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Role of REL/NFκB members in v-rel induced transformation

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November 1996

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science.

Résumé:

Parmis les rétrovirus à très haut pouvoir transformant, le virus REV-T est le plus virulant. L'activité transformante du virus implique l'oncogène d'origine virale v-rel, le premier membre de la famille croissante des régulateurs transcriptionnels REL/NF-κB à avoir été identifié. Le mécanisme de la transformation des cellules aviaires mais aussi de mammifères par v-rel n'est pas encore clair. Cependant, il a été montré par immunoprécipitation avec des anticorps spécifiques de v-rel que les protéines pp40 IκB alpha, p68 c-rel, hsc70 et les précurseurs p124 et p115 respectivement des sous-unités p50 et p52 de la famille REL/NF-κB sont associés à v-rel. Ces mêmes protéines sont aussi associées avec le proto-oncogène c-rel dans les cellules normales. Les membres de la famille REL/NF-κB ont été impliqués dans la régulation de la croissance, la différenciation et l'activation des lymphocytes, ce qui suggère que la transformation par v-rel impliquerait une perturbation de la fonction normale de REL/NF-κB. Nous avons étudié différents systèmes de régulation génétiques dans le but de mieux comprendre la fonction des molécules aviaires. Plus précisément, nous avons testé et employé des oligonucléotides couplés au phosphorothiorate ainsi que des vecteurs permettant l'expression d'ARN antisens afin de bloquer l'expression de certains gènes dans les cellules aviaires. Nous avons testé le système de vecteurs RCAS (Replication competent avian leukosis LTR & splice acceptor) pour l'infection de cellules de poulet et la dissémination du gène cloné. L'utilisation potentielle de systèmes dépendant de la tétracycline dans les cellules aviaires a aussi été étudiée. Ces nouveaux systèmes de régulation nous permettront de comprendre le rôle joué par les membres de la famille REL/NF-κB dans la transformation cellulaire.

Abstract

The REV-T virus is the most virulent of all the known acutely transforming retroviruses. The transforming activity of the virus resides within the virally encoded v-rel oncogene, the first member identified of a growing number of transcriptional regulators called the REL/NFκB family. The mechanism of v-rel mediated transformation of avian as well as mammalian cells remains elusive, however immunoprecipitations performed with anti-v-rel antibodies clearly reveal associated proteins which include pp40 IκB alpha, p68 c-rel, hsc70 and the p124 and p115 precursors for the p50 and p52 subunits of the REL/NFκB family, respectively. In normal cells these same proteins are also found associated with the proto-oncogene c-rel. REL/NFκB members have been implicated in the regulation of lymphocyte growth, differentiation and activation suggesting that v-rel mediated transformation involves the deregulation of normal REL/NFκB function. In order to better understand v-rel protein function, we have investigated several gene regulatory systems. Specifically, we have tested and used phophorothioate oligonucleotides as well as vectors producing antisense RNA for the purpose of disrupting gene expression in avian cells. We have tested the RCAS system (Replication competent avian leukosis LTR & splice acceptor) of vectors to infect and disseminate within a chicken effectively producing somatically transgenic chickens where cloned genes may be expressed from the infected cells. Also investigated was the feasability of using the tetracycline regulated gene expression system in avian cells. These new regulatory systems will allow us to understand the role played by individual members of the REL/NFκB family in cellular transformation.

Acknowledgements

Of course the first on my list would have to be Mike Ratcliffe, not only for the financial support granted, but teaching me about science, respect and attitude, through mostly his example.

It is necessary that credit is given where it is deserved and indeed much of the support through the years have come from my mother, Angela Calzada, always there to listen, understand and just be there. My brothers and sister have also constantly rewarded me through these years with their rhetoric, jokes, support, stories and their warmth as only one you grew up with can give.

I have encountered many a character in the process of getting my Master's thesis. This would include the members of the irrepressible and unimpressable Immunological Journal Club. Not only was there "merriment and song" but scientific insight was the greatest reward for the experience. Characters jump to mind like Michael Julius, Trevor Owens, Vince Dodelet, Julie (The), Joanne, and others.

However, the pleasure of coming to the lab day in and day out lay in the people of the "Ratcliffe Lab" (the noisy group). Every one of them deserves mention....Mostly Alice Coumidis and Karen Jacobsen for their complete support and unending joviality. Other people like Eustache Paramithiotis, Toufic, Mina Marmor, Jennifer Wilshire, Bessy, Sandra Desmaries, Camil Sayegh all deserve my thanks. Also thank you Luchino Cohen for the résumé.

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Abbreviations

2-ME : 2-mercaptoethanol

BCR : B cell receptor

BSA : Bovine serum albumin

BSS : Balanced salt solution

C : Celsius

CAT : Chloramphenical acetyl transferase

CEF : Chicken embryo fibroblasts

CSV : Chicken syncitial virus

D'PBS : Dulbecco's phosphate buffered saline

DNA : Deoxyribonucleic acid

FCS : Fetal calf serum

H : heavy

Ig : Immunoglobulin

L : light

NCS : Normal chicken serum

PAGE : polyacrylamide gel electrophoresis

PBL : Peripheral blood lymphocytes

RCAN : Replication competent avian leukosis LTR & no splice acceptor

RCAS : Replication competent avian leukosis LTR & splice acceptor

Rev-T : Reticuloendotheliosis virus strain T

RSV : Rous sarcoma virus

SDS : sodium dodecyl sulfate

TCR : T cell receptor

Tet : tetracycline

Tris : Trizma base

GENERAL INTRODUCTION

In the year 1911, Peyton Rous made an important discovery which was to revolutionize our understanding of the factors which give rise to tumerogenisis. The landmark discovery was made in the avian system and demonstrated for the first time that tumor formation could be transmitted from one chicken to another in cell free extracts. The transmissible agent, was the Rous Sarcoma virus. This tumor virus contained a vital gene, named v-src (reviewed by Brickell, 1992), required for the transforming activity of the virus and thus identified the virus as the first member of the acutely transforming retroviruses.

Oncogenic Retroviruses

The acutely transforming and latent viruses represent the two categories of oncogenic retroviruses. The latent retroviral group of which the parental virus (Avian Leukosis virus) of the Rous Sarcoma virus belongs to, contains no intrinsic transforming activity. Instead, the transforming property of the latent viruses rests in the strong promoter activity of the 3' LTR (Boerkoel et al 1992). Incorporation of the virus into the genome of the host upstream or downstream of a proto-oncogene may lead to the deregulated expression of that host gene. The virus must integrate their viral genome immediately adjacent of a cellular proto-oncogene, a chance event, allowing for a long latency period between viral infection and tumor formation.

Recombination between an infecting retroviral genome and host sequences allow the incorporation of host genes into the virus. Acutely transforming viruses have had proto-oncogenes incorporated into the

viral genome. This recombination event between viral and host sequences lead to the disruption of viral genes (the sole exception being RSV) resulting in a defective virus which now requires a helper virus to propagate. The incorporated oncogene, inevitably mutated by the viral error-prone polymerase, enhances the tumerogenic potential of the virus (Hrdlickova et al 1994a).

