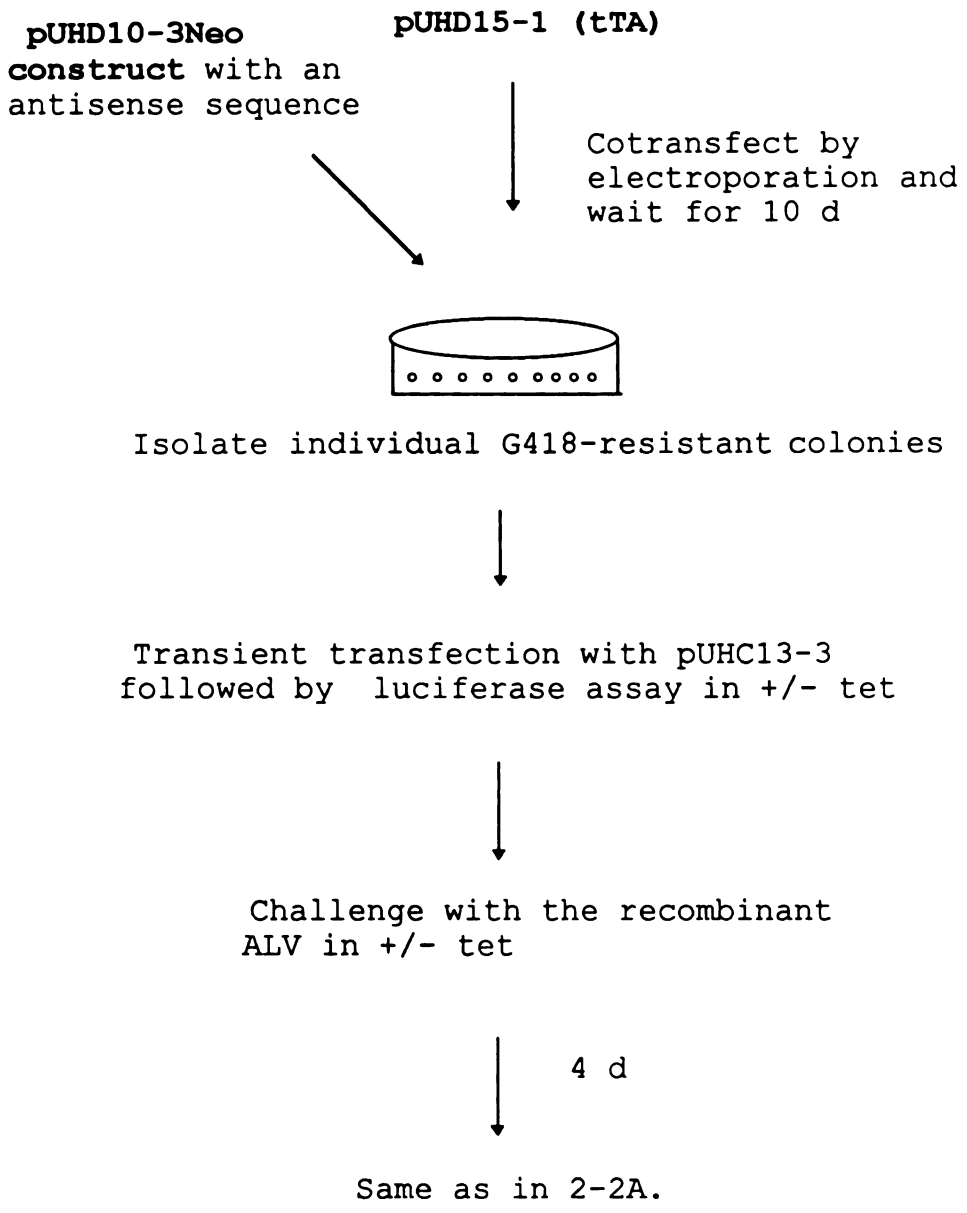


Figure 2-2B. Diagram illustrating the procedure employed to screen antisense-expressing colonies using the tet-regulatable expression system.

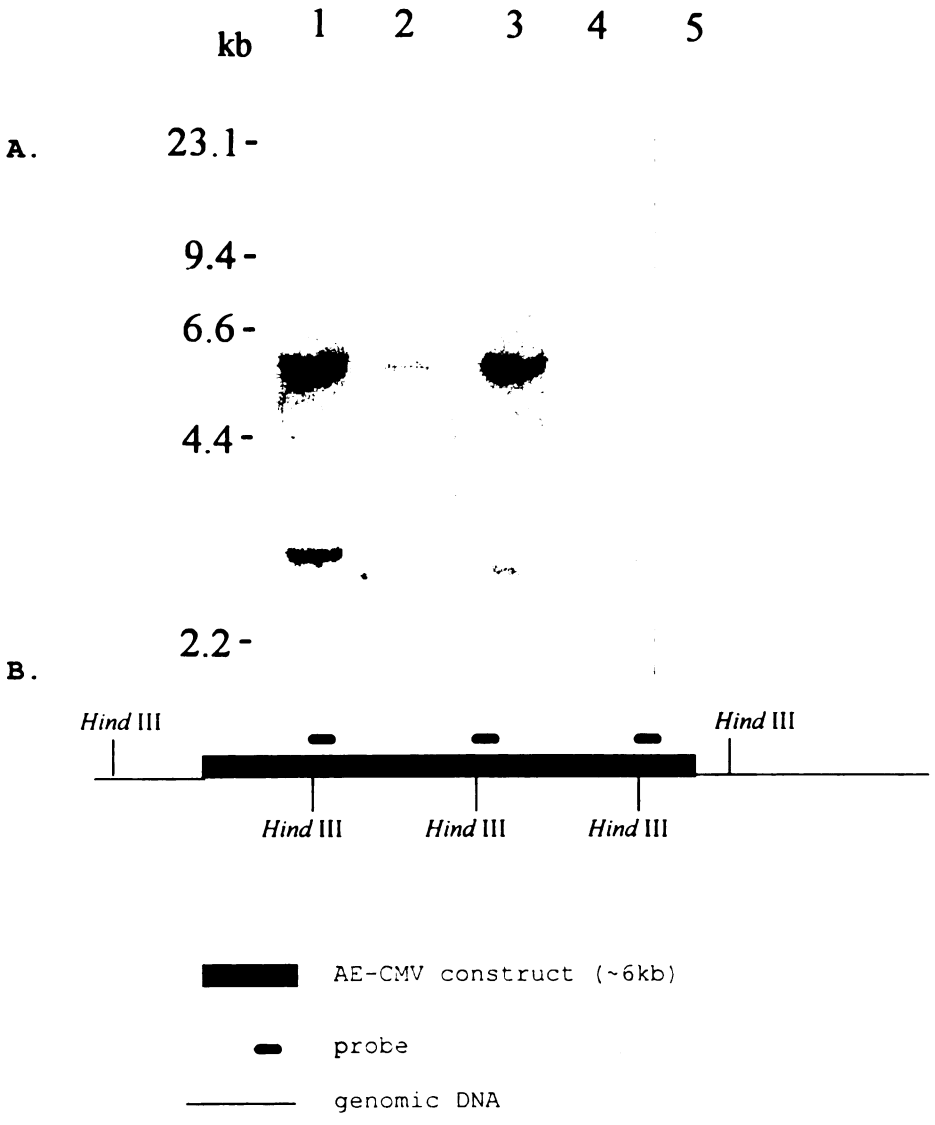


Figure 2-3. Southern blot analysis of represntative clones.
A. Genomic DNA was extracted and digested with *Hind*III, fractionated on an agarose gel, blotted and hybridized to a ^{32}P-labeled AE-DNA fragment. The molecular sizes in kb are indicated. Lanes:1, a sense clone; 2, an antisense clone(3C5); 3, an antisense clone (3B3); 4, a vector-alone clone; 5, RP30 cells.
B. Diagram of probable orientation of transfected DNA in 3B3.

Table 2-2. Effects of the transfectants on ALV replication.

		cell-free p27/mg prot		cell-asso. p27/mg prot	
	clone	average	SE	average	SE
I	RP30	20	6	29	5
	vector 1	13	7	41	5
	AE-sense	22	4	55	10
	3B3	0	0	3	0
	1	79	1	110	3
	2	44	12	74	2
	3	39	17	75	7
	4	29	6	58	0
	5	38	17	67	8
	6	44	9	34	8
	7	14	5	25	3
	8	16	2	42	3
	9	36	24	71	63
II	vector 1	86	3	87	22
	10	120	3	60	1
	11	110	19	60	3
	12	120	8	59	4
III	RP30	71	7	27	1
	13	150	0	34	2
IV	RP30	120	14	15	2
	vector 2	71	15	49	2
	3B3	6	1	7	2

I; samples collected on day 5 post infection
II; samples collected on day 4 post infection
III and IV; samples collected on day 6 post infection.
SE; standard error
vector 1 and vector 2; transfectants with the vector-alone.

Table 2-3. The effects of clone 3B3 on replication of ALV.

Virus	moi	% p27 control		% virus titer control
		cell-associated	cell-free	
RCOSBPCAT(A)	0.01	15 +/- 1[a]	18 +/- 1[a]	2[a]
	0.1	13 +/-5	16 +/- 15	ND
	1	12 +/- 2	12 +/- 9	ND
RCASBPCAT(A)	0.01	37 +/- 10	58 +/- 43	ND
	0.1	59 +/- 14	64 +/- 7	ND
	1	63 +/- 2	72 +/- 4	ND
RAV-49	0.01	22 +/- 2	34 +/- 5	2

ND; not done.
% p27 control was calculated by dividing the p27 ELISA OD per 10^6 cells (or mg protein[a]) of 3B3 by the p27 ELISA OD per 10^6 cells (or mg protein[a]) of control RP30 cells.
% virus titer was calculated by dividing the viral titer per 10^6 cells (or mg protein[a]) of 3B3 by the viral titer per 106 cells (or mg protein[a]) of control RP30 cells.

3 kb band, which most likely represents the integration junction fragment containing the probe region, we estimate that there are 3-5 AE-constructs tandemly integrated in the genomic DNA of 3B3. Northern blot analysis was performed with total RNA from 3B3 to detect the expression of an antisense RNA, but no such transcript was observed (results not shown), suggesting that the steady-state level of antisense AE transcript in this transfectant may be very low. However, a substantial amount of AE transcript was detected from the clones harboring the sense AE-construct. Therefore, RT-PCR was performed to increase the sensitivity of RNA detection. Figure 2-4, lane 4, demonstrates a detectable level of RT-PCR product AE fragment templated by 3B3 cDNA. As expected, the same fragment was generated using a small amount of the transfecting plasmid DNA (lane 5) as template and when cDNA was used from an AE-sense direction transfectant known to express detectable levels of RNA (lane 3). The control which received no RT in the cDNA reaction did not generate any detectable RT-PCR product (Figure 2-4, lane 1), thereby demonstrating that the fragment did not arise from genomic DNA that could have contaminated the 3B3 RNA.

RCASBPCAT(A) has the same antisense target region sequence as RCOSBPCAT(A) except for 19 nucleotides (nt) in

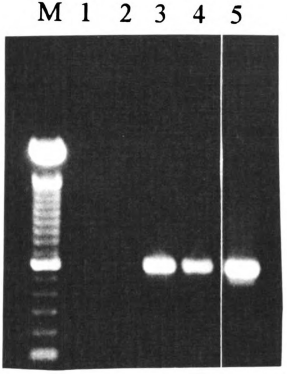


Figure 2-4. RT-PCR analysis of clone 3B3. After PCR amplification of the first-strand cDNA reverse-transcribed from the total RNA extracted from a vector-alone clone (lane 2), sense clone (lane 3) and clone 3B3 (lane 4) as described in MATEIRALS AND METHODS in Chapter 2. Lane 1 is a no-DNA control where no reverse transcriptase was added in RT reaction. Lane 5 is a positive control where the AE-CMV plasmid was used as the template for PCR reaction. Marker (M) was 100-bp ladder. A PCR product of correct size, 540-bp is present in lane 3, 4, and 5.

the leader region. Due to its more active LTR, the RCAS virus gives at least ten fold higher titers than RCOSBPCAT(A). Clone 3B3 was challenged with RCASBPCAT(A) and showed a reduced inhibitory effect on RCASBPCAT(A) replication at several MOIs tested (Table 2-3). Thus, the inhibitory effect observed in transfectant 3B3 is a partial one, primarily observed when assaying the more slowly replicating RCOSBPCAT(A). This may relate to the limited amount of antisense RNA detected in 3B3 and the ability of a more active virus to make excess viral RNA or mRNA.

Reduced ALV susceptibility of 3B3 as determined by titer

Clone 3B3 was further tested for its reduced ability to grow the RCOSBPCAT(A) target virus by direct titration of virus grown on these cells (Table 2-3). While 3B3 still can grow the RCOS virus, the titer of virus produced from 3B3 was much lower than that from control RP30 cells. This indicates that viral spread was significantly impaired in 3B3 cells. Interestingly, the reduction in virus titer is more dramatic than that in p27 production. Whatever the block to replication in 3B3 cells, it may result in the production of the non-infectious empty virus particles which lack RNA but still contain p27 (Han *et al.*, 1991).

Reduced susceptibility of 3B3 to subgroup C ALV

Since only one of 20 antisense transfectants showed a significant inhibition of ALV replication, we questioned whether this inhibition was due to antisense RNA expression or a random clonal variation. A likely possibility for the latter would be the fortuitous loss or mutation of the subgroup A-specific receptor on the cell membrane for ALV (Bates *et al.*, 1993). Rous-associated virus 49 (RAV-49) belongs to subgroup C and, therefore, recognizes a different receptor on the cell surface, but it retains the same antisense target sequence as that of RCOSBPCAT(A). 3B3 was challenged with RAV-49 and still showed a significant reduction in virus replication in both ELISA assays and viral titer (Table 2-3). This suggests that the inhibition of viral growth in 3B3 cells occurs after the attachment and entry of the virus into the cells. At this time we have been unable to design a test to unambiguously distinguish between antisense RNA expression and a fortuitous host mutation that alters a later step in viral growth and spread as the cause of the 3B3 resistance. Unfortunately, we have been unable to identify a test virus which lacks the target antisense sequence but still replicates well in the control RP30 cells. If such a virus were to grow normally on 3B3, it

would provide some (but not conclusive) support for the possibility that 3B3 exerts its effect through antisense expression.