The REV-T retrovirus

The most virulent of all the acutely transforming retroviruses, REV-T, induces the malignant transformation of avian lymphoid and myeloid cells causing a fatal lymphoma within 7-10 days of infecting a young chicken. It is a type C retrovirus and is the sole acutely transforming virus of a small group of avian retroviruses which include the parental REV-A and chicken syncitial viruses, both of which can act as helper viruses. The transforming activity of REV-T resides in the v-rel oncogene, a mutated form of the c-rel proto-oncogene (reviewed in Kabrun and Enrietto 1994, Bose Jr. 1992).

The REV-A retrovirus incorporated the turkey c-rel proto-oncogene within its ENV region, creating the REV-T virus (Wilhelmsen and Temin 1984). REV-T also features a truncated GAG region and a nearly complete deletion of the POL region. The v-rel protein itself has 11 ENV-derived amino acids replacing the first two turkey c-rel amino acids and 18 ENV-derived amino acids replacing the last 118 amino acids from the c-rel protein. Also, v-rel contains numerous amino acid substitutions and three small in frame deletions. The resulting mutated viral protein is expressed at high levels by the strong REV-T LTR.

REV-T cellular targets of v-rel mediated cell transformation

The cells targetted for transformation by the viral oncogene depend in part on the REV-T and helper virus tropism and on cytopathology (Barth and Humphries 1985). For the most part, those cells transformed *in vivo* in the context of helper viruses CSV or REV-A reflect those cells which would be transformed *in vitro*. REV-T(REV-A) infected chickens contain transformed cells that express myeloid or T cell markers with no evidence of immunoglobulin chain rearrangements. On the other hand, REV-T(CSV) infection produces IgM positive tumors which differ in the extent of immunoglobulin diversification. Recently, v-rel was shown be involved in mammalian transformation events. Mice were made transgenic for v-rel whose expression was controlled by the lck promoter (mostly T cell specific). These mice developed immature T cell leukemias/lymphomas and died before reaching 10 months of age (Carrasco et al 1996).

v-rel is a member of the REL/NFκB family

The v-rel oncogene was the first identified member of a group of transcriptional regulators called the REL/NFκB family. Members of this family are defined by a stretch of homologous sequence near their 5' ends termed the REL Homology Domain (RHD) (reviewed in Baeuerle 1990, Grilli et al 1993). As illustrated in Figure 1, members include the subunits of the NFκB transcription factor p65 and p50. Within and around the RHD reside the motifs required for DNA binding, protein dimerization and IκB inhibitor binding. Individual members are distinguished by alternate C-terminal sequences. Both c-rel and p65 contain a transcriptional activation domain, while others such as p124 contains ankyrin repeats. p124 is one of the few REL/NFκB members which undergoes post-translational modification in the form of a cleavage event. The C-terminal end of the protein is cleaved away from the N-terminal region allowing the new

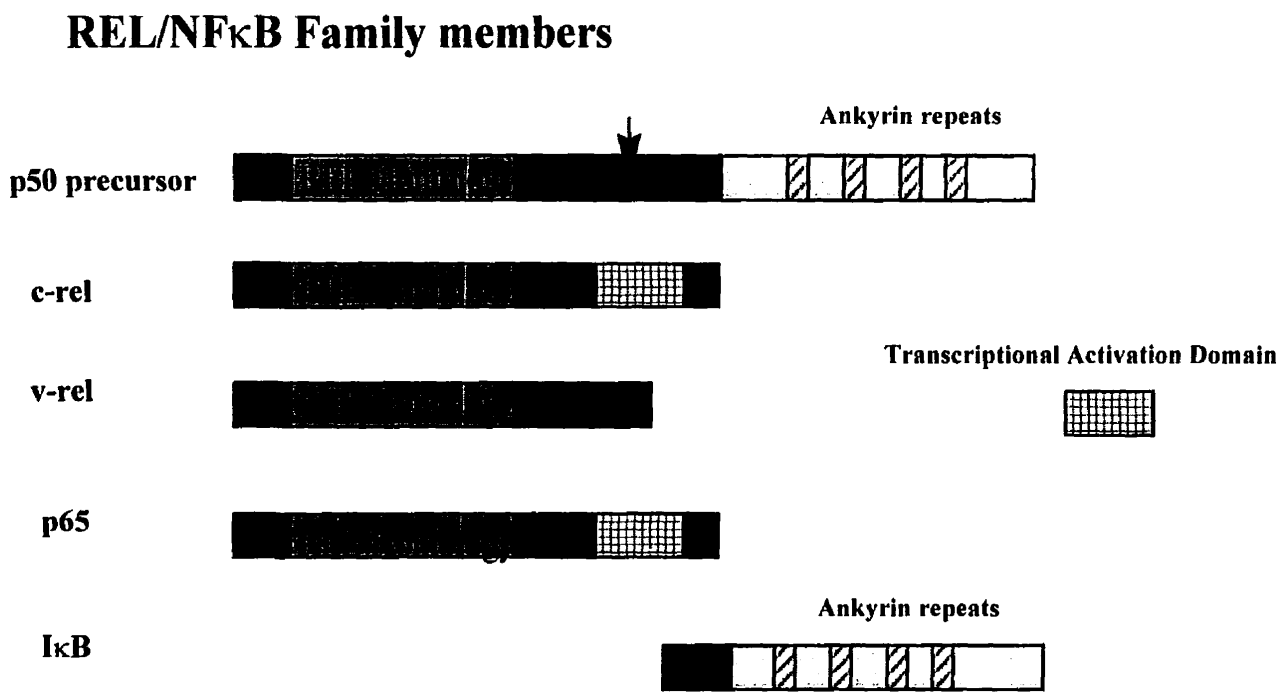


Figure 1. REL/NFκB members. Members of this family are characterized by a sequence of homology at their amino terminal end named the REL homology domain. This domain encompasses an area of the protein involved in DNA binding, dimerization and binding to the IκB inhibitors. The p50 precursor is thought to function as an inhibitor perhaps with the aid of the C-terminal end of the protein harbouring some ankyrin repeats also found in the IκB inhibitors. The arrow schematically depicts the cleavage site of the protein.

amino terminal half of the protein (p50) to perform a different function. The p124 protein is hypothesized to play a dual role, partly due to the presence of the ankyrin repeats at the C-terminal end of the protein. Ankyrin repeats are generally thought to mediate protein protein interactions in a variety of different systems. The unprocessed form containing the ankyrin repeat is thought to bind and inhibit the action of other REL/NFκB members (general discussion in Dobrzanski et al 1994) while the smaller N-terminal end of the processed protein dimerizes with p65 to form the NFκB transcription factor.

REL/NFκB function and regulation

REL/NFκB members are well suited for the purpose of rapid signal transduction. The NFκB transcription factor, first identified as the protein complex binding the κB enhancer site of the murine κ light chain (Sen and Baltimore 1986), resides within the cytoplasm of many hematopoetic cells, in an inactive form. The p65-p50 heterodimer is held in check by an inhibitor protein, IκB, which possibly masks the DNA binding and nuclear translocation sites as well as holds the protein in the cytoplasm by binding the cellular cytoskeleton through its ankyrin repeats. Upon stimulation, the cell sends a signal which ultimately leads to the phosphorylation of the IκB molecule, resulting in IκB ubiquitinilation. The ubiquitinilation of the IκB molecule will then flag the molecule for destruction by proteasomes thus releasing the transcription factor (Henkel et al 1993). NFκB will quickly translocate across the nuclear membrane, bind to the decameric κB concensus binding sites and transactivate target genes. Therefore, labile IκB molecules function to restrain functionally competent REL\NFκB transcriptional regulator complexes in such a manner as to allow the cell a

rapid mechanism of signal transduction, which does not require *de novo* protein synthesis.

Situations which require a rapid cellular response make use of the NFκB family of transcription factors. Inflammatory, stress and acute phase responses are in part mediated by the action of REL/NFκB transcription factors. For example, in the acute phase response, liver cells respond through the REL/NFκB family for the production of acute proteins (Freedman et al 1992, Brasier et al 1996). Inflammatory signals also use REL/NFκB members to transcriptionally upregulate lymphokines such as IL-2, IL-6 and receptors such as TCR (beta chain) and BCR (kappa light chain)(reviewed in Baeuerle 1991). Although REL/NFκB members are well suited to play the role of rapid effectors resulting from cellular signalling, they play an even greater role in the physiological makeup of higher organisms.

REL/NFκB members are involved in tissue growth and differentiation

REL/NFκB members play a key role in tissue growth, development and differentiation within multicellular organisms. For example, the Dorsal protein from Drosophilia regulates dorsal-ventral morphogenisis in the early developmental stages of the fly (discussed in Gilmore 1990). NFκB members play an especially important part in the development and regulation of lymphoid tissue. This is evident from mice generated lacking the RelB transcription factor (another REL/NFκB member) (Burkly et al 1995). These mice are devoid of a specific type of medullary epithelial cell found in the thymus, very similar to dendritic cells and demonstrate a general impairment in antigen presentation. The added observation of excess granulocyte and macrophage production strengthen the author's

suggestion for a role of RelB in regulating lineage commitments in the immune system. C-rel itself is expressed ubiquitously in the developing avian embryo (Abbadie et al 1993) and possibly plays a role in both differentiation and immune cell proliferation.

c-rel is involved in the regulation of lymphocyte proliferation

C-rel's role in growth regulation was first inferred when it was observed to be the cellular homologue of v-rel, the mediator of REV-T virus induced transformation. It was further demonstrated that transcripts of c-rel are upregulated maximally 4 to 8 fold 1 hour after mitogen or serum stimulation of murine fibroblasts and T cells (Bull et all 1989, Grumont et al 1990). This clearly indicated that c-rel belonged to a specialized family of genes known as immediate-early genes, implicated in growth regulation. Immediate-early proteins are among the earliest proteins upregulated by growth factor (contained in serum) and mitogenic stimulation and include other important protooncogenes such as c-fos.

The c-rel null mouse illustrates the importance of REL/NFκB members in immune cell proliferation (Kontgen et al 1995). Mice devoid of c-rel contained B and T cells that were unresponsive to immune receptor stimulation. An impairment in the humerol response was observed as well as a deficiency in the production of IL-2, an important lymphokine growth factor. Maximal IL-2 promoter stimulation has been shown to be dependant on adequate levels of serum response factor and c-rel (Pierce et al 1995).

REL/NFκB members are involved in B lymphocyte differentiation

B cell differentiation correlates with an observed change in the levels of different REL/NFκB members. Immunoglobulin receptor regulation is tightly linked to the regulation of B cell differentiation. Both BCR levels and B cell differentiation (Singh 1994) are regulated, in part, by the REL/NFκB family. Specifically, Ig κB consensus sequence binding as assessed by band shift analyses reveal different mobility patterns dependant on the differentiation state of the B cell lysate. This occurs because B cell differentiation manifests a change in the major REL/NFκB transcription factor species (Grumont and Gerondakis 1994, Miyamoto et al 1994), as induced by lipopolysaccharide (LPS). This REL/NFκB transcription factor species in pre B cells is NFκB (p50 and p65 heterodimer), in mature B cells is the p50 / c-rel heterodimer and in plasma cells is p52 and REL B. Also, recent evidence suggests that v-abl inhibits REL/NFκB function (Singh 1994), therefore explaining the immature B cell phenotype of the murine v-abl-transformed cells. Thus, REL/NFκB expression patterns in B cells are directly involved in the process of B cell differentiation.

REL/NFκB members form multisubunit transcriptional regulators

Within and around the RHD, certain polypeptide sequences are responsible for the protein-protein interactions and DNA binding ability of REL/NFκB members. Thus REL/NFκB members exist as multisubunit species where the individual subunits participate in defining the function of the transcriptional regulator (Perkins et al 1992). A well known example is the NFκB transcription factor, a potent transcriptional activator. The potent transactivity of the NFκB factor is donated by the p65 subunit, since the p50 subunit lacks a transactivation domain. However, the p50 subunit plays a part in determining the specificity of the transcription factor by

regulating the affinity of NFκB for certain κB sites in the enhancers of specific genes. For example, p50-p65 heterodimers bind pentameric κB sites with slight differences, such that the p50 subunit binds preferentially to one half site while p65 binds to the other. By contrast, the p50 homodimer preferentially binds pentameric κB sites which are palindromic. In such a manner, different genes are transcriptionally regulated under circumstances where different hetero or homo REL/NFκB dimers are activated.

The mechanism of v-rel mediated cellular transformation remains elusive

The exact mechanism by which c-rel regulates cell growth is unknown. This creates a hole in our understanding as to how v-rel may mediate cellular transformation. The difficulty of defining a mechanism for v-rel is exemplified by the observation that v-rel, detected in both the cytoplasm and nucleus of the transformed cells may transform cells irrespective of its subcellular location (discussed in Bose Jr. 1992). Although the molecular mechanism of v-rel transformation remains elusive, knowing the nature of REL/NFκB function may allow us to hypothesize the method v-rel uses to transform an immune cell.

Cellular transformation is associated with the corruption of REL/NFκB function

The v-rel protein is likely to cause the transformation of cells by altering the function of REL/NFκB complexes. V-rel protein is produced at high levels in transformed cells and is found associated with other members of the REL/NFκB family. Immunoprecipitations with anti-v-rel antibody reveal many associated proteins (Morrison et al 1989): p40 IκB a,

p68 c-rel, hsc70 and the p124 and p115 precursors for the p50 and p52 subunits of the REL/NFκB family, respectively. Also associated, are three recently described proteins and one protein related to RelA (Xu and Gelinas 1995). Interestingly, synchronization experiments revealed a change in the proteins expressed and/or associated with v-rel in different phases of the cell cycle (Xu and Gelinas 1995)

V-rel, the corrupted form of the c-rel protein, once introduced into a cell by the REV-T virus is free to then associate with other members of the REL/NFκB system and change the activity and /or specificity of the multisubunit transcriptional regulators. This functional association may explain the observed perturbation of host gene expression when cells are transformed with v-rel. These include genes partly regulated by REL/NFκB members such as MHC II, MHC I and the Il-2 receptor (Benetar et al 1991, discussed in Humphries and Zhang 1992). The levels of expression of these and other proteins not mentioned are severely altered in REV-T transformed avian cells. Determining the mechanism of transformation is complicated by the fact that v-rel associates with other different REL/NFκB members. This may alter the function of many of these transcriptional regulators all at once leading to the deregulation of many genes.