Tetracycline-regulatable antisense expression system

As the analysis of transfectant 3B3 demonstrates, it is difficult to definitively distinguish putative antisense RNA effects from potential unrelated clonal genetic variance. However, if antisense RNA is expressed from an experimentally inducible promoter, and viral resistance is demonstrated to be similarly inducible, each cell line provides its own genetically identical internal control. Therefore, we decided to study the effect of antisense RNA by tightly regulating its transcription using the tet-regulatable expression system described by Bujard *et al.* (Gossen and Bujard, 1992; Gossen *et al.*, 1995). This system has several advantages over other regulated gene expression systems (Gossen *et al.*, 1993). First, it generally shows a lower baseline expression level and a higher level of induction than others such as lactose or heavy metal inducible systems. Second, most vertebrate cells can tolerate tet to a certain extent. In this system, gene expression is regulated by a hybrid transcription factor, tTA, that consists of the transactivation domain of herpes simplex virus VP16 fused

to the carboxy terminus of the tetracycline-repressor (tetR). The gene of interest is placed downstream of a minimal promoter linked to seven tandem copies of the tetR-binding site (tetO). The activation of transcription from this promoter depends on the binding of tTA to the tetO site, a process which is tightly regulated by the presence or absence of the drug. In the presence of tet, binding of the tTA to tetO is blocked and gene expression is silent or greatly reduced. Upon removal of tet, tTA binds to tetO and induces a high level of transcription.

Figure 2-2B illustrates the procedure used to screen effective antisense colonies in the tet-system. As before, upon electroporation with the constructs, G418-resistant RP 30 cells were selected and single colonies were expanded. Each was then screened for its level of transactivation activity by transient transfection with the *luc* plasmid (pUHC13-3). This plasmid employs the same tet-operator/minimal CMV promoter as pUHD10-3Neo to drive expression of *luc*. Therefore, the level of luciferase induction after transient transfection provides confirmation that the regulatable expression system is operating effectively in any given clonal cell line. Since the antisense RNA cassette is driven by an identical promoter to that of *luc*, it is likely that transcription

initiation of the antisense gene will show a similar level of inducibility. Of course, post-transcriptional effects likely will cause the relative expression level of antisense RNA to differ from that of luciferase activity, but the transactivation test allows one to eliminate cell clones in which inducible promoter control is non-functional or poorly functional (presumably due to integration effects on the pUHD15-1 plasmid).

Fourteen G418-resistant cell transfectants harboring the AE sequence in the antisense orientation were screened. Seven of these showed significant transactivation activity when grown in the media without tet (Figure 2-5). However, none of those seven showed a significant inhibition of viral growth upon challenge with RCOSBPCAT(A) when grown in the media without tet verses that observed with tet (Figure 2-5).

Two cell lines harboring the AD portion of the target sequence (Figure 2-1) in the antisense orientation showed a significant transactivation activity, but, again, neither of these showed a tet-regulated inhibition of viral replication (Figure 2-6). Similarly, 15 stably-transfected cell lines containing the AC (Figure 2-1) sequence in the antisense orientation showed a high level of tet-inducible luciferase activity without any

Figure 2-5. The effect of cell lines harboring the AE antisense sequence in the tet-repressible system. % p27 in -tet was calculated by this formula: [(p27 ELISA OD/10^6cells in -tet)÷(p27 ELISA OD/10^6 cells in +tet)]x100. Fold activation* was based on luciferase activity and calculated by this formula: (luciferase activity /10^6 cells in -tet)÷(luciferase activity /10^6 cells in +tet).

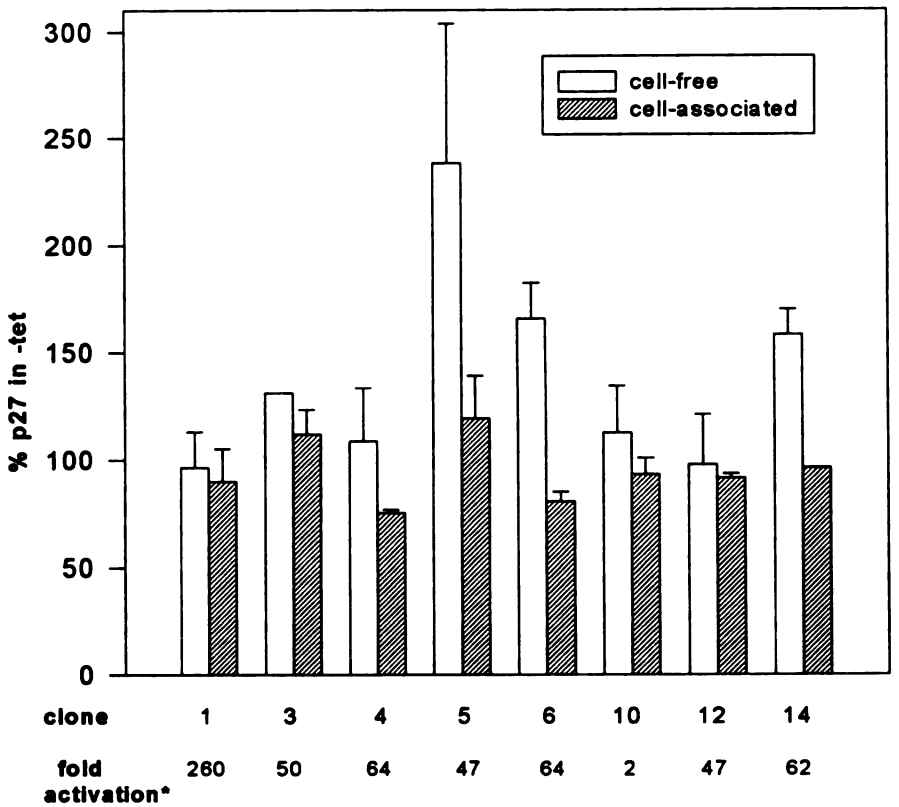

300
250
200
150
100
50
0
% p27 in -tet
cell-free
cell-associated
clone 1 3 4 5 6 10 12 14
fold activation* 260 50 64 47 64 2 47 62

Figure 2-5

Figure 2-6. The effect of cell lines harboring the AD antisense sequence in the tet-repressible system. % p27 in -tet was calculated by this formula: [(p27 ELISA OD/10^6cells in -tet)÷(p27 ELISA OD/10^6 cells in +tet)]x100. Fold activation* was based on luciferase activity and calculated by this formula: (luciferase activity /10^6 cells in -tet)÷(luciferase activity /10^6 cells in +tet).

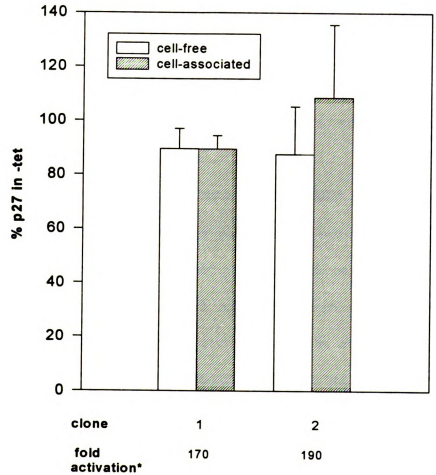

140
120
100
80
60
40
20
0
% p27 in -tet
cell-free
cell-associated
clone
1
2
fold activation*
170
190

Figure 2-6

statistically significant reduction in viral replication (Figure 2-7).

Northern blot analysis of two cell lines harboring the AD sequence (Figure 2-1) demonstrated the substantial expression of an antisense transcript in a tet-regulated fashion (Figure 2-8). Similarly, specific transcription of AE-antisense RNA was detected in two transfectants only in the absence of tet (Figure 2-9). As described above, none of these transfectants demonstrated tet-regulated viral resistance.

CF antisense RNA expression did not induce ALV resistance

The results described in the previous section demonstrate that several different conserved regions of the ALV genome have no anti-viral effect, even when they can be shown to be expressed as antisense RNA at relatively high levels. Others (Goodchild *et al.*, 1988; Sczakiel *et al.*, 1992) have shown that the choice of a target region for antisense inhibition can be critical to its efficacy. As an experimental method to enhance our choice of a target, we employed antisense oligonucleotides in hopes of identifying sequences that are particularly sensitive to antisense inhibition. These results are described in Chapter 3 of this thesis. While antisense oligonucleotides did not show a dramatic anti-viral

Figure 2-7. The effect of cell lines harboring the AC antisense sequence in the tet-repressible system. % p27 in -tet was calculated by this formula: [(p27 ELISA OD/10^6cells in -tet)÷(p27 ELISA OD/10^6 cells in +tet)]x100. Fold activation* was based on luciferase activity and calculated by this formula: (luciferase activity /10^6 cells in -tet)÷(luciferase activity /10^6 cells in +tet).

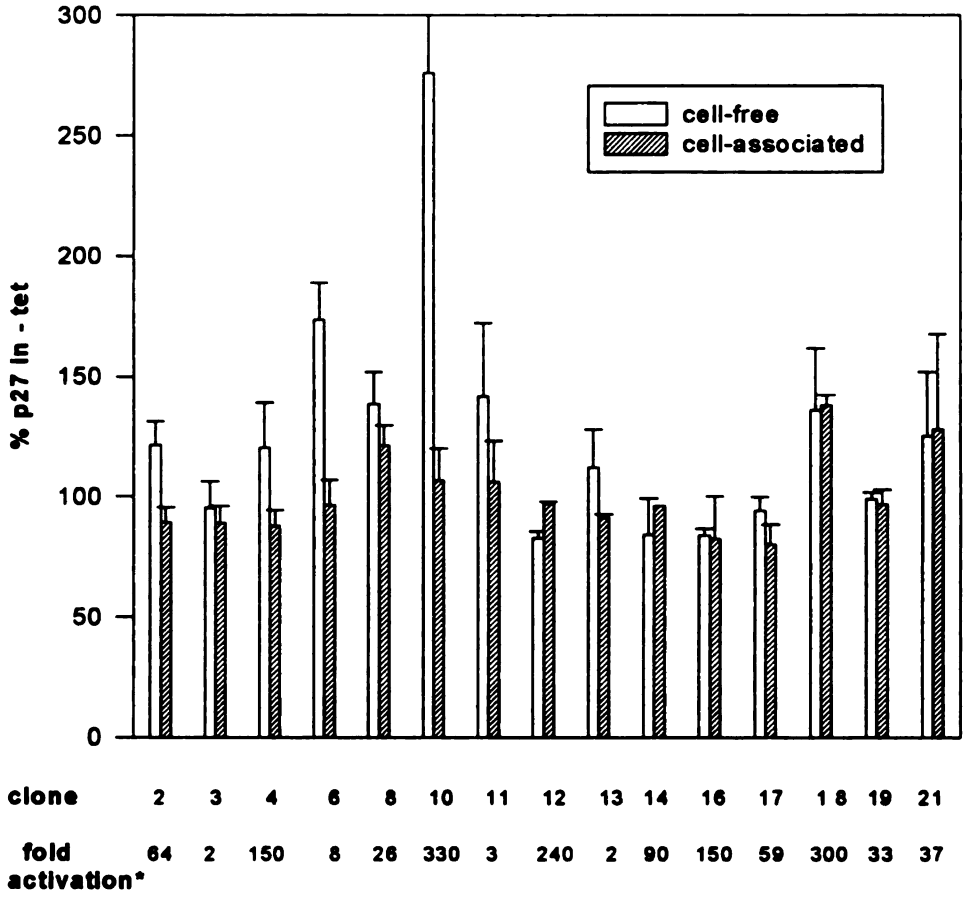

300
250
200
150
100
50
0
% p27 in - tet
cell-free
cell-associated
clone 2 3 4 6 8 10 11 12 13 14 16 17 18 19 21
fold activation* 64 2 150 8 26 330 3 240 2 90 150 59 300 33 37

Figure 2-7

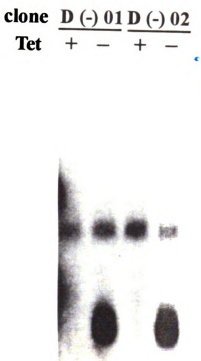


Figure 2-8. Northern blot analysis of the AD-tet clones. Each clone (D(-)01 and D(-)02) was grown in the presence or absence of tet (4 μg/ml) 2 d prior to total RNA extraction as described in MATERIALS AND METHODS. Thirty μg of each RNA was electrophoresed, blotted and hybridized with ^{32}P-labeled AD-DNA fragment. A band of approximately 400 nt was detected.

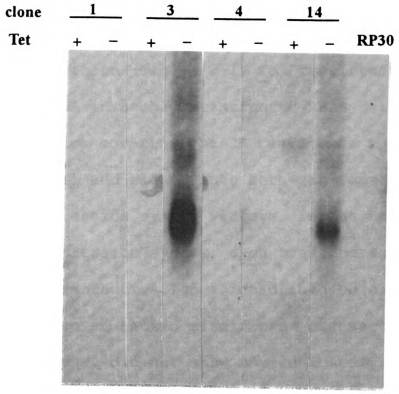


Figure 2-9. Northern blot analysis of the AE-tet clones. Each clone was grown in the presence or absence of tet (4 μg/ml) for 2 d prior to total RNA extraction as described in MATERIALS AND METHODS. Thirty μg of RNA per lane was electrophoresed, blotted and hybridized with a ^{32}-P labeled AE-DNA fragment.

effect, statistically significant inhibition was observed with oligonucleotides targeted to a short sequence ranging from the PBS to the middle of the leader sequence (Table 3-4, Chapter 3). Based on these results, we chose to target the CF region (Figure 2-1) for regulated expression of antisense RNA in stably transfected RP30 cells.

The sequence covering the CF region was amplified by PCR (Figure 2-1) and cloned into both tet-repressible and -inducible expression system vectors. In the tet-repressible expression system, each transfectant was screened for transactivation as described before. Six transfectants which showed significant transactivation activity, however, did not show any inhibitory effect on viral replication as assayed by ELISA (Figure 2-10). In the tet-inducible expression system, the plasmid pUHD172-1Neo contains a mutated tetR fused with the transactivation domain of VP16, so that, in the presence of dox (a derivative of tet), tTA binds to the tetO in the pUHD10-3 vector and induces a high level transcription of the gene located downstream of the promoter (Gossen *et al.*, 1995). Using this system, RP30 cells were cotransfected with CF-10-3/puro and pUHD172-1Neo. Eight transfectants which are both G418- and puromycin-resistant were selected. These cell lines were

Figure 2-10. The effect of cell lines harboring the CF antisense sequence in the tet-repressible system. % p27 in -tet was calculated by this formula: [(p27 ELISA OD/10^6cells in -tet)÷(p27 ELISA OD/10^6 cells in +tet)]x100. Fold activation* was based on luciferase activity and calculated by this formula: (luciferase activity /10^6 cells in -tet)÷(luciferase activity /10^6 cells in +tet).