The possible mechanisms of v-rel mediated transformation

V-rel may mediate the transformation of avian cells through two possible mechanisms. The first mechanism may involve the deregulated activation of a REL/NFκB transcription factor. This ACTIVE process could possibly be mediated by the participation of v-rel in a multisubunit transcription factor by simple association or replacement of an existing REL/NFκB member (possibly c-rel). This newly formed transcription factor

would then be able to mediate the inappropriate expression of those genes required for the transformation event to take place. The second mechanism would involve a DOMINANT NEGATIVE role for v-rel. The association of v-rel to a REL/NFκB complex would lead to a loss of function for that complex. The function of that REL/NFκB complex would be critical for the maintenance of the normal physiological state of that cell and would be lost because of the association of the v-rel protein. Both of these mechanisms are equally probable and lend themselves to certain predictions when critical subunits from the transcriptional regulatory complex are eliminated. In other words, if a critical component of the complex containing v-rel is functionally eliminated the outcome of this functional elimination can be predicted from the two different mechanisms of transformation already mentioned.

The investigation of v-rel mediated transformation

Thus, dissection of the roles played by the different REL/NFκB members may illuminate their functional contribution to the transformation event. Elimination of individual members of REL/NFκB in a step by step process may allow us to determine the mechanism by which v-rel causes cellular transformation. Theoretically, the expression of different NFκB members may be inhibited by the use of antisense technology. By eliminating key REL/NFκB members one may demonstrate the mechanism by which v-rel can transform a cell.

Predicting the outcome of eliminating key components of the v-rel containing REL/NFκB transcription factor

Several predictions may be made as to the outcome of eliminating the role of a key component of the v-rel containing REL/NFκB transcription factor. ACTIVE model: Since the v-rel containing complex is actively participating in a function which is critical towards the transformation event then eliminating a key participant in the complex could lead to the abolishment of the transforming process. By eliminating the key component of the complex and eliminating the transforming activity of the transcriptional regulator one may predict that there should be some reversal from the transformed phenotype of the cell in question. One could predict that a clear change in cellular phenotype should be observed such as a change in growth regulation or changes in the expression levels of certain genes.

If the DOMINANT NEGATIVE model were true then the functional elimination of a key player would lead to a different outcome. It is likely that if an important protein that formed part of the v-rel containing complex were eliminated that the phenotype of the cell in question would not change. If v-rel causes the loss of function of the complex to which it becomes associated then the elimination of one of the subunits of that complex would most probably be inconsequential for the maintenance of the transformed state. However, the functional elimination of the same protein in a normal cell, may itself lead to a transformation event. Both models make different predictions as to the outcome of a specific experiment in which one eliminates a key component of the v-rel containing complex.

Dissection of the roles played by individual REL/NFκB members

The transcriptional regulators of the REL/NFκB family are composed of individual subunits. To better understand the role played by c-rel and v-rel

proteins in different cellular processes, these transcriptional factors were targetted by antisense methods.

Antisense technology for the purpose of disrupting gene expression

A variety of means have been developed by which one may elucidate the function of a particular gene. One recently developed method makes use of antisense oligonucleotides for the purpose of disrupting gene expression. The hybridization of antisense transcripts or oligonucleotides to the sense transcript of the gene leads to the inhibition of that gene's expression. This allows one to assess the consequence of eliminating the protein from the system in question. The approach is particularly appealing because of it's exquisite specificity and the fact that disruption of gene expression, quite often, presents a clearer understanding of the proteins' biological role, as compared to other methods such as overexpression of the protein.

Antisense technology is relatively new and is largely based on the observation that nucleic acid duplexes that contain at least 1 molecule of RNA lead to mechanisms that destroy this potentially "dangerous" material (Reviews: Stein and Cheng 1993, Milligan et al 1993, Nellen and Lichtenstein 1993). The actual mechanism is unknown, however RNases such as Pac1 (Nellen and Lichtenstein 1993) and RNase H have been implicated. RNase H has been widely touted, but not proven (Rosolen et al 1993, Gao et al 1992) to play a role in the actual recognition and cleavage of the material (Dash et al 1987, Dagle et al 1991, Boiziau et al 1992, Larrouy et al 1992). It is also unclear whether RNase H acts by itself to degrade the duplex and whether complete degredation of unhybridized areas of the product also occur as part of the process.

Two general methods are used to inhibit the expression of targetted genes. The first method employs the use of an expression vector which produces antisense transcripts against the gene in question. The second method uses short antisense phosphorothioate oligonucleotides from an extracellular source to inhibit the expression of the targetted gene.

Retroviral expression of antisense transcripts

Disrupting gene expression by using antisense transcripts has already been used to better understand the functions of several proteins (for example: *c-myb*, Raschellà et al 1992, *poly(ADP-ribose) polymerase*, Ding et al 1992). Typically, the cDNA of the gene of interest has been expressed in the antisense orientation from a vector, including retroviral vectors (examples: *c-abl*, Daniel et al 1995; *p53*, Ishiwatari et al 1994) that feature a strong promoter. The use of a strong promoter is absolutely required because one needs the production of antisense transcripts at a high enough level that will disrupt gene expression. Retroviral vectors have come under scrutiny as of late because of their potential use in the future as the vehicles of choice for *in vivo* gene therapy through antisense technology (reviewed in Uckert and Walther 1994)

Phosphorothioate antisense oligonucleotides for the elimination on REL/NFκB member gene expression

Phosphorothioate antisense oligonucleotides have also been used in the past to inhibit the expression of a plethora of genes . The advantage of using phosphorothioate linkages between the sequence nucleotides is that the oligonucleotide is more resistant to cleavage by endogenous nucleases (Stein and Cheng 1993) . There is also an unexplained and empirical observed advantage in using these specialized oligonucleotides in that they

may increase the efficiency with which the cell nucleases destroy the RNA-containing duplexes formed (Stein et al 1988). Disadvantages are also apparent. These oligonucleotides are in general not as easily accepted into the target cells as are normal oligonucleotides. Also, the efficiency with which they may hybridize to the target RNA is lower compared to oligonucleotides with regular linkages (Stein et al 1988). In general though, the ability of these phosphorothioate nucleotides to withstand cleavage by endogenous nucleases outweighs the use of normal oligonucleotides for the purpose of disrupting gene expression.

Factors which govern successful disruption of gene expression

Several important factors govern whether an oligonucleotide can disrupt gene expression. 1) the oligonucleotide must be able to enter the target cell in a quantity which can disrupt gene expression. The ability of an oligonucleotide to enter the cell is determined by A) the type of cell being used, B) the type of oligonucleotide (type of linkages for example) being used and C) the length of the oligonucleotide (the longer the less efficient). 2) the oligonucleotide must be able to interact with the target RNA with minimal crosshybridizations which decrease the efficiency of specific gene disruption. A) The length of the oligonucleotide is very important. Oligonucleotides which lack the proper specificity for their target transcript end up disrupting other transcripts at the same time. Short oligonucleotides are not specific enough for obvious reasons, however long oligonucleotides may have regions which match short segments of non-target transcripts decreasing the efficiency of target transcript disruption. B) the specific sequence is important in terms of i) The GC and AT content which affects the efficiency of hybridization (especially with respect to phosphorothioate oligonucleotides) and ii) the target on the transcript, whether it is accessible to hybridization or is closed off by the secondary structure of the RNA.

The empirical nature of antisense technology

The ability of a set of phosphorothioate oligonucleotides or an expressed transcript to successfully disrupt gene expression must be determined by empirical means. Considering the empirical nature of the antisense technology, several tests must be performed with the antisense constructs against genes which will have a predictable phenotype. For example, since v-rel mediates the transformation of avian cells it stands to reason that by inhibiting its expression one would inhibit the efficiency with which avian cells are transformed by the REV-T virus.

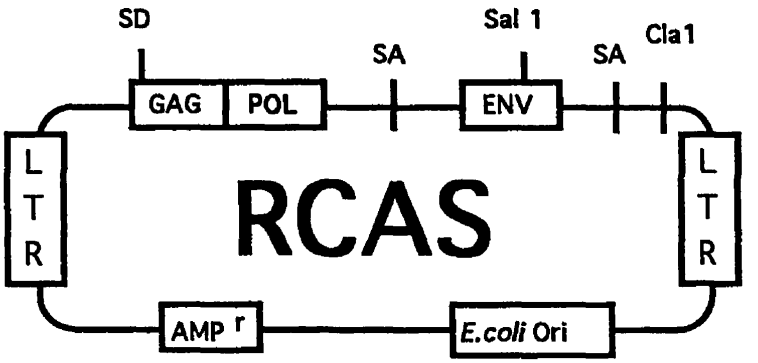
Expression Systems

This thesis made use of two expression systems. The first expression system was developed in Stephen H. Hughes' laboratory in Maryland and uses the avian rous sarcoma retrovirus lacking the v-src gene as a replication competent retroviral vector (Petropoulos and Hughes 1991). The second system is an inducible system where the level of gene expression is dependant on the concentration of exogenous tetracycline (Gossen and Bujard 1992).

The RCAS system of retroviral vectors

The RCAS replication competent avian retroviral gene transferring cassettes developed by Steve Hughes (Frederick, MD) are a set of retroviral vectors capable of expressing inserted genes both *in vivo* and *in vitro*. The original prototype virus, the SR-A strain of Rous sarcoma virus had been consistently modified and improved to yield the final forms used in this thesis (Hughes et al 1987) namely RCAS and RCAN. These retroviral vectors (Figure 2) have had the *src* gene replaced with a unique cloning site

RCAS System of Retroviral Vectors



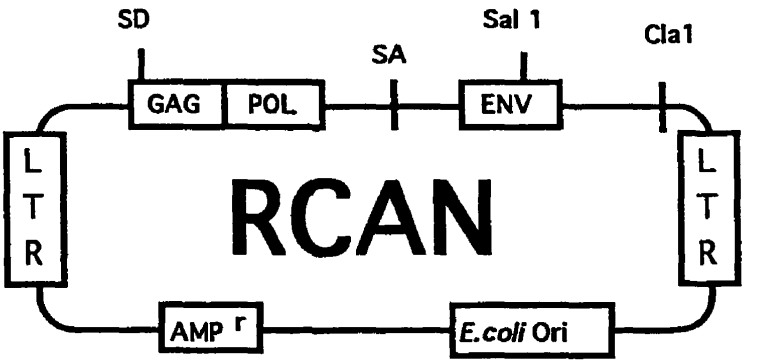


Fig.2 RCAS System of Vectors. The RCAS system of vectors were built upon the SR-A strain of rous sarcoma virus. They have had the *v-src* gene replaced by a unique cloning site (Cla 1). The RCAN provirus, unlike the RCAS provirus, lacks a second splice acceptor located immediately "upstream" of the cloning site. These vectors feature a strong ALV LTR, the Bryan "high titer" polymerase, and an *E.coli* replicon which allows the production of proviral DNA within bacteria. Importantly, there exist several types of ENV regions allowing for the infection of a single cell with multiple viral vectors.

(Cla 1). These vectors have been engineered as provirus in an E.coli replicon which allows the production of proviral DNA from bacteria. The strong promoter in the RSV LTR drives the production of viral transcripts. Importantly, transcripts spliced from the donor site to the second splice acceptor site produce the transcripts competent for the translation of the gene of interest.

Differences in the viral vectors potentiate the usefulness of the system. For instance, the RCAS and RCAN provirus differ in that RCAN lacks a splice acceptor "upstream" of the cloning site. Therefore, genes cloned into the Cla1 site of the RCAN vector will not be efficiently expressed. This creates the possibility of inserting a gene along with its own promoter into the Cla1 cloning site. This would allow the more controlled expression of the inserted gene.

Importantly, viral vectors with different ENV regions permit the super-infection of a single cell by more than one type of virus. The super-infection of a single cell potentially allows the expression of two different genes from within the same cell.

RCAS vectors are productive viruses which have a tropism for a wide variety of avian cells. Viral infection of chickens with these viruses lead to the dissemination of infectious particles throughout the infected individuals. This allows their potential use in creating somatically transgenic chickens, where infected cells express the gene of interest.

The Tetracycline Responsive System

The tetracycline resistance operon encoded in the Tn*10* of *E.coli* has been used to create an efficient regulatory system for gene expression (Gossen and Bujard 1992). A Tet repressor was fused to the activating

domain of the herpes simplex virus virion protein 16 (C-terminus of VP16, Trienzenberg et al 1988), transactivator. This transactivator (tTA) drives transcription initiated at a minimal promoter derived from the CMV IE promoter by binding to an adjacent upstream set of Tet operator sequences (Hillen and Wissmann 1989).

Upon the addition of a minimal amount of tetracycline, transactivator function is inhibited, silencing the promoter. The advantages of this system include the following: 1) using the herpes virus VP16 transactivation domain ensures strong transactivation (up to five orders of magnitude as compared to basal levels in Hela cells), 2) only small amounts of Tet are needed to almost completely disable any transcription initiated from the Tet promoter element (1µg/ml for Hela cells) 3) the Tet molecule is relatively free of the pleitropic effects caused by other well known inducers or repressors (for example elevated temperatures, glucocorticoid hormone action, Lee et al 1988) and 4) the tTA protein binds with specificity and high affinity to the Tet promoter element. Hence, this system allows stringent control of gene expression as compared to other systems used in higher eukaryotes such as the lacR/O/IPTG system (Wyborski et al 1991) which acts slowly and inefficiently.