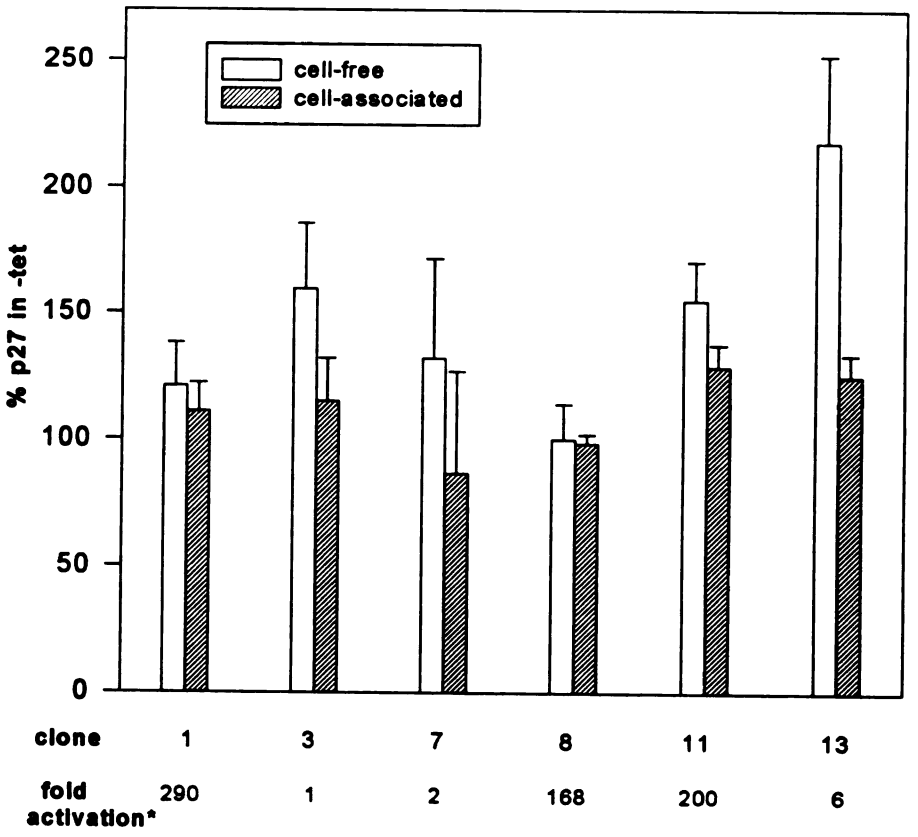

250
200
150
100
50
0
% p27 in -tet
cell-free
cell-associated
clone
1
3
7
8
11
13
fold activation*
290
1
2
168
200
6

Figure 2-10

tested for transactivation activity as previously described. The results shown in Table 2-4 identified six transfectants with low baseline luciferase expression and high levels of transactivation. The fold of activation was in a range of 150 to 1000, which was significantly higher than that of the tet-repressible expression system (ranging up to 300 fold). Although detectable antisense RNA levels were observed in a few transfectants (Figure 2-11), none of these showed an inhibitory effect on viral replication, as shown in Figure 2-12.

DISCUSSION

In this report, the potential inhibition of ALV retroviral growth and spread by expression of antisense RNA was examined. In preliminary studies, one cell line, 3B3, was detected that was significantly more resistant to the RCOSBPCAT(A) test strain than the RP30 parental cell line. 3B3 expresses an antisense transcript at very low levels which is complementary to most of the regulatory signals at the 5′ end of ALV, including the PBS, Ψ, the leader sequence, the ATG translation initiation signal and the SD. 3B3 showed a considerably greater reduction of RCOS virus titer than of capsid protein production compared to the controls. This observation suggests that a

Table. 2-4. The level of transactivation of the CF-tet inducible clones.

clone	fold activation[a]
1	150
2	2
3	940
4	370
5	330
6	300
8	160
10	1

[a] Fold activation was calculated as described in Figure 2-12.

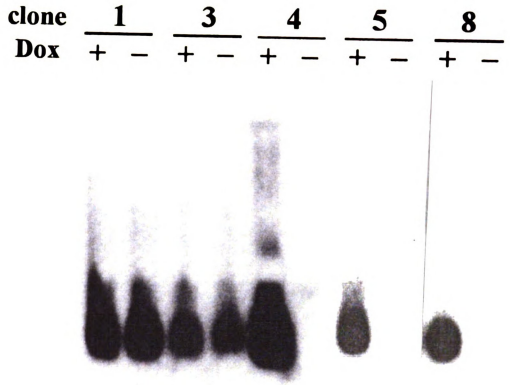


Figure 2-11. Northern blot analysis of the CF-clones in the tet-inducible expression system. Each clone was grown in the presence or absence of dox (1 µg/ml) for 1 d prior to total RNA extraction as described in MATERIALS AND METHODS. Thirty µg of RNA per lane was electrophoresed, blotted and hybridized with a ^{32}-P-labeled CF-DNA fragment. The predicted size of the transcript is approximately 200 nt. In clone 1 and 3, the antisense transcript was detected despite the absence of inducer, possibly due to cointegration of CF-pUHD10-3puro and pUHD172-1Neo, resulting in deregulated transcription of the CF sequence.

Figure 2-12. The effect of cell lines harboring the CF antisense sequence in the tet-inducible system.
% p27 in +dox was calculated by this formula: [(p27 ELISA OD/10^6cells in +dox)÷(p27 ELISA OD/10^6 cells in -dox)]x100. Fold activation* was based on luciferase activity and calculated by this formula: (luciferase activity /10^6 cells in +dox)÷(luciferase activity /10^6 cells in -dox).
dox=doxycycline

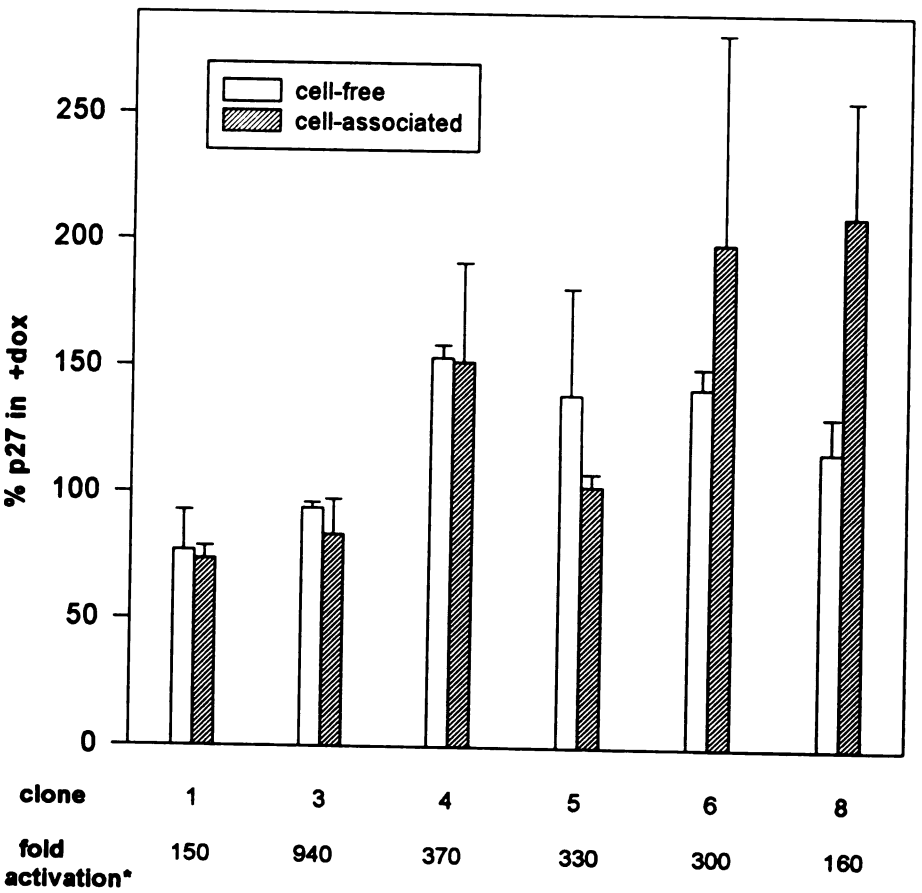

cell-free
cell-associated
% p27 in +dox
250
200
150
100
50
0
clone
1
3
4
5
6
8
fold activation*
150
940
370
330
300
160

Figure 2-11

defect in viral growth on 3B3 cells might occur in the packaging step, resulting in the production of non-infectious particles. Southern blot analysis showed at least three copies of the antisense plasmid constructs were integrated tandemly in the 3B3 genome. It has been shown that Ψ is recognized by a specific motif (Cys-His box) of the NC protein during viral assembly (Aronoff *et al.*, 1993;
Dupraz *et al.*, 1990), and this sequence could therefore act as a potential target for antisense inhibition. Alternatively, the interaction between antisense RNA and its target could lead to the induction of double-strand specific cellular RNases, resulting in the degradation of both RNAs and in production of empty virus particles. Since only one antisense RNA-producing transfectant demonstrated reduced viral susceptibility, it is certainly possible that the inhibitory effect in 3B3 cells may derive from a clonal variance unrelated to antisense RNA production. In other words, the 3B3 line might have incurred an unrelated mutation which reduces its ability to grow the target virus. The replication of ALV, like that of other retroviruses, depends on the host gene expression machinery. The best characterized genetic mechanism for host cell resistance to ALV involves changes

in the subgroup-specific receptor genes such as *tva* and *tvb* (Crittenden, 1991). In this case our test virus is subgroup A and if 3B3 had acquired a mutation in *tva*, one would expect no change in its susceptibility to RAV-49, a subgroup C virus. Our results showed that 3B3 also had a reduced ability to grow RAV-49, and, again, the titer of the virus was reduced more significantly than was production of the capsid protein. Since it is unlikely that 3B3 spontaneously acquired mutations in both loci encoding the subgroup A and subgroup C receptors, receptor variance does not appear to explain viral resistance in 3B3. Unfortunately, the converse control, using a virus of subgroup A with an altered antisense target sequence that grows in RP30 is not presently available. However, challenge of 3B3 with the RCAS virus showed much reduced, if any, viral resistance. This could be due to overcoming the antisense effect with the larger amount of viral RNA generated by the stronger promoter in the RCAS virus. Alternatively, 3B3 may provide a reduced level (relative to parental RP30 cells) of a trans-acting host factor required for replication of RAV-0-based viruses but not by the RSV-A-based viruses. To further examine the properties of 3B3 cells, the line was transfected with a sense RNA-expressing (AE) construct cloned in the tetracycline-

repressible expression system in hopes of overcoming the viral resistance, if it is due to antisense RNA. Pools of sense-transfected 3B3 cells were analyzed in bulk. If anything, removal of tet to allow expression of AE sense RNA led to a further reduction of viral growth rather than relief of the 3B3 viral resistance (Figure 2-13). While these results do not conclusively prove that antisense expression is not the cause of the 3B3 viral resistance, they reinforce doubts raised by our other experiments. By employing tet-regulatable expression systems, we could control for possible complications due to clonal variance. Although specific, regulated transcription of antisense RNA was obtained in several transfectants, no corresponding reduction in virus replication was observed. It is especially intriguing to note that the antisense RNA from the CF sequence, which seemed to be the optimal target region for an antisense ODN (Chapter 3), had no antiviral effect. One explanation for this discrepancy might relate to the need to deliver the antisense nucleic acid to the appropriate subcellular compartment. Although northern blots demonstrate the presence of substantial antisense RNA within several cell lines, it is possible that this RNA is confined to the nucleus and needs to reach the cytoplasm to exert the described effect on the

Figure 2-13. The effect of cell lines harboring the CF antisense sequence (without poly A signal) in the tet-inducible system.
% p27 in -tet was calculated by this formula: [(p27 ELISA OD/10^6cells in -tet)÷(p27 ELISA OD/10^6 cells in +tet)]x100. Fold activation* was based on luciferase activity and calculated by this formula: (luciferase activity /10^6 cells in -tet)÷(luciferase activity /10^6 cells in +tet).