Experimental Objectives

Crucial to advancing our notions of how certain avian proteins function, is the development of systems that control gene expression. The possibility of protein expression within a controlled environment would present a more clear view of the role played by the protein. To control gene expression, we investigated the possibility of using antisense phosphorothioates in avian cells as well as the using antisense producing RCAS vectors for the purpose of disrupting specific gene expression. This would allow us a powerful system which we could then adapt towards

better understanding v-rel mediated cell transformation. We also tested the RCAS system of replication competent retroviruses for the production of somatically transgenic chickens where infected cells could express the gene of interest. And finally, we also endeavored to demonstrate that the tetracycline-sensitive inducible system (already demonstrated to function in mammalian cells) could function in avian cells.

MATERIALS AND METHODS

Chicken Strains

Chicken strains used in this work were SC line chickens (Hyline International, Dallas Center, IO) an F1 hybrid of S and C line chickens and line 0 chickens. (a chicken line devoid of endogenous retroviral loci)(RPRL, East Lansing)

Cell Suspensions

Peripheral blood lymphocytes were prepared by drawing chicken blood into syringes filled with 1/10 volume heparin in saline. 3 volumes of Hanks BSS was added to 1 volume of blood and centrifuged at 321g for 10 minutes. The pellet was then resuspended in Hanks BSS and the suspension was underlayered with a lympholyte (Cedarlane, Hornby, Ont.) gradient and subsequently centrifuged at 1075g for 15 minutes at 14°C. The cells from the interface (PBLs) were collected, washed once at 387g for 10 minutes, and then washed twice more at 321 g for 10 minutes before being used.

Spleen and bursal cell suspensions were prepared by disruption through a wire mesh. The solution was pippetted into a tube and debris was allowed to sediment at 1g for 5 minutes at 0°C. The supernatant was taken and washed in Hank's BSS. Red cells were removed by underlayering with lympholyte and spinning at 1075g for 15 minutes at 14°C. The cells at the interface were washed once at 387g for 10 minutes and then twice at 321g for 10 minutes prior to use.

CEF preparation

Chicken embryonic fibroblasts were aseptically prepared in the following manner.10 day, line 0 (chicken line devoid of endogenous retroviral loci)(RPRL, East Lansing) or SC line (Hyline International, Dallas Center, IO) chicken eggs were cracked above the air sac. The embryos were lifted through the resulting hole and placed into 100mm plate containing cold D'PBS. After removing the embryo neck, arms and legs, the torsos were placed into a small beaker and minced with scissors. The pieces were left to settle in the cold D'PBS and the blood was decanted away. This was repeated a few times. Tissue fragments were then transferred into a flask containing 15 mls of a 0.025% trypsin solution and incubated at 37° C for 15 minutes with intermittent swirling. Supernatant containing cells was poured off, through a sterile gauze, into a centrifuge tube. More trypsin was added to the flask and the above-mentioned procedure was repeated twice more. The cells were then washed several times in D'PBS at 321 g and plated at a concentration of approximately 5×10^6 cells per plate in a total of 10 mls of media and incubated at 37°C under 5% CO_2. At confluence, the CEFs were harvested, washed, resuspended in freezing media (RPMI [Gibco/BRL, Burlington,Ontario] supplemented with 10% DMSO and 20% FCS) and aliquoted (approximately 3-6 million CEFs per tube) for storage at -150°C.

Cell Lines and Maintenance

Vials of CEFs and COS-7 cells were thawed quickly, washed once and plated onto 3 100mm plates. CEFs and COS-7 cells were generally passaged twice a week.

CEFs and COS-7 cells were harvested in the following manner. The media was aspirated from the plates and cells washed with 5 mls of PBS A (Dulbecco's phosphate buffer without calcium and magnesium). A volume of 2 mls per plate of prewarmed (37° C) 0.125% tissue culture grade trypsin (Boeringher Mannheim, Laval, Québec) solution (0.125% Trypsin, 0.7mM EDTA in PBS A) was added and along with consistent tapping, was used to suspend the adherent cells. Media containing serum was then added to limit further trypsinizing of the cells. The resulting suspension was pippetted up and down to disperse any clumped cells, transferred to a centrifuge tube, and washed once prior to use.

Rev-T transformed bursal cells were prepared by using an *ex vivo* SC chicken bursal cell suspension transformed with Rev-T(CSV) virus (Benatar et al, 1991)

Cos-7 cells (ATCC, Rockville, Maryland)(kindly given by Dr. Allan Cochrane) were derived from an african green monkey kidney cell line, CV-1. They have a defective SV40 virus which solely expresses large T antigen.

TLT-1 is an avian leukosis transformed B lineage tumor that grows in suspension (Calnek et al, 1978).

Tissue Culture

All tissue manipulations were performed in Iscove's modified Dulbecco's medium (Gibco/BRL, Burlington, Ontario), supplemented with 3.025 g/L $NaHCO_3$, 100 U/ml penicillin, 100 mg/ml streptomycin (Gibco/BRL), $5x10^{-5}$ 2-ME, 5% heat inactivated FCS and 2% NCS (Gibco/BRL) for transformed cells, 1% NCS for CEFs or a total of 10% FCS

for COS-7 cells. Cultures were incubated at 37°C with 5% CO_2 in humidified air.

Competent DH5α

DH5α *E.coli* cells were grown in 1X LB broth (1% bactotryptone, 0.5% yeast extract, 1% NaCl pH 7.5) to an OD_{595} of 0.2-0.4. Cells were then chilled on ice for 20 minutes and then centrifuged at 2000g for 10 minutes at 4°C. Cells were then resuspended in 100mM $CaCl_2$ and incubated 20-24 hours. Competent cells were then frozen at -70°C in 10% glycerol.

Plasmid Preparation

Mini-Prep

Mini-preps were performed on plasmid containing DH5α cells grown overnight in 3mls of 1X LB broth supplemented with 100mg/ml of ampicillin. Cells were pelleted in 1.5 ml tubes, resuspended in 200ml TEG buffer (25mM Tris pH 8, 50mM Glucose, 10mM EDTA) and incubated for 5 minutes at room temperature. After adding 400ml of freshly made alkaline solution (0.2N NaOH, 1% SDS) each tube was inverted several times and incubated at 0°C for 5 minutes. High molecular weight debri was removed by adding 300ml of a 7.5M ammonium acetate solution to each tube which was then gently shaken, incubated for 10 minutes at 0°C and microfuged 13 000g for 5 minutes. The supernatant was then added to 0.6 volumes of isopropanol which precipitated the plasmid after at least a 10 minute incubation. The DNA was then pelleted, washed with 70% ethanol, resuspended in either water or TE pH 8 and quantitated using a DNA fluorometer (Hoefer Instruments, San Francisco, California)

Maxi-Prep

A large overnight culture of DH5α cells (250 mls) was used as the starting material for each maxi-prep performed with Wizard Maxipreps (Promega, St.Laurent, Québec). Briefly, cells were pelleted, resuspended in 15 mls resuspension buffer (50mM Tris-HCL pH 7.5, 10mM EDTA, 100µg/ml RNase A), lysed with 15mls lysis buffer (0.2M NaOH, 1% SDS) at room temperature for approximately 5 minutes (when the solution clarifies) and neutralized with 15 mls neutralization solution (1.32M potassium acetate, pH 4.8). The debri was then pelleted away and the supernatant was poured through a filter into 0.6 volumes of isopropanol, mixed and the resulting precipitate was pelleted and resuspended in 2 mls TE pH 8. After 10 mls of DNA purification resin was mixed into the solution, the slurry was poured into a column and pulled into the matrix by a vaccum source. The matrix was then washed, twice with with column wash (200mM NaCl, 20mM Tris-Hcl pH 7.5, 5mM EDTA), once with 80% ethanol and then air dried. The DNA, eluted with approximately 1 ml of preheated (65-70°C) water was centrifuged at 1 300g from the column into 50ml tube and then transferred into a 1.5 ml tube. The final concentration of DNA was generally 300-500µg/ml.