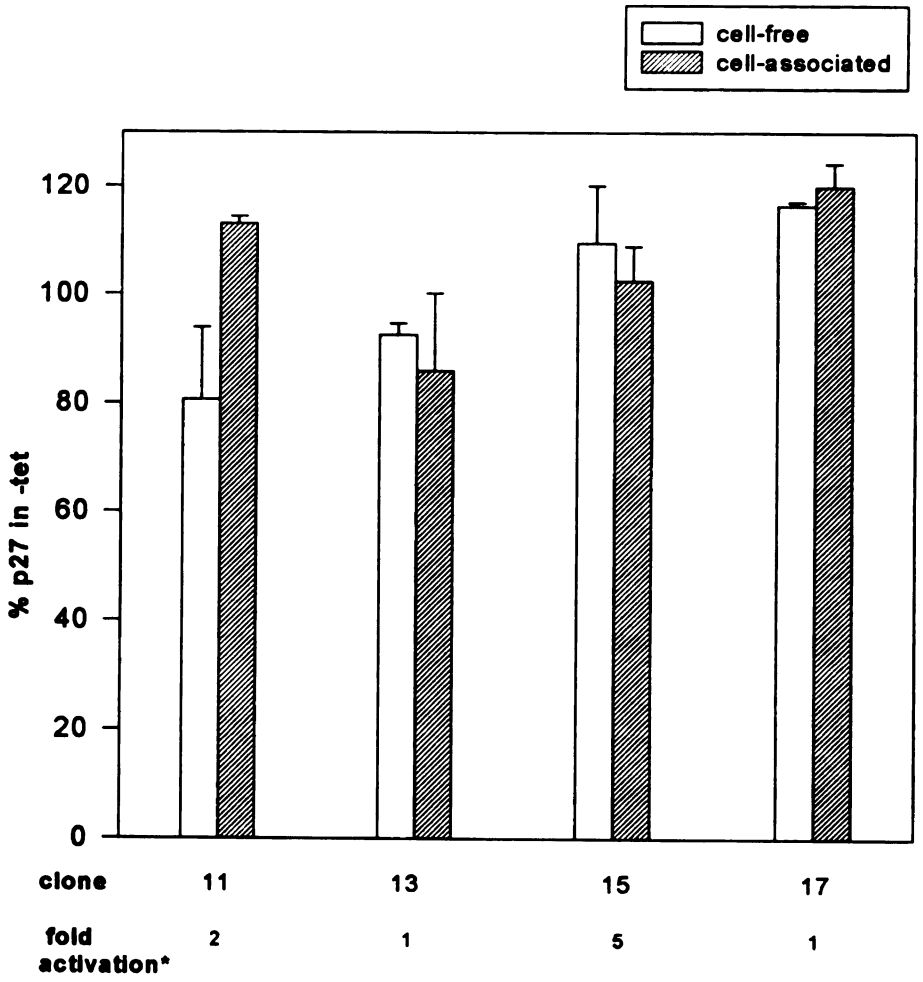

cell-free
cell-associated
120
100
80
60
40
20
0
% p27 in -tet
clone
11
13
15
17
fold activation*
2
1
5
1

Figure 2-13

virus. However, since a variety of vector systems were used to express antisense RNA, all with no increased viral resistance, this seems an unlikely explanation. Another factor may be that *in vivo*-expressed antisense RNA is considerably longer than the antisense ODN, containing sequences flanking the presumed optimal target site, including the 3′ poly A tail. Longer RNA has a greater potential to form higher-order structures, which could block interaction with the presumptive antisense target. We have transfected RP30 cells with a CF-pUHD10-3Neo construct devoid of the poly A signal. However, the level of transactivation as measured by assaying the luciferase acvtivity was significantly reduced. And when these trasnfectants were assayed for ALV resistance, again, they showed no inhibitory effect on ALV replication (Figure 2-13). Peng *et al.* (1996) have suggested from studies on HIV that shorter antisense RNA expressed from a stronger promoter, in their case, a Pol III system, might be more effective. A third explanation for the difference in ODN and antisense RNA results would be that ODN-generated inhibition (Chapter 3) has a very limited effect on viral growth, and the transfected cell system may be insufficiently sensitive to detect such an effect, either

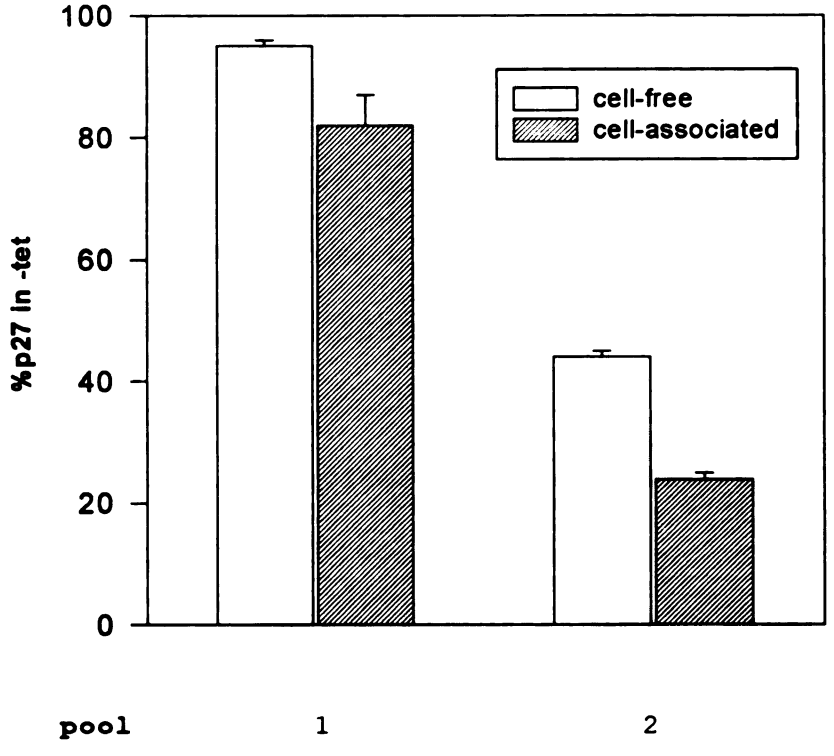


Figure 2-14. The effect of pooled-cell lines derived from clone 3B3 by transfecting a sense tet-repressible AE-construct.

in individual or pooled colonies. This lack of sensitivity may relate to the inherent clonal variability of the transfectants, the limited number of transfectants that can reasonably be tested or the potential masking effect of tet or dox on cell growth.

In conclusion, several cell lines have been obtained which have been shown to express substantial amounts of antisense RNA in a regulated fashion but which have no significant effect on their ability to support growth of ALV. A variety of conserved viral sequences at the LTR and the 5′ end of the virus have been targeted, including regions which showed limited, but significant inhibition when used as targets for antisense ODN (Chapter 3), all without significant effects on viral growth. In addition, several different vector systems have been used to generate antisense RNA, both with and without poly A addition. While it would certainly be possible to test many other regions of the ALV genome as antisense targets, the well-known ability of retroviruses to rapidly mutate (Coffin, 1991) is likely to confound this approach, at least for any attempt to create chickens with resistance to a wide range of field strains of ALV. At best only limited conclusions can be drawn from predominantly negative results, like those shown in this thesis, but the

clear suggestion from our experiments is that *in vivo* expression of antisense RNA is unlikely to be an effective way to generate transgenic poultry that are resistant to field strains of the virus.

In our experiments involving constitutive expression of antisense RNA, one cell line (3B3) was obtained that consistently demonstrated significantly reduced susceptibility to the target RCOS-based virus and to an ALV (RAV-49) of a different subgroup. It remains possible that the effect observed was due to antisense RNA expression, but this conclusion is placed in doubt by the following observations: 1. Only very low levels of antisense RNA were detected in 3B3, 2. Little or no resistance was evidenced against a more virulent ALV (RCAS) with nearly the same antisense target sequence as RCOS, 3. Numerous attempts to replicate this observation in other antisense-expressing cell lines failed, and 4. Attempts to overcome the resistance observed in 3B3 by counter-expressing sense RNA failed. Therefore, it seems likely that the effect observed in 3B3 derived from a mutation in this clone of cells that may be unrelated to antisense expression, perhaps in the expression of some trans-acting host factor required for RCOS replication but dispensable for RCAS replication.

Chapter 3.

Antisense Oligodeoxynucleotides as Inhibitors of ALV Growth

ABSTRACT

Antisense oligodeoxynucleotides (ODN) are short stretches of synthetic DNAs made to be complementary to a target RNA. Numerous cases of ODN inhibition of viral replication have been reported. In Chapter 2 of this thesis, we describe the general ineffectiveness of expressed antisense RNA in attempts to inhibit ALV replication in different expression systems. In this chapter, we have employed antisense ODN in hopes of finding the most effective antisense target in the 5′ end of ALV genome. Eight different target sites were selected based on their potential capacity to block key processes that occur during viral replication. A short region from the primer binding site (PBS) to the middle of the leader/packaging sequence seemed to be the most effective antisense target in these experiments.

INTRODUCTION

Antisense oligodeoxynucleotides (ODN) are short synthetic DNAs made to be complementary to a target RNA in hopes of blocking the function of that RNA. Their effects were first demonstrated when a 13-mer antisense ODN complementary to the R region of the long terminal repeat (LTR) sequence was found to inhibit Rous sarcoma virus (RSV) replication in chicken embryo fibroblasts (CEF) (Zamecnik and Stephenson, 1978). Biological efficacy of antisense ODN is generally considered to depend on their stability against nucleases, their ability to penetrate the cell membrane and their binding affinity to the specific target sequence (Peyman *et al.*, 1995). Antisense ODN have a negatively charged phosphate backbone structure which is expected to hamper their uptake by cells, and they may also be susceptible to DNase-mediated degradation. Modifications of the phosphate backbone are often employed to increase cellular uptake and stability. These include thiolation and alkylation of phosphodiester linkages and also conjugation of the ODN with peptides, such as poly(L-lysine) and low-density lipoprotein (Bongartz *et al.*, 1994; Lemaitre *et al.*, 1987; Mishira *et al.*, 1995). Most modifications (phosphorothioate being the exception) result in at least a partial reduction in

nuclease susceptibility (Wagner, 1995). In addition, antisense ODN with partial modifications (i.e., modification of terminal linkages) have been tested in various systems and found to be more effective than the ones with total modification (Chavany *et al.*, 1995). The direct mechanism of ODN uptake is not yet known. Experiments with FITC-conjugated ODN show that, upon internalization, they appear as speckles inside the cytoplasm, and most of them are localized in the nucleus (Geselowitz and Neckers, 1992; Wagner, 1995). These results suggest that the majority of ODN are encapsulated in vesicles, and these vesicles may play a role in transport to the nucleus. Several studies have reported inhibition of viral gene expression in cultured cells by phosphorothioate ODN (Lisziewicz *et al.*, 1994; Matsukura *et al.*, 1987). Clinical trials to evaluate the efficacy of antisense ODN against human immunodeficiency virus (HIV) and human cytomegalovirus (HCMV) are in progress (Agrawal and Tang, 1992; Lisziewicz *et al.*, 1994).

Avian leukosis virus (ALV) is a retrovirus belonging to the avian leukosis-sarcoma group. This virus infects chickens and induces lymphoid leukosis. In the previous chapter of this thesis, several conserved regions of the ALV genome were examined as potential targets for inducing

viral resistance via expression of antisense RNA in stably transfected cell lines. With one possible exception, we were unable to detect an antiviral effect when 540 nucleotides (nt) at the 5′ end of ALV genome were used as a target for antisense RNA expression. To explore this region in a more detailed manner for the most effective target sequence for antisense RNA, we chose to examine the effect of antisense ODN on ALV replication.

MATERIALS AND METHODS

ODN synthesis

ODN were synthesized using an Applied Biosystems model 394 DNA synthesizer at the DNA Core Facility of Marshall University, Huntington, WV 25704. All ODN were precipitated in ethanol prior to use.

Cell culture and ODN treatment

RP 30-5 cells were maintained as described previously (Chapter 2). Cells were washed once with serum-free media prewarmed to 37°C and counted. $2x10^6$ cells were seeded onto a 35mm plate (Sarstedt Inc., Newton, NC 28658) in 0.8 ml of the serum-free media. Twenty µl of Lipofectin reagent (1 mg/ml, Life Technologies, Gaithersburg, MD 20877) was mixed with 80 µl serum-free media and incubated for 30 min. Meanwhile, each ODN was also diluted in 100 µl of the serum-free media at five different concentrations (0, 2, 5, 10, and 20 µM). The diluted Lipofectin reagent was mixed with each ODN preparation and incubated for 15 min at room temperature. The mixture was added to each corresponding plate of cells. After 13 hr at 40°C, cells were pelleted by centrifugation for 3 min using the IEC clinical centrifuge. The cell pellet was resuspended in 4

ml of complete media, and the cells were incubated for an additional 6 hr prior to infection with the target ALV vectors, RCASBPCAT(A) and RCOSBPCAT(A) (Hughes and Kosik, 1984; Hughes *et al.*, 1987).

Virus infection

Cells which were treated with antisense ODN-liposome mixtures were counted. $4x10^5$ cells were seeded onto each 60 mm plate in triplicate and infected with $4x10^3$ infectious units (iu) of RCOSBPCAT(A) or RCASBPCAT(A). The antisense ODN solutions were re-added to the cells at their original concentrations, and additional antisense ODN were added 48 hr post-infection, again at the stated concentrations. Cells and culture supernatants were collected after 4 d and assayed by ELISA as previously described (Chapter 2).

RESULTS

Dosage-dependent inhibition of antisense ODN on replication of ALV

RP30 cells were transfected with antisense ODN and infected with recombinant ALV test strains followed by measurement of p27 viral capsid production as illustrated

in Figure 3-1. The 5′ end of the ALV genome was the primary focus of these experiments, as it contains numerous conserved sequence elements critical to viral replication (Chapter 2, also see Figure 3-2). The sequence of each antisense ODN is shown in Table 3-1. Each ODN was evaluated at five different extracellular concentrations (0, 2, 5, 10 and 20 μM) in triplicate for its effect in RP30 cells on replication of RCOSBPCAT(A) or RCASBPCAT(A). Both RCOS and RCAS are recombinant ALV vectors but differ in the U3 of their LTR, which in RCAS contains a strong enhancer element. The two test viruses also differ in their leader/packaging region sequences by 19 nt. Therefore, antisense ODN #5 RCOS and #3 RCOS are specifically complementary to the RCOS viral RNA , whereas #5 RCAS and #3 RCAS are complementary to RCAS viral RNA. The results from the initial test are shown in Table 3-2. In this experiment, the RCOS-specific ODNs were tested with RCOS virus and the RCAS-specific ODNs were tested with RCAS virus. Both cell-free and cell-associated p27 production were assayed by ELISA as previously described (Chapter 2) and normalized to total cell count. The effect of each antisense ODN is compared to controls which were treated only with the Lipofectin reagent at the transfection step.