Cloning

Restriction Digests

Typically, digests were performed in the appropriate buffer with 5 units of enzyme and 1 µg of DNA at 37°C for 1-2 hours or overnight with the exception of a few enzymes which may exhibit star activity or require more stringent conditions such as Bam H1 (1 hour incubation only), Eco R1 (1 hour incubation also) and Sma 1 (1 hour, 30°C). Analyses of the resulting fragments was performed by separating them on agarose gels and viewing under UV light. The DNA was run on 1% agarose 14.5 X 14.5 cm gels

containing 0.5 mg/ml ethidium bromide for 1-1.5 hours in 1X TBE (0.89 M Tris, 0.89 M boric acid, 0.02 M EDTA pH 8) containing ethidium bromide (0.5µg/ml) at approximately 120 Volts, after which it was photographed.

Band Isolations from Agarose gels

Typically, a maxi digest would yield a fragment (to be isolated) containing 0.7-0.4 µg. This band, visualized by standard agarose gel separation techniques with ethidium bromide was isolated in the following manner. Running buffer was drained and replaced with 1X TBE without ethidium bromide and at a height which was level with the top of the gel. Some running buffer was then added to a small trough which was cut in front of the band to be isolated. An applied voltage eluted the band into the trough and the buffer containing the DNA was collected. The procedure was repeated until no band was visible under a hand-held UV lamp. The buffer collected was then spun at 10 000g for 5 minutes to pellet away any pieces of agarose carried over. Ethidium bromide was then extracted twice, from the solution collected, with equal volumes of butanol. The DNA was precipitated with 0.25 M sodium acetate pH 5.2 and 2 volumes of 95% ethanol for either 1 hour or overnight at -30°C. After pelleting the DNA, it was washed with 70% ethanol, resuspended in 6-15µl of water and quantitated using the DNA fluorometer.

Ligation Reactions

Ligation reactions were performed in a total volume of 10 µl in either 1X OPA+ buffer (Pharmacia, Baie D'Urfé, Québec) or 1X T4 ligation buffer (Gibco/BRL, Burlington, Ontario) containing 1mM ATP, either 2-7 Weiss units T4 Ligase (sticky ended ligation) or 7-15 Weiss units of T4 Ligase (blunt ended ligation). Insert would be added at either a 1-3:1 ratio of insert

to vector (sticky ended) or 6-8:1 ratio of insert to vector (blunt ended) such that the final concentration of vector was 10ng/ml. This would then be incubated for either 4 hours at room temperature or overnight at 12-16°C.

Transformations

Transformations were performed under aseptic conditions. Competent DH5α were thawed and quickly added (50ml) to pre-cooled (0°C) tubes containing 5-50ng of DNA in a volume of 2-5ml. The mixture of DNA and cells was incubated for minimum 30 minutes at 0°C and then heat shocked at 43°C for 3 minutes. 200ml of pre-warmed 1X LB without antibiotics was added to each tube and incubated at 37°C with agitation for approximately 1.5 hours. The resulting cells were then plated onto 100mm sterile petri dishes containing 10mls of 1X LB, 1.5% bactoagar and 100μg/ml of ampicillin. Plates were then incubated upside down at 37°C overnight.

Colony Screening

Colony lifts

Colony lifts were performed as suggested in Molecular Cloning (Sambrook, Fritsch and Maniatis, 1989). DH5αs growing on 100 mm agar plates were incubated at 37°C for approximately 14-16 hours so that colonies were still small (about 1mm) and then were placed at 4°C for at least 30 minutes. In aseptic conditions, nitrocellulose Protran filters (Schleicher & Schuell, Feene, NH) were placed directly onto the lawn of bacterial colonies until thoroughly moistened. Filter orientation on the agar plate was then established by making holes strategically at three corners of the filter through to the agar. The filter was then slowly lifted, along with some cells from each colony, and allowed to air dry. The original plates were then

placed into a 37°C incubator until the original colonies regrew to a respectable size and then were parafilmed and stored at 4°C.

In Situ Colony lysis

In situ colony lysis, directly on the filter, was performed as suggested by Molecular Cloning. For each filter, puddles of various buffers were made directly onto Saran wrap, upon which each filter was directly incubated (colony side up) at room temperature. The filters were then moistened evenly by stretching the Saran wrap under them. Colonies were lysed when filters were placed on a puddle of 750ml of 0.5N NaOH for 3 minutes. The filter was then blotted on a paper cloth and the procedure was repeated once more with fresh lysis buffer and incubations of only 2 minutes. The filters were then placed on 750ml of 1M Tris-Cl pH 7.4 , incubated for 5 minutes and blotted. This was also repeated with fresh buffer. Lastly, filters were incubated on 750ml of 1.5M NaCl, 0.5M Tris-Cl pH 7.4 for 5 minutes, blotted and air dried for at least 20 minutes. The DNA was then crosslinked onto the filter by UV irradiation (100 000-140 000 mJ/cm^2 filter) in a UV Stratalinker (Stratagene, La Jolla, CA)

Colony Selection

Plasmid DNA from each colony crosslinked to the membranes was then screened through hybridization reactions using the appropriate probes. Those colonies determined to be positive for such reactions, were then traced back to the original plate lifted. The clone was then picked, grown up in 1X LB, prepared appropriately and diagnosed however required.

Probe Labelling and Hybridization

Nick Tanslation

Nick translations were carried out using 0.5mM dNTP, 10 μCi/ml a^{32}P-dCTP, 0.25U DNAase (prediluted in enzyme buffer [20mM Tris pH 7.5, 500 μg/ml BSA, 10mM 2-ME]), 10.5U DNA polymerase and 25ng DNA all in 25μl of DNA polymerase 1 buffer (0.05M Tris pH 7.5, 0.01M $MgCl_2$, 1mM DTT, 0.05mg/ml BSA) for 1 hour at 16°C, subsequently stopped with a 2.6% final volume of 0.75M EDTA pH 8. The probe was separated from unincorporated radio-nucleotides by passing through a 2ml G-50 Sephadex column and eluting with TE pH 8. Out of 12, 200ml fractions collected, monitered by geiger counter, only the first fractions that peaked in radioactivity were pooled. The probe was then boiled and ready to be used.