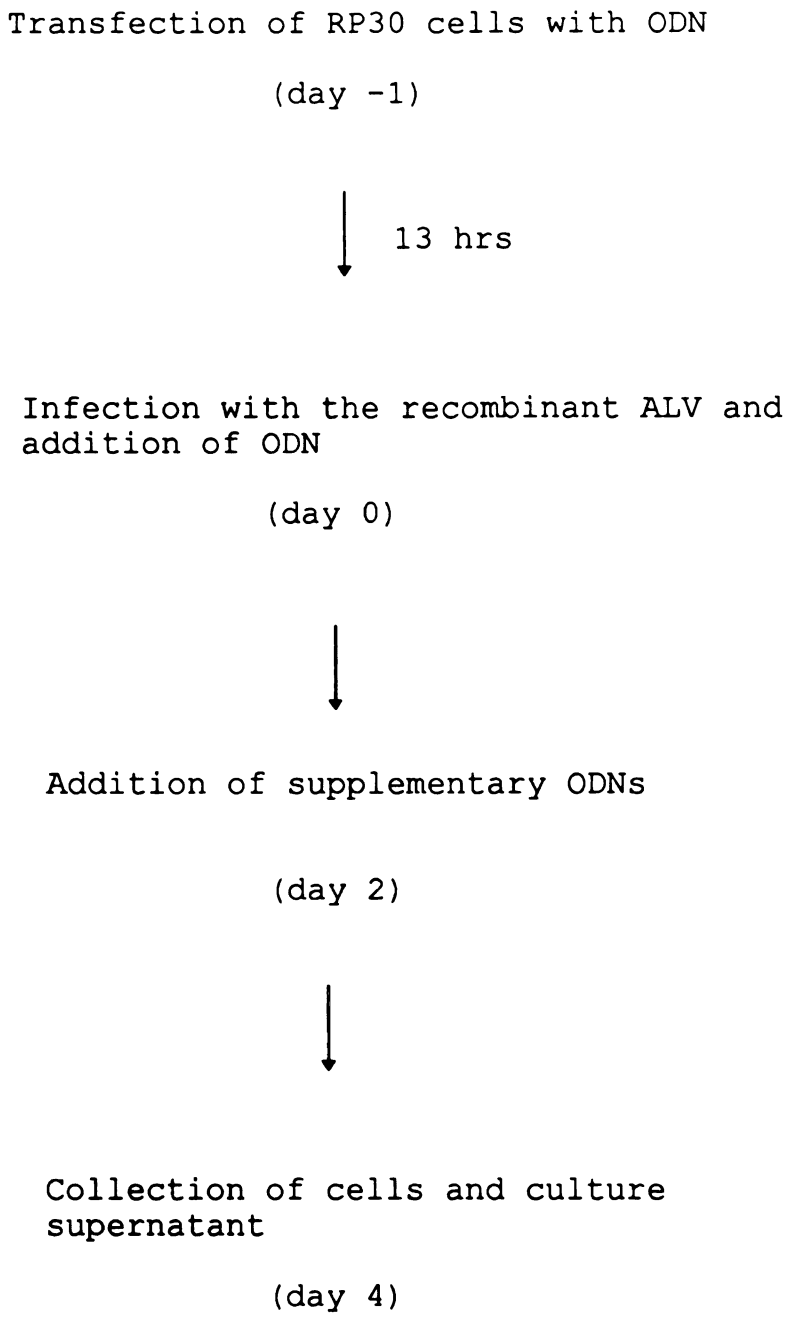


Figure 3-1. A diagram illustrating the procedure used to test antisense ODN inhibition of viral replication

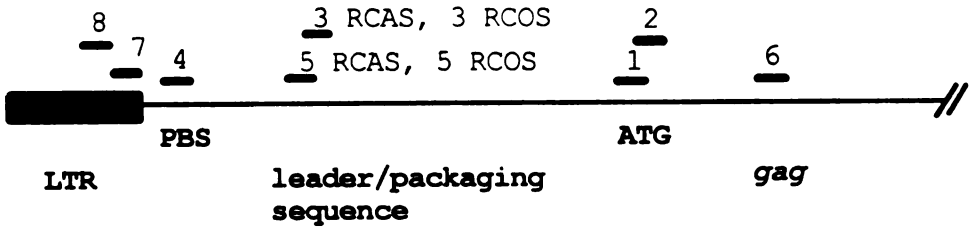


Figure 3-2. The regions targeted by antisense ODN. LTR, long terminal repeat; PBS, primer binding site.

Table 3-1. Sequence of antisense oligodeoxynucelotides

ODN	sequence
1	TGA CGG CTT CCA TGC TGG AT
2	CTT AAT GAC GGC TTC CAT GC
3 RCOS	CGT TAA GCG AGA CGG ATG AG
3 RCAS	CGA TAG ACG AGA CGG ATG GA
4	ATC ACG TCG GGG TCA CCA AA
4A	GGT CAC CAA ATG AAG CCT TC
4B	TTC CCT AAC TAT CAC GTC GG
5 RCOS	ATG AGG GCA GGA TCG CCA CG
5 RCAS	ATG GAG ACA GGA TCG CCA CG
6	TAA GCA ACC CTT CCT TTT GT
7	CAT GCA GGT GCT CGT AGT CG
8	GGT GAA TGG TAA AAT GGC GT

Table 3-2. Dose response of inhibition of ALV replication by antisense ODN

	% control				% control			
	cell-associated				cell-free			
antisense ODN	2 μM	5μM	10 μM	20 μM	2 μM	5 μM	10 μM	20 μM
1	160	150	50	55	120	110	110	66
2	60	60	45	ND	150	130	120	ND
3RCOS	71	70	63	51	68	65	78	44
3RCAS	96	64	7	2	70	82	16	9
4	92	45	48	22	120	88	90	65
5RCOS	67	33	33	27	63	50	25	25
5RCAS	160	120	38	6	210	220	59	5
6	61	38	28	19	85	78	48	25
7	140	65	46	32	100	63	50	36
8	88	75	38	16	86	130	62	52

% control was calculated by dividing the ELSIA OD per 10^6 cells treated with antisense ODN by the ELISA OD per 10^6 cells of untreated cultures. Averages of triplicate samples are shown.
ND; not done.

First, a dosage-dependent inhibitory effect of each antisense ODN was observed. In other cases high concentrations of ODN exhibited toxic effects on cell growth (Gao *et al.*, 1991; Stein, 1995). In this experiment, the highest concentration of ODN (20 μM) had no effect on cell growth, as determined by microscopic observation and cell counting (data not shown). However, every ODN had some inhibitory effect on viral replication at increasing concentrations, suggesting a non-specific effect. However, the levels of virus replication appeared to be lower in the presence of some antisense ODN than others. To control for the non-specific inhibitory effects of ODN, selected ODN were further investigated by comparison to those of random sequence control ODN.

Specificity of the inhibitory effect of antisense ODN

Phosphorothioate (PS)-oligonucleotides have been shown to induce non-specific effects by binding to cell surface proteins or other intracellular regulatory factors (Neckers *et al.*, 1995; Stein, 1995). For example, PS-oligos, in a length-dependent but relatively sequence-independent manner, are known to bind to soluble CD4 at or near the HIV-1 binding site (Yakubov *et al.*, 1993), and they also bind to the v3 loop of the HIV-1 envelope glycoprotein, gp120 (Stein *et al.*, 1993). It was also

suggested that the non-specific effect is dependent on the number of phosphorothioate linkages in a given length of ODN (Cheng *et al.*, 1991). Therefore, random sequence control ODN with the same base composition and modifications were designed to analyze the specific effect of a given antisense ODN on virus replication. Based on our preliminary results, random combinations of antisense ODN #3, #4, #5 and #6 were synthesized and tested. Table 3-3 shows the sequence of each control random sequence ODN. Each antisense ODN was then tested in parallel with its control ODN for inhibition of virus replication (Table 3-4). Antisense ODN #4 demonstrated an inhibitory effect on viral replication when compared to that of its control at all concentrations tested ($p<0.05$). ODN #6 showed a slight, but statistically insignificant, decrease in viral replication when compared to that of its control. Interestingly, ODN #5 showed a significant reduction of viral replication compared to the control only at 10 and 20 μM. ODN #3 did not show any significant reduction of viral replication compared to the control. None of these ODN had an inhibitory effect on cell growth during the time course of the experiment. However, both antisense and control ODN still showed non-sequence-

Table 3-3. Random sequence control oligodeoxynucleotides.

ODN	ODN sequence
#4 random	AGG ATA CGC ATC GTA CAC GC
#5 RCOS random	AGA CTG CGT GCC GGA GAG AC
#5 RCAS random	AAG ACG GCG TCA ATC GAC GG
#6 random	TTC GAT TAT TCC CAT CGA TC
#3 RCOS random	AGC CGT CGA AGT AGT AGA GG
#3 RCAS random	AAG GGT ATG GAG ACG ACC AG

Table 3-4. Effects of antisense ODNs compared to their randomers.

antisense ODN	% control cell-associated				% control cell-free			
	2μM	5μM	10μM	20μM	2μM	5μM	10μM	20μM
4[a]	33	22	31	33	65	39	46	46
5RCOS	100	100	62	56	100	100	38	31
5RCAS	100	100	68	40	100	100	100	41
6[b]	85	89	81	91	88	61	49	85
3RCOS	100	100	86	100	100	100	100	100
3RCAS	100	100	23	100	100	100	100	100

% control was calculated by dividing the ELISA OD per 10^6 cells of cultures treated with antisense ODN by the ELISA OD per 10^6 cells of cultures treated with the randomers. Average of duplicated samples is shown. By this calculation, percentage $\geq$ 100 is expressed as 100%, which represents no reducion of p27 production.
[a]$p<0.05$ and [b]$p>0.05$ (by the Student t-test)

specific inhibitory effects on virus replication, especially at higher concentrations.

The primer binding site as an ODN target

The results above suggested that antisense ODN #4 exhibited the most consistent reduction of viral replication. As a further control, a cocktail of randomers (rather than a single oligonucleotide whose sequence was chosen at random) with the same base composition and modification pattern as antisense ODN #4 was synthesized. This "super randomer" theoretically contains 4^{20} different sequences. RP30 cells were treated with antisense ODN #4, randomer #4 and the super randomer simultaneously and infected with RCOSBPCAT(A) followed by the p27 ELISA assay. Again, RP30 cells treated with antisense ODN #4 showed inhibition of virus replication at 5 and 10 μM (Figure 3-3). Little or no effect was observed at 2 and 20 μM. At 2 μM the ODN concentration may have been too low to generate a significant inhibition, while at 20 μM the generic inhibition observed for all ODN may have obscured any sequence-specific effects. Furthermore, the effect of the super randomer on viral replication was similar to that of the single #4 randomer. These results suggest that

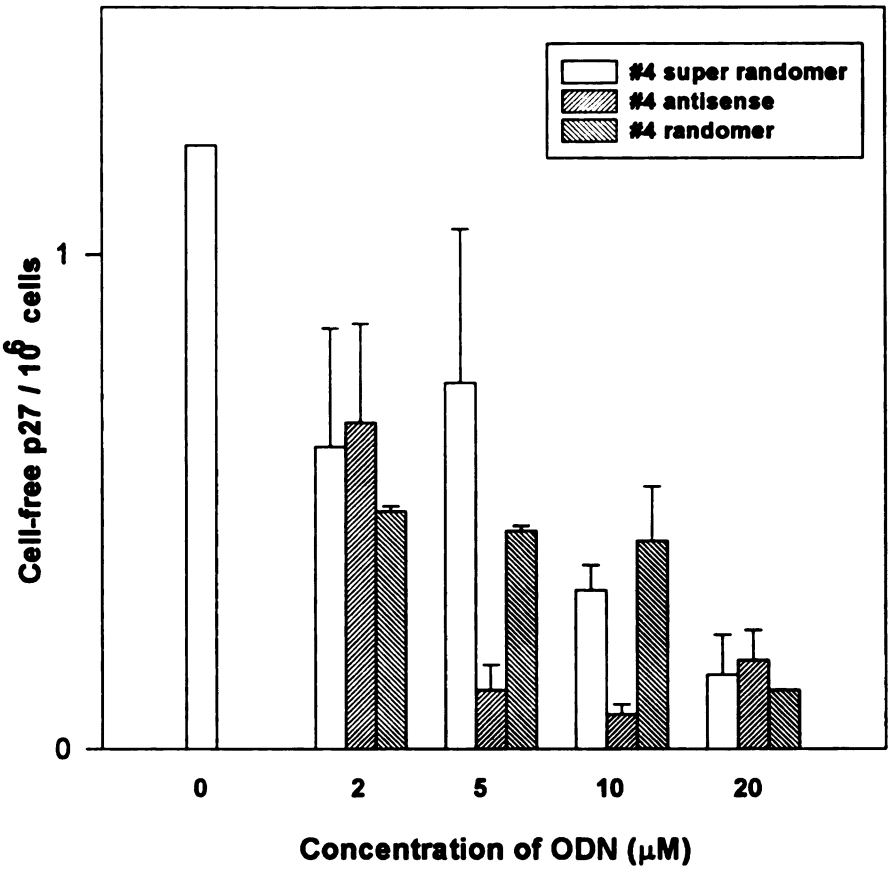


Figure 3-3.A. Effects of ODN #4 compared to the controls. Cell-free p27 ELISA OD was normalized to 10^6 cells.

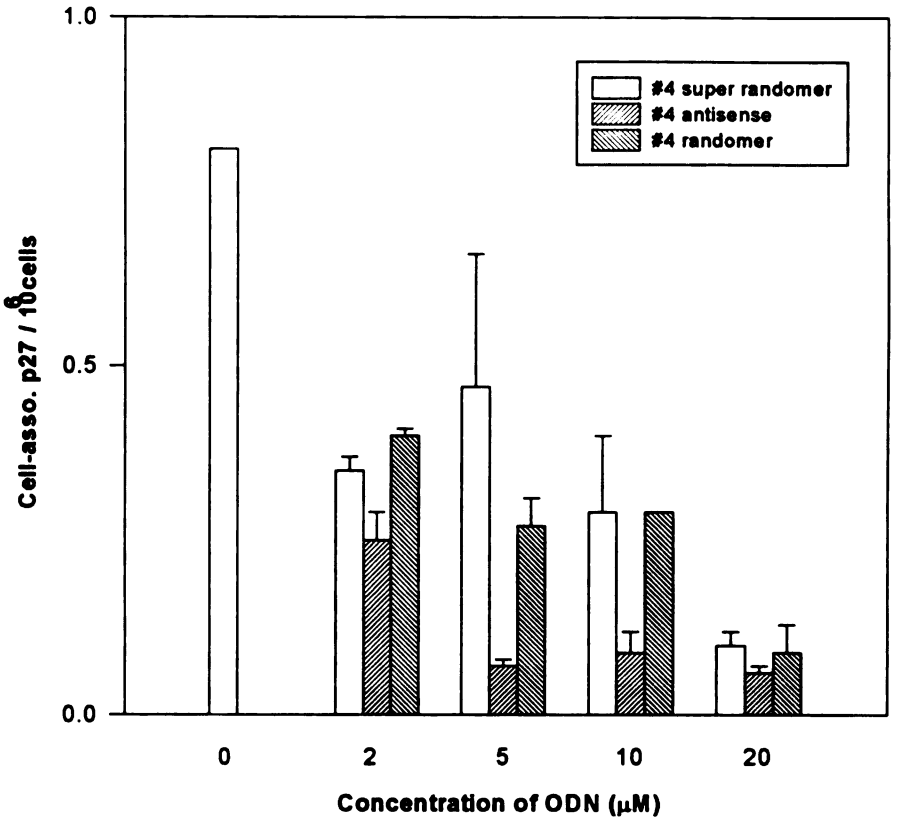


Figure 3-3.B. Effects of ODN #4 compared to the controls. Cell-associated p27 ELISA OD was normalized to 10^6 cells.

the inhibitory effect of antisense ODN #4 is sequence-specific.

Since antisense ODN #4 is complementary to the complete sequence of the PBS, we decided to explore its effect further by using ODN which are partially overlapping its target region. The relative location of each ODN is shown in Figure 3-4. ODN #4A overlaps #4 by 10 nt at the 3′ end, and #4B, by 10 nt at the 5′ end. Direct comparison of the effect of each ODN with one another demonstrated that #4A was not as effective as #4 (Figure 3-5). However, #4B showed a similar reduction in virus replication as #4 at all concentrations tested.

DISCUSSION

Since its first demonstration (Zamecnik and Stephenson, 1978), the antisense ODN approach has been widely employed to inhibit target gene expression in various systems (Cowsert, 1993). It has been particularly useful in inhibiting retrovirus replication, including that of HIV (Goodchild *et al.*, 1988; Lisziewicz *et al.*, 1994; Matsukura *et al.*, 1987). In fact, some antisense ODNS are in trials as potential anti-HIV therapies (Lisziewicz *et al.*, 1994). While there is no imaginable prospect that antisense ODN would ever be a cost-effective

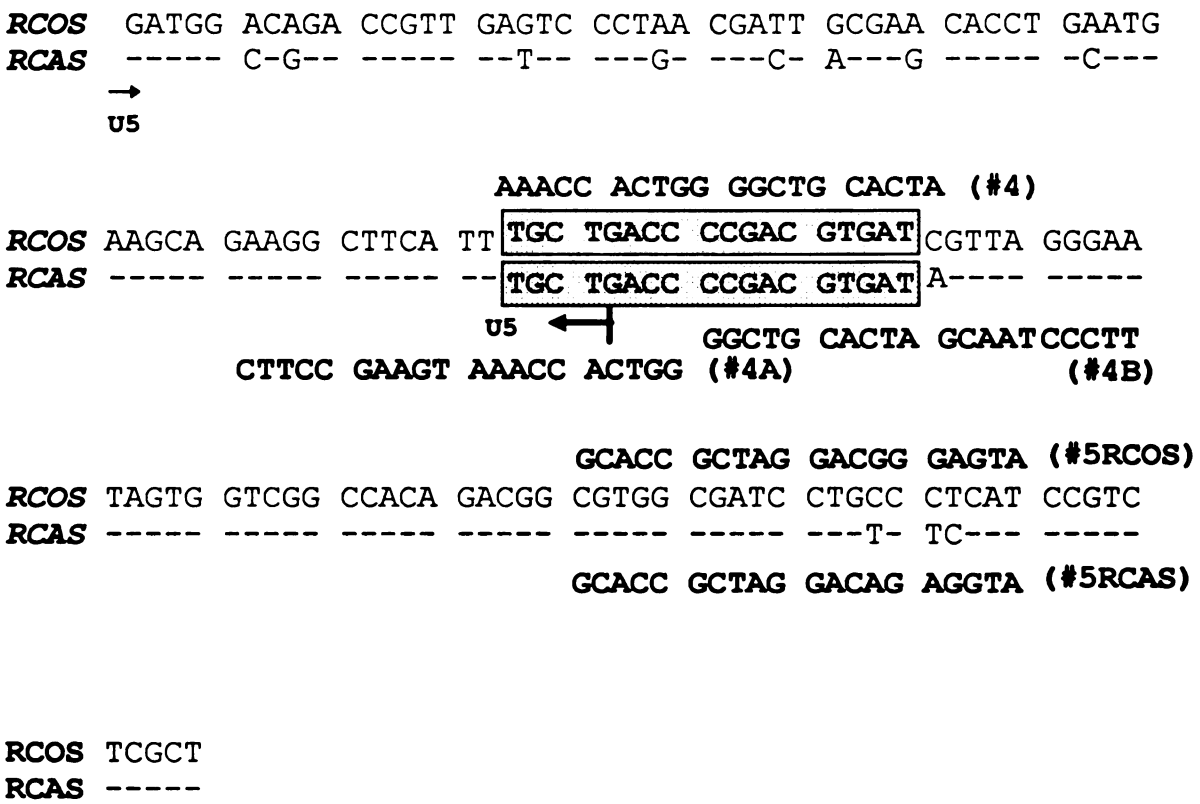


Figure 3-4. Sequence of the region near the PBS of the RCOS and the RCAS viral RNA targeted by antisense ODN #4, #4A and #4B. The PBS is marked by [ ]. The sequence of viral RNA is shown in regular characters and the sequence of each antisense ODN is shown in bold characters. The common nucleotides between the RCAS and the RCOS are indicated as -.

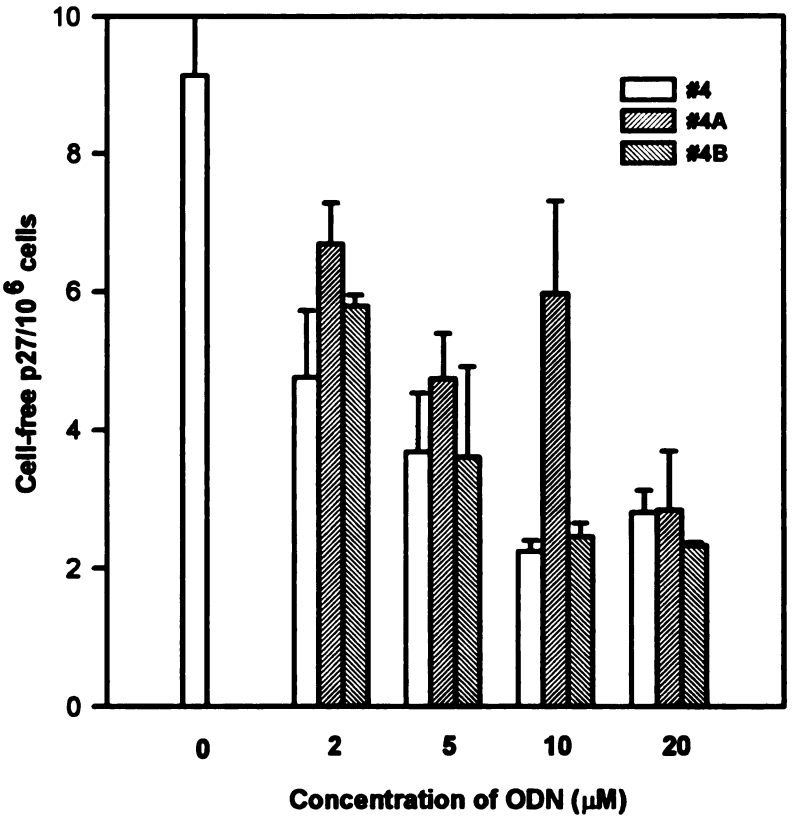


Figure 3-5. A. Effects of ODN #4, #4A and #4B. Cell-free p27 ELISA OD was normalized to 10^6cells.

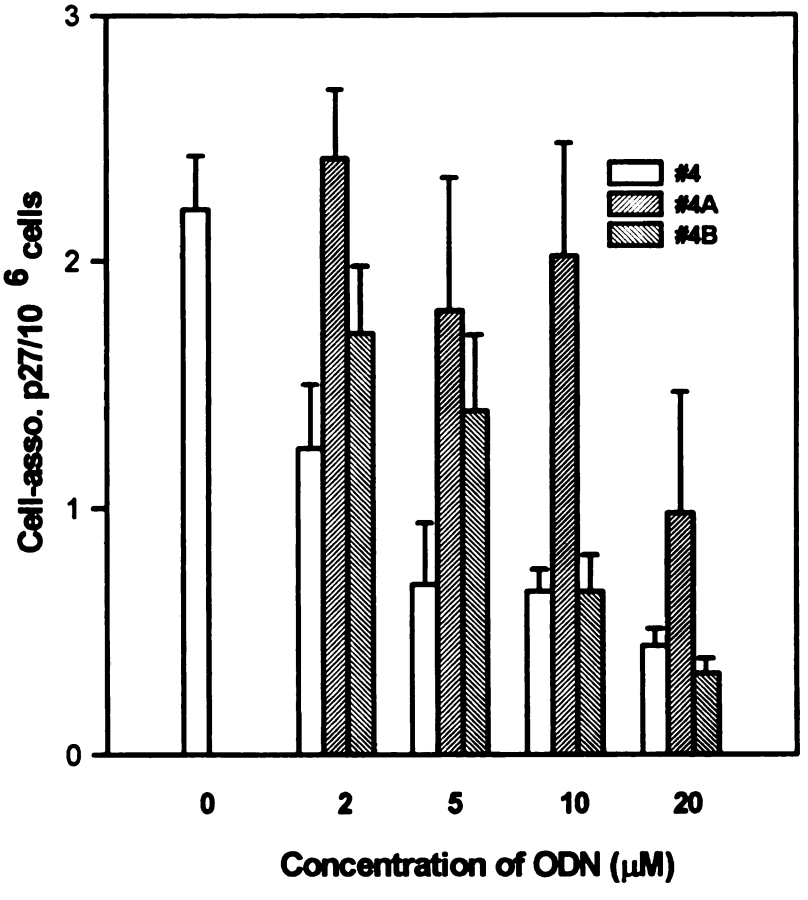


Figure 3-5. B. Effects of ODN #4, #4A and #4B. Cell-associated p27 ELISA was normalized to 10^6cells.

antiviral therapeutic in domestic poultry, we chose to examine the effect of antisense ODN on ALV replication in RP30 cells in hopes that it might guide us in the construction of antisense RNA-expressing vectors (Chapter 2).

Each antisense ODN in our experiments was modified by thiolation at two linkages at the 5′ end and four linkages at the 3′ end to improve its resistance to cellular nucleases. The internal linkages were kept as normal phosphodiester bonds to promote efficient hybridization with the target RNA. After an initial, uncontrolled survey, four of the original 8 target sites were selected for further study. (This included two sites at which RCAS and RCOS differ in sequence.) Each of these ODN was examined in parallel with an appropriate random sequence control ODN. Each control ODN had the same length, base composition and modification pattern as its antisense counterpart. Of the 4 sites tested versus control ODN, 3 continued to show inhibition of viral replication, but one, ODN #4, appeared to be most consistently effective. ODN #4 is complementary to the PBS region of ALV and therefore has the potential to compete with the host $tRNA^{trp}$ primer for binding to the viral RNA at the PBS. In the event that this antisense ODN displaces the tRNA

primer, this could impair either the generation of proviral DNA or the subsequent synthesis of functional viral RNA. Furthermore, the correct secondary structure at the 5′ end of ALV RNA near the PBS plays a critical role in the initiation of reverse transcription as shown by mutational analysis (Aiyar *et al.*, 1994). Therefore, the presence of ODN #4 instead of the tRNA primer at the PBS could also potentially block the initiation step of reverse transcription. However, other studies have shown that the ODN accumulate preferentially in the nucleus (Wagner, 1995). Therefore, we cannot exclude the possibility that antisense ODN #4, for unknown reasons, is preferentially bound by ALV RNA in the nucleus leading to its degradation (Cowsert, 1993). Furthermore, the region including the PBS also contains an element of the secondary structure which was proven to be critical in efficient encapsidation of ALV genomic RNA (Knight. *et al.*, 1994). Therefore, antisense ODN #4 could also disrupt this secondary structure leading to inefficient packaging of viral RNA.

Antisense ODN showed no inhibitory effects on cell growth as determined by microscopic inspection and cell counting. However, all ODN exhibited a generic inhibitory effect on virus replication at higher concentrations,

suggesting non-sequence specific effects. Non-specific antiviral effects of PS-ODN have been described in several reports (Chavany, 1995; Gao. *et al.*, 1991; Krieg and Stein, 1995), further stressing the importance of comparing antisense effects to appropriate control ODN. However, there is some difficulty in choosing an ideal control. We have generally used a single, randomly chosen permutation of the antisense ODN sequence. Since there is an extremely large number of possible choices (sequences with similar stretches to the antisense ODN or likely problems in synthesis are eliminated), and since all ODN have some effect on viral replication, it is impossible to completely rule out the possibility that a fortuitously "good" control has been chosen (leading to a false positive antisense effect) or a fortuitously "bad" control has been chosen (leading to a false negative). For ODN #4, we also used a "super randomer" control, a mixture of all possible ODN sequence. While this is a better control in some ways, it could be argued that the effective concentration of each of the 4^{20} control ODN sequences is so low that it is an inappropriate comparison to a large concentration of a single ODN sequence. We have employed both control ODN approaches with essentially equivalent results with respect to ODN #4. In addition, by comparing

the effect of ODN #4 to flanking sequences in ODN #4A and #4B, the different ODN studied in effect act as the controls for each other.

The maximum antiviral effect that was observed with ODN #4 was approximately 80%. In other cases (Lisziewicz *et al.*, 1994, Matsukura *et al.*, 1987), particularly of HIV, the antiviral effect ranged up to 99%. GEM91 (Lisziewicz *et al.*, 1994) was a 25-nt long phosphorothioate ODN complementary to the ATG initiation signal of the *gag* reading frame of HIV. However, we did not observe a significant inhibition of ALV replication with ODN #6 which was complementary to the ATG signal of ALV *gag*. Goodchild *et al.* (1988) have shown in studies of HIV that a 20-mer phosphodiester ODN complementary to the PBS demonstrated 30-60% inhibition, and the most effective inhibition was obtained with ODN complementary to the R region and to certain splice sites (85% and 80%, respectively). Furthermore, cellular uptake efficiency in each experiment is expected to account for at least part of the variation in effectiveness. Standifer *et al.* (1995) have shown that up to 1.7 % of total labeled ODN could be found intact and associated with neuronal cells, and this amount reduced target mRNA levels by 25-30%. However, an approximately 18-fold of increase in uptake was shown when

cells were incubated with an ODN in the presence of Lipofectin reagent (Bennet *et al.*, 1992). Therefore, we employed the lipofection technique to create an intracellular "reservoir" of antisense ODN and re-added ODN every 2 days to replace degraded ODN. At this moment, we do not have information on the cellular uptake efficiency of ODN in our system. However, since the cells were treated with an antisense ODN in parallel with its control randomer, which also has the same modification pattern, it seems unlikely that there was an extensive variation in the uptake efficiency between them. Hoke *et al.* (1991) suggested from their studies with herpes simplex virus that the reduced efficacy of partial compared to fully PS ODN in HeLa cells may result from increased degradation of the mixed phophodiester/PS-oligos. Therefore, the effects of different antisense ODN are likely to depend on several variables, such as the length, the modification, the target site, the mode and activity of viral replication and the host cells used.

In conclusion, we have found a region from the PBS up to the middle of leader sequence to be an effective target for antisense ODN. In particular, antisense ODN #4, which is complementary to the PBS, showed the most consistent inhibition (maximum of 80%), relative to its controls. In

Chapter 2 of this thesis, we have tested the antiviral effect of this region in a stable antisense RNA expression system, without significant antiviral effect.

Chapter 4.

Antisense RNA complementary to subgroup A ALV receptor mRNA expressed in a quail cell line: test for effects on virus susceptibility

INTRODUCTION

A critical step in the life cycle of an enveloped virus is the binding of the virus to the host cell. This process is mediated by specific interactions between the viral envelope glycoprotein (SU) and the cell-surface receptor (Coffin, 1990). Avian retroviruses of the avian leukosis virus (ALV) group have been divided into several subgroups. The subgroups of ALV are defined by host range in chicken cells that differ in susceptibility to infection, patterns of receptor interference, and virus neutralization (Crittenden, 1991). These properties are all regulated by differences in the viral envelope surface glycoprotein, SU (gp85). There are five major subgroups of ALV (subgroup A to E), which are determined solely by amino acid differences in the variable regions of SU. The susceptibility of chickens to ALV subgroups is controlled by three genetic loci, *tva*, *tvb*, and *tvc* (Crittenden, 1991). *tva* and *tvc* alleles are thought to be linked and encode receptors for subgroup A and subgroup C viruses, respectively. Different alleles of *tvb* might encode receptors for subgroups B, D, and E.

Recently, the gene encoding the receptor for subgroup A ALV has been cloned in a quail cell line (QT6) and shown to be the product of the *tva* locus (Bates *et al.*, 1993, L.

Crittenden, personal communication). *tva* cDNAs of two different sizes (800 and 950 nt), named pg800 and pg950, respectively, were identified, which were shown to be generated by alternative splicing. The deduced amino acid sequences predicted that the extracellular domain of the receptor had some sequence homology to a ligand-binding domain of the low-density lipoprotein receptor. Although neither their mRNAs nor protein products are detectable in chicken cells, these cDNAs can confer susceptibility to infection by subgroup A ALV to an otherwise resistant cell line. The cellular function of this receptor, however, remains unknown. A truncated form of the putative subgroup A ALV receptor (without the transmembrane domain) was able to protect the cells from infection, by binding to the viruses and blocking attachment (Connoly *et al.*, 1994).

The *tva* gene can exhibit both susceptible and resistant alleles, so it was of interest to see if viral resistance could also be induced by blocking expression of the gene. Since "knock-out" chicken technology has yet to be perfected, one attractive method was to try to use antisense techniques to block *tva* expression. The corresponding mRNA is expressed at undetectable levels in most avian cells, so it might be relatively easy to overcome its function by expressing small to moderate

amounts of stable antisense RNA. Our attempts at expressing ALV antisense RNA (Chapter 2) were generally unsuccessful, possibly due to some inherent properties of viral RNAs. Thus we wished to try the antisense approach against a host cell target mRNA.

We have expressed antisense RNAs against the message for subgroup A ALV receptor in hopes to block the viral infection at its entry step. To date, our focus has been on the 5′ end of this gene. The transcription start site has not been mapped yet, but there are two putative TATA boxes 300 and 200 bp upstream of the ATG translation initiation signal. Two DNA fragments which covered the ATG signal including the 5′ untranslated region were amplified by the Polymerase Chain Reaction (PCR). The effect of antisense RNA complementary to this 5′ region was examined in a tetracycline(tet)-regulatable gene expression system.

MATERIALS AND METHODS

Cell culture

QT6 is a chemically transformed quail fibroblast cell line (Moscovici *et al.*, 1977). This cell line and all of its derivatives were maintained in Leibovitz L-15/ McCoy's 5A medium (Life Technologies, Gaithersburg, MD 20877) under 5% CO_2 containing 10% chicken serum, 5% fetal bovine serum, 2.5% tryptose phosphate broth supplemented with gentamycin (10 µg/ml) and amphotericin B (2.5 µg/ml).

Transfection

Transfection was performed using Lipofectin as described by the manufacturer (Life Technologies). Briefly, cells were split one day prior to transfection into 60 mm plates. Six h before transfection, the media was replaced with fresh. In one tube, 2.5 µg of each plasmid construct was mixed with 5 µg of pUHD15-1 (transactivator-encoding plasmid) in 150 µl of serum-free media. In another tube, 45 µl of Lipofectin (1 mg/ml) was diluted to 150 µl with serum-free media and incubated for 30 min at room temperature. The Lipofectin solution was then added to the DNA solution and incubated for 15 min. Meanwhile, cells were washed once with 4 ml of serum-free

media. Serum-free media was added to the DNA/liposome mixture to 2 ml, and this mixture was then added to cells and incubated for 16 h at 40°C. At this point cells were changed into complete media and incubated for an additional 24 h. Cells were then split in to two 10 cm plates in selection media containing 0.4 mg/ml of neomycin (G418-sulfate, Life Technologies) and 4 µg/ml of tet. The G418-resistant colonies developed in 10 d. Each colony was expanded and tested for transactivation by transient transfection with the *luc* construct, pUHC13-3.

Luciferase assay

Each G418-resistant transfectant was split into 60mm plates containing media with and without tet one d prior to transfection with pUHC13-3. Lipofection was performed as described above using 1.8 µg of pUHC13-3 and 20 µl of Lipofectin (1 mg/ml) in 300 µl of serum-free media. Cells were washed twice with phosphate buffered saline (PBS), lysed in 300 µl of 1x lysis buffer (diluted from 5x with water; Promega, Madison, WI 53706), and spun at 14k at 4°C briefly to remove cell debris. The supernatant was diluted 100-fold with 1x lysis buffer and 10 µl was mixed with 100 µl of the substrate at room temperature. The luciferase

activity was then measured using a Turner TD-20e luminometer (Turner Designs, Inc., Sunnyvale, CA 94086).

Plasmid construction

The quail genomic clone (Q5.5-pBSks(-)) containing the subgroup A ALV receptor gene was provided by Paul Bates (Bates *et al.*, 1993). Figure 4-1 illustrates the region targeted by antisense RNA. Contigs 2 and 6 are regions previously sequenced by Dr. Bates' lab, who provided the sequence to us. The "gap" between these contigs was sequenced using the primer pRec 5 by the dideoxy chain termination method (Sanger *et al.*, 1977). The length of this gap was determined to be 125 base pairs (bp). The PCR primers used are listed in Table 4-1 and their positions are shown in Figure 4-1. PCR reactions were performed as described (Sambrook *et al.*, 1989) with appropriate pairs of primers as indicated in Figure 4-1. Each PCR fragment (QR2 or QR3) was directly cloned into the TA vector (Invitrogen, San Diego, CA 92121) and sequenced. The fragments were then transferred to pUHD10-3Neo by *Eco*RI digestion and ligation. General procedures used for subcloning were as described (Sambrook *et al.*, 1989).

Table 4-1. The primers used in PCR amplification

primer	sequence
pRec 2	5′ TTA CCG GAC CCG TTA CCG 3′
pRec 5	5′ GCG CCA TGT CGG TAC CGC 3′
pRec 6	5′ AGT TTC AGC TGG GCA CGT 3′
pRec 7	5′ TTG GGC CGC TGT TCG CTC 3′

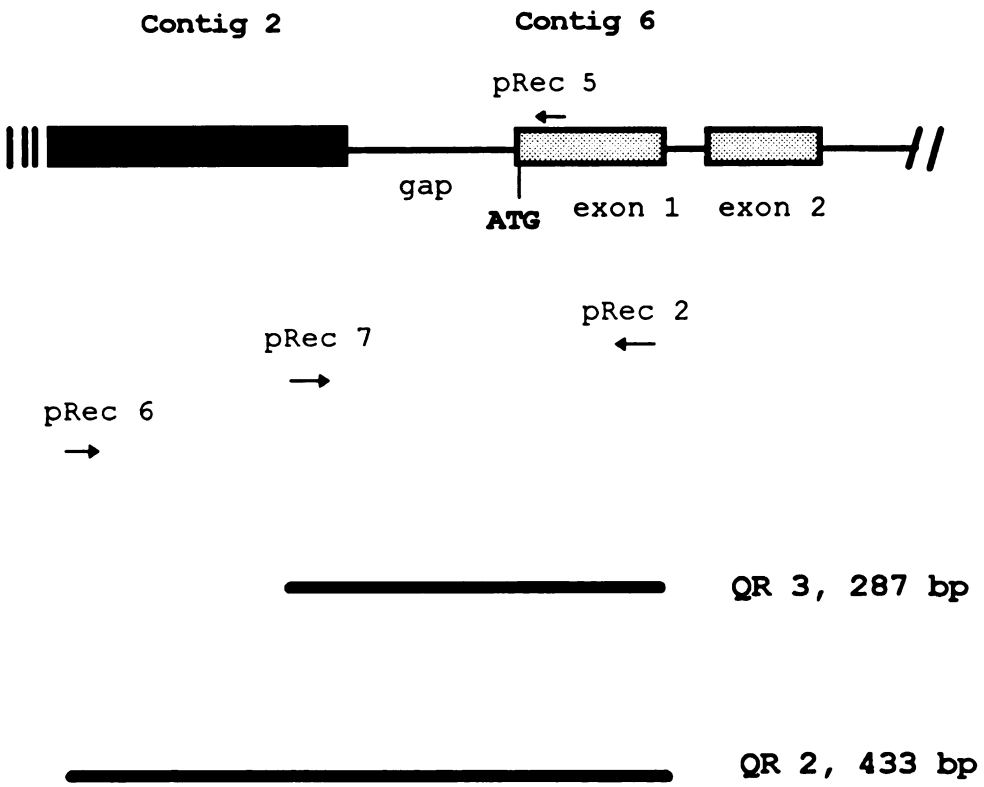


Figure 4-1. The region targeted by antisense RNA. Contig 2 (798 bp) and contig 6 (281 bp) were sequenced by Dr. Bates. Contig 6 contains exon 1 and contig 2 contains two putative TATA boxes. Gap was sequenced as described in MATERIALS AND METHODS and determined to be 125 bp in length. The 5′ untranslated region is expected to be included in the 3′ end of contig 2 and in gap.

Virus preparation

RCASBPAP(A) is a subgroup A recombinant ALV vector carrying the alkaline phosphatase (AP) gene at the 3′ of *env* (Federspiel *et al.*, 1995). Line 0 chicken embryo fibroblasts (CEF) infected with RCASBPAP(A) were obtained from Mr. Bill Payne (Department of Microbiology, Michigan State University, East Lansing, MI 48824). The culture supernatant of these CEF was collected and spun at 2000 rpm at 4°C for 5 min to remove any cellular materials. Its titer was determined by infecting QT6 cells at limited dilution and assaying for *AP* activity. The virus stock was kept at -70° C.

Infection with RCASBPAP(A) and the AP assay

G418-resistant quail cell transfectants with significant transactivation levels were split and grown in media with and without tet (4 μg/ml) for 4 d prior to infection with RCASBPAP(A). AP activity was assayed by either direct cell staining or by a soluble assay. AP staining: $1x10^5$ cells of each cell line in +/- tet were seeded into 6-well plates in triplicate 1 d before infection. The media was replaced with 1 ml of fresh and $1x10^3$ infectious units (iu) of RCASBPAP(A) was added to each well. After 3 h of incubation, the cell monolayer

was washed twice with PBS to remove the input virus, overlayed with 3 ml of media containing 0.6% low-melting point (LMP) agarose (Life Technologies) and incubated for 3 d. The agarose overlay was then removed, and the cells were stained for AP activity as described (Rong and Bates, 1995). Soluble AP assay: $2x10^6$ cells of each transfectant were seeded into 10 cm plates in duplicate one d prior to infection. The media was removed from the cells and 9 ml of new media was added. $2x10^6$ iu of RCASBPAP(A) was added to each plate and incubated for 48 hr. Two d post-infection, the cell monolayer was washed twice with PBS and the AP assay was performed as described (Berger *et al.*, 1987).

Southern hybridization

Genomic DNA was isolated as described in Chapter 2 of this thesis. Ten µg of each genomic DNA was digested with *Cla*I and subjected to 0.8% agarose gel electrophoresis, followed by transfer to a nylon membrane (MSI, Inc., Westborough, MA 01581) and hybridization as described (Sambrook *et al.*, 1989). The hybridization probe was a 2 kilobase pair (kb) fragment released from the RCASBPAP(A) plasmid by *Cla*I digestion or a ^{32}P-labeled QR2 DNA fragment prepared by digestion of QR2-10-3/Neo with *Eco*RI

^{32}P-labeled by the random primer extension method (Sambrook *et al.*, 1989).

Northern hybridization

Total RNA was isolated by lysis using Trizol (Sigma, St. Louis, MO, 63178) as described by the manufacturer. 30 µg/lane of RNA was run on a 1.2% agarose gel as previously described (Chapter 2). Blots were hybridized as described above for Southern blot analysis.

RESULTS

Generation of QT6 clones harboring an antisense sequence complementary to the mRNA for the subgroup A receptor

DNA fragments from the cloned subgroup A receptor gene were amplified and cloned into the antisense expression vector pUHD10-3Neo in the appropriate orientation as described in MATERIALS AND METHODS. Each construct was then cotransfected into QT6 cells with the plasmid pUHD15-1 which constitutively expresses tTA, the transactivating protein. Eighty G418-resistant colonies were selected in media with tet (to keep the expression of antisense RNA uninduced state, in case repression of *tva* expression might be deleterious to the cells). Each G418-

resistant colony was screened for its level of transactivation by transient transfection with pUHC13-3 (*luc*-encoding plasmid), followed by an assay of luciferase activity in the presence and in the absence of tet. A total of 40 G418-resistant colonies were found to show strong transactivation activity, one of them ranging up to 200 fold. Table 4-2 shows 29 colonies which had transactivation activity and the results from AP staining.

Unfortunately, neither the message nor the protein expressed by the subgroup A receptor gene has been detectable by typical northern or immunoblot analysis (P. Bates, personal communication). Therefore, the level of ALV infectivity is most sensitive and, to date, the only effective assay for receptor expression. G418-resistant QT6 cell lines with high levels of transactivation were further tested by two infectivity assays. Since these experiments were performed in QT6 cells, we employed a more facile assay for viral infection based on newly developed AP-expressing ALV vectors (Federspiel *et al.*, 1995)

AP staining of the infected cell lines

RCASBPAP(A) is a recombinant ALV vector carrying an alkaline phosphatase (AP) gene at the 3′ of *env*. It was postulated that those QT6 transfectants in which antisense

Table 4-2. A. The effect of QT6 clones harboring the QR2 antisense sequence on viral infectivity.

Clone		Fold activation[a]	Number of Infection Foci			
			+tet	SD	-tet	SD
QR2	5	2	49	2	16	8
	8	45	30	8	14	5
	9	75	30	7	25	3
	11	60	41	3	38	10
	12	7	28	9	34	6
	13	21	36	3	30	14
	15	190	47	2	73	9
	17	140	8	2	70	14
	19	80	14	5	18	2
	28	85	23	4	14	8
	29	64	38	5	38	6
	32	13	39	10	10	6
	36	37	51	12	43	8
	42	16	28	7	33	15
	52	36	2	3	0.3	0.6
	53	18	14	4	33	12
	54	7	5	1	6	4
	56	22	8	7	6	2
	58	53	4	2	3	1
	60	20	27	7	15	3
	62	20	10	4	9	2

[a] Fold activation was based on luciferase activity and calculated by this formula: (luciferase activity in - tet)÷(luciferase activity in +tet).
Cells were grown in the presence and absence of tet (4 μg/ml) for 4 d prior to infection.
Average number of infection foci in triplicate wells are shown.
SD; standard deviation

Table 4-2. B. The effect of QT6 clones harboring the QR3 antisense sequence on viral infectivity.

clone		Fold activation[a]	Number of infection foci			
			+tet	SD	-tet	SD
QR3	3	96	11	6	3	2
	16	3	56	6	27	5
	17	4	21	4	13	4
	28	110	41	6	43	1
	31	150	28	5	38	4
	33	59	41	5	53	6
	40	2	3	1	3	2
	42	51	0.3	0.6	1	2

[a] Fold activation was based on luciferase activity and calculated by this formula: (luciferase activity in - tet)÷(luciferase activity in +tet).
Cells were grown in the presence and absence of tet (4 μg/ml) for 4 d prior to infection.
Average number of infection foci in triplicate wells are shown.
SD; standard deviation

RNA inhibits the expression of the subgroup A ALV receptor should generate a reduced number of infected cell centers upon infection with RCASBPAP(A) followed by staining for AP. The G418-resistant quail transfectants which showed significant levels of transactivation were split and incubated for 4 d in media with and without tetracycline. Although the ALV subgroup A receptor is likely to be rather stable, given its very low levels of expression, we hypothesized that this time period would be long enough to observe any decrease in the synthesis of new receptor molecules that might result from antisense inhibition. 10^5 cells of each transfectant were infected with RCASBPAP(A). Three days later, the cells were stained for AP activity. Each QT6 transfectant was assayed in triplicate and the results are shown in Table 4-2. The data demonstrate that the QT6 transfectants remained susceptible to infection with the RCAS virus, but some of the transfectants showed a limited decrease in foci in the absence of tet.

Transcription of antisense RNA in the absence of tet

To confirm the presence of regulated expression of an antisense transcript, total RNA was extracted from each transfectant after growth in the presence and absence of tet for 4 d. Total RNA was analyzed by Northern blot using

a QR2-specific probe (Figure 4-2). Detectable levels of antisense QR2 transcripts were found in the absence of tet in transfectants #9 and #52. Transfectants #5, #8, and #60 did not have a detectable level of antisense QR2 transcript, and were not further examined.

Soluble AP assay

A few transfectants appeared to exhibit a decreased susceptibility to RCASBPAP(A) infection in the absence of tet as assayed by histochemical AP staining (Table 4-2). However, the major drawback of this assay is that it is based on a relatively limited number of foci, leading to potentially large variances in the results (e.g., QR2-15 and QR2-17, Table 4-2A). Therefore, another type of AP assay was performed which measured the total AP activity by spectrophotometry. In this assay, a crude cell extract containing AP was prepared and assayed by measuring the amount of AP enzyme reaction product (*p*-nitrophenol) (Berger *et al.*, 1987). A QT6 transfectant which becomes less susceptible to RCASBPAP(A) infection due to decreased expression of *tva* engendered by antisense RNA should display decreased AP units/mg cell protein, in the absence of tet compared to the presence of tet. Each cell line was infected with RCASBPAP(A) and, on day 2 post-infection, the cells were harvested and an AP-enriched fraction was

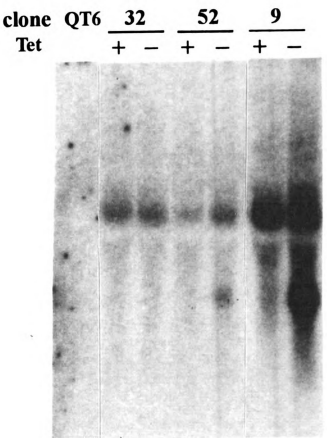


Figure 4-2. Northern blot analysis of antisense QR RNA. Total RNA was extractedn electrophoresed, transferred, and hybridized with a DNA fragment specific to QR2 and QR3 RNA as described in MATERIALS AND METHODS.
NV; no virus

Table 4-3. Assay of AP activity

clone		tet	AP units / mg cell protein exp 1	exp 2	exp 3	exp 4
QR 2	9	+	36[a]	100 +/- 11	87 +/- 13	50 +/- 18
		-	42	100 +/- 15	100 +/- 14	58 +/- 0
	32	+	45[a]	95 +/- 27	107 +/- 41	110 +/- 25
		-	59[a]	70 +/- 8	86 +/- 27	110 +/- 18
	52	+	42 +/- 7	62 +/- 3	58 +/- 14	76 +/- 17
		-	24 +/- 3	62 +/- 1	75 +/- 9	70 +/- 3
QR 3	16	+	63 +/- 7	100 +/- 5	160 +/- 25	130 +/- 10
		-	52 +/- 2	93 +/- 2	84 +/- 1	100 +/- 22
QT6		+	ND	94[a]	150[a]	130[a]
		-	ND	130[a]	170[a]	130[a]

AP units/mg protein is expressed as an average of duplicate +/- SE.

[a] not performed in duplicate.

ND; not done.

Clone #5, #8 and #60 were not analyzed by this method because QR2 RNA was not detectable.

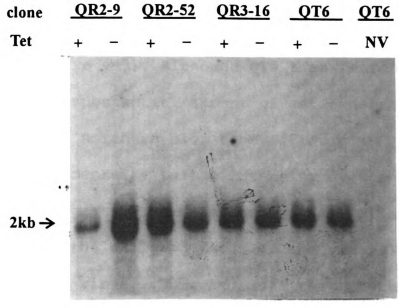
obtained as described in MATERIALS AND METHODS. The results of the AP assays are shown in Table 4-3. Although some of these transfectants seemed to have a reduced infectivity when assayed by histochemical AP staining and two of them showed relatively high levels of inducible antisense *tva* transcript, no significant difference in AP activity was detected ± tet in several tests of these transfectants.

Detection of proviral DNA

Once a retrovirus enters its host, it copies its RNA genome into a double-stranded DNA and this DNA copy integrates into the host genome to establish infection. It was reported that QT6 cells don't produce ALV at a high titer, although they can be normally infected (Friis, 1972). Therefore, we decided to examine the potential influence of receptor interference at an earlier stage of the viral life cycle, i.e., that portion up to the integration step. The time required for a virus to enter a host cell and to complete the generation of a proviral DNA has been reported to be 4-8 hr (Coffin, 1990).

The G418-resistant QT6 transfectants were carried in media with and without tet for 4 d prior to infection with RCASBPAP(A) at 0.5 MOI. Ten hours post infection, genomic DNA was isolated and digested with *Cla*I. This released an

A.



B .

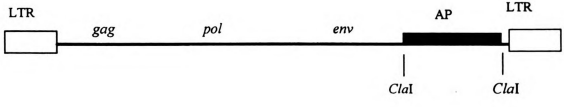


Figure 4-3. A. Detection of proviral DNA. Genomic DNA was isolated 10 hr post infection, digested with *Cla*I, electrophoresed, transferred, and hybridized with 2kb-DNA fragment specific to AP gene as described in MATERIALS AND METHODS.

B. The genome organization of RCASBPAP(A) provirus

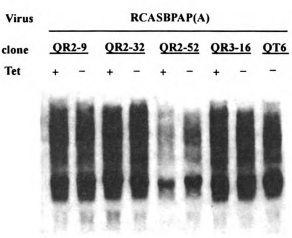
internal 2-kb fragment from proviral DNA which corresponds to the *AP* gene (Figure 4-3). Southern hybridization with a probe specific to *AP* demonstrated that, as expected, the retroviral DNA of RCASBPAP(A) stably integrated into the host genome (Figure 4-3). Although QT6 transfectants #9 and #52 showed a substantial amount of antisense transcript in the northern blot assay (Figure 4-2), they did not show any decrease in the intensity of the proviral DNA fragment, suggesting that antisense RNA expression had had no effect on the early portions of the viral life cycle of the test virus. This includes the attachment phase which we might have expected would have been influenced by the expression of antisense to the receptor mRNA.

Analysis of viral gene expression

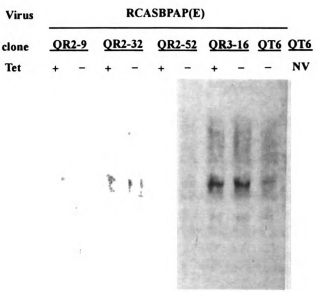
Since it was not feasible to examine virus spread by p27 ELISA due to inefficient virus production from QT6, we decided to investigate viral mRNA synthesis using northern blotting with a virus-specific probe. On d 3 post-infection of QT6 transfectants with RCASBPAP(A), total RNA was extracted and hybridized to a probe specific for a spliced AP message (Figure 4-4). Again, the results did not demonstrate any reduction in the amount of AP message generated when cells were carried in the media without

Figure 4-4. Northern blot analysis of AP mRNA expression. Total RNA was extracted 3 d post infection, electrophoresed, transferred, and hybridized with a DNA fragment specific to AP gene as described in MATERIALS AND METHODS. A: infection with RCASBPAP(A) and B: infection with RCASBPAP(E).
NV; no virus

A


Virus
RCASBPAP(A)
clone
QR2-9
QR2-32
QR2-52
QR3-16
QT6
Tet
+ − + − + − + − −

B


Virus
RCASBPAP(E)
clone
QR2-9
QR2-32
QR2-52
QR3-16
QT6
QT6
Tet
+ − + − + − + − − NV

tet. RCASBPAP(E) recognizes a different receptor and, therefore, was included as a control. Previous experiments indicated that subgroup E ALV infects QT6 cells considerably less efficiently than does subgroup A ALV (data not shown). This was confirmed in Figure 4-4B showing a much lower abundance of the AP message from RCASBPAP(E)-infected cells. As expected, where it could be detected, there was no difference in *AP* gene expression ± tet.

DISCUSSION

Here we report our attempts to use an antisense RNA approach to suppress the expression of the subgroup A ALV receptor in the QT6 cell line. We have employed the tet-regulated gene expression system to generate antisense *tva* RNA. It was expected that, in cells which induced high levels of antisense QR2 or QR3 RNA in the absence of tet, expression of the receptor would be suppressed, leading to a decreased susceptibility to ALV. Results of northern blot analysis (Figure 4-2) indicate that, in a few QT6 transfectants, substantial amounts of antisense RNA were transcribed specifically in the absence of tet. However, these cells were still equally susceptible to infection by subgroup A ALV, as judged by DNA provirus formation or production of AP enzyme or mRNA. While it might be argued

that the receptor protein could be extremely stable, delaying the effect of inhibiting its synthesis, cells were grown through numerous doublings over 4 d, which should allow for a substantial reduction in receptor level on cell surfaces. The affinity between the virus and the receptor may be very high, such that only one or a few functional receptors would be required for efficient infection, thereby making the threshold level at which we would observe a decrease in viral susceptibility very low. However, since the protein itself has proven to be virtually undetectable, one assumes it is present at extremely low amounts in the first place, and if antisense inhibition were working that an influence on viral replication would be detected.

In conclusion, we have obtained several QT6 cell lines which express an antisense transcript complementary to the *tva* message in a regulated fashion. However, none of these cell lines demonstrated a significant decrease in susceptibility to subgroup A ALV infection. Several of the many possible explanations for the failure of antisense inhibition are discussed in Chapter 2 and need not be repeated here.

Summary and Conclusions

1. Various regions at the 5′ end of the ALV genome were targeted for expression of antisense RNA.

2. One stably transfected cell line (3B3) showed a significant inhibition of viral replication in a constitutive expression system. A low level of antisense transcript was detected in this transfectant by RT-PCR. A very significant decrease in the titer of ALV grown on 3B3 was observed for ALV members of two different subgroups (A and C). However, attempts to attribute the decrease in virus susceptibility in 3B3 to the production of antisense RNA were unsuccessful, leading to the suggestion that this cell line may be a random clonal variant.

3. In the tet-regulatable expression systems, a substantial amount of antisense transcript was detected in the presence of inducer or in the absence of repressor. However, a significant inhibition of viral replication was not observed in these transfectants despite the use of several expression vectors or antisense target regions.

4. Antisense oligodeoxynucleotides (ODN) were employed in hopes of finding the most effective antisense target region near the 5′ end of the ALV genome. A short region including the PBS was demonstrated to be the most effective antisense ODN target with the inhibitory effects of up to 80%. However, an antisense RNA complementary to this region did not show any inhibitory effect on viral replication when tested in a stable expression system.

5. The 5′ end of the message encoding the cellular receptor for subgroup A ALV in a quail cell line has been employed as a target for antisense RNA expression, in hopes of specifically reducing susceptibility to subgroup A ALV. Although specific expression of antisense RNA was detected in a few transfectants, again, no reduction in infectivity was observed.

6. Overall, *in vivo* expression of antisense RNA appears unlikely to be an effective way to generate transgenic poultry that are resistant to field strains.

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