Kinase Reaction

Radioactive labelling of primers was carried out by ^{32}P-labelling the 5' end of oligonucleotides through a kinase reaction. Reactions containing buffer (Tris pH 7.6, 0.01M $MgCl_2$, 5mM DTT, 0.1mM Spermidine, 0.1mM EDTA), 25pmol/ml of primer to be labelled, 10U/ml T4 kinase enzyme and 10μCi/ml γ^{32}P-dATP, were carried out at 37°C for 1 hour. The probe was now ready to be used.

Hybridization

Nytran and nitrocellulose filters were prehybridized for 2 hours in 0.2ml/cm^2 filter of prehybridization solution, 0.5M $NaPO_4$ pH 7.2, 1mM EDTA, 1% BSA and 7% SDS.

Hybridization of the probe to DNA was carried out in hybridization buffer: 30% formamide, 0.2M $NaPO_4$ pH 7.2, 1mM EDTA, 1% BSA and 7%

SDS overnight with agitation. The filter was then washed 4 times for 15 minutes each time with 40mM $NaPO_4$ pH 7.2, 1mM EDTA, 1% SDS and either put up on Kodak X-OMAR film or placed into a phosphorimager cassette for the required time. Membranes incubated with nick translated probes were prehybridized, hybridized and washed at 65°C. When probed with primers, filters were prehybridized, hybridized and washed at 37°C.

Cell Transfection

Calcium-phosphate mediated transfections

A calcium phosphate-DNA coprecipitate was prepared by adding 220ml of DNA (40µg/ml in 0.1X TE[pH8.0]) to 250ml of a sterile solution of 2X Hepes buffered saline. Slowly 31µl of 2M $CaCl_2$ was added and then gently bubble-mixed with the pipettor. This was incubated for approximately 20 minutes room temperature to allow the precipitate to form. The precipitate was added, with gentle rocking, to a fibroblast plate already containing 5 mls of media. This was incubated for four hours before glycerol shocking the cells. Briefly, the media was aspirated away and replaced with 2 mls of normal media supplemented with 15% glycerol and incubated for 2 min. at 37°C. The monolayer was then washed and incubated with normal media.

Lipofectamine mediated transfections

A lipofectamine-DNA coprecipitate was prepared by adding 100ml of serum free medium containing 2µg of DNA to 100ml of serum free media containing 7µl (when transfecting CEF) of lipofectamine (Gibco/BRL, Burlington, Ontario) or 10µl (when transfecting COS-7 cells) of lipofectamine. This was incubated at room temperature for 15-45 minutes and then slowly added to 70-90% confluent cells in 35mm plates with 800µl

of serum free media. This was incubated for at least 4-6 hours and the media was either replaced with 2 mls of normal media, in the case of COS-7 cells, or supplemented with 1 ml of normal growing media, in the case of CEFs. Cells were then harvested 48 hours later for CAT expression assays.

Virus Stocks

The S_2A_3 cell line produces Rev-T in the context of a CSV helper virus, Rev-T(CSV). Virus was harvested from S_2A_3 cells cultured at a concentration of $5x10^6$ cells/ml in fresh media for 4 hours. The collected supernatant was then filtered through a 0.22μm filter, aliquoted and stored at -70°C.

RCAS virus stocks were produced by replacing the media in confluent infected CEFs plates with 5 mls of IMDM supplemented with both 5% FCS and 2% NCS and incubating the plates for 4 hours. The supernatant collected was spun at 321g for10 min. and then aliquoted and stored at -70°C.

Cell Infection

CEFs, 50 to 80% confluent, were infected by incubating with 50% virus supernatant for 4 hours, which was then diluted to 25%, until confluence was reached and the media was replaced.

Suspension cells were infected with 75% virus supernatant which was replaced only when the cells required splitting.

Avian Embryo Infection

CEFs, whose supernatant tested positive for reverse transcriptase activity, were harvested, washed and loaded into 1 ml syringes at a concentration of 1 million cells/ml in IMDM. 3 day old chicken eggs were then injected sterily into the air sac using a 2 cm long needle with 0.5 million CEFs per egg. The eggs were then immediately replaced in the egg incubator. Candling was performed determine embryo viability less than two weeks later.

Reverse Transcriptase Assays

Supernatants, to be assayed for reverse transcriptase activity, were harvested, spun at 10 000g in a 1.5 ml tube microfuge for 5 minutes and then frozen at -30°C. At the time of assay, 10 ml of freshly thawed supernatant was added to 50 ml of a reverse transcriptase cocktail containing 50 mM Tris pH 7.8, 75 mM KCl, 2 mM dithiothreitol, 5 mg/ml polyadenylic acid (Pharmacia, Baie D'Urfé, Québec), 1.575 mg/ml pd(t) 12-18 Oligo dT (Pharmacia, Baie D'Urfé, Québec), 0.05% NP-40 and approximately 1-2mCi/ml of Thymidine 5' [α-^{32}P] triphosphate (ICN, Mississauga, Ontario) in a 96 well U-bottom microtiter plate. This was incubated with gentle agitation at 39°C for 90 min. 10 ml of the reaction was dotted onto DE81 paper (Whatman International Ltd, Maidstone, England) and air dried for about 20 min. The paper was then washed 4X for 5 minutes each time, with 2X SSC on a rotator. Two more washes were performed in 95% ethanol and the DE 81 paper was air dried and placed into a phosphorimaging cassette for at least 2 hours. Radioactivity was measured with a Phosphorimager (Molecular Dynamics, Sunnyvale, California) using ImageQuant software.

CAT Assay

CAT ELISA

Cells were assayed 48 hours after transfection with the appropriate plasmid(s) and/or provirus for the presence of CAT enzyme using a CAT-ELISA. (Boeringher Mannheim, Laval, Québec) In brief, Cos-7 and CEFs were lysed directly on the plate, the nucleus, intact, adhered to the plate, while the cytoplasmic contents were collected and large debri spun away. Protein concentrations of the lysates were determined using a Bio-Rad (Bio-Rad, Richmond, California) protein assay and a BSA standard curve. Equal volumes of each sample lysate of similar concentration (250 μg/ml total protein) were then incubated for an hour at room temperature in anti-CAT antibody coated wells. Washes were performed, after which the assay was developed with first anti-CAT digoxigen antibodies (2μg/ml, 1 hour, room temperature) and anti-digoxigen antibodies alkaline phosphatase (20 U/ml, 1 hour, room temperature) with washes performed inbetween and at the end of each step. Substrate was added and incubated for approximately 20 minutes allowing a green blue color to develop. Dual wavelength (wavelength 405nm with a reference wavelength of 490nm) readings were taken using a Microplate reader (Bio-Rad, Richmond, California) and compared with a standard curve, constructed with each assay, using purified CAT protein at known concentrations, to determine the exact concentration of CAT protein per sample assayed.

Standard CAT assay

Cells (5 million/ assay) to be tested for CAT activity were washed in D'PBS, resuspended in 100ml of 250 mM Tris pH 8 and lysed by the freeze-thaw method (cells were frozen in a dry ice-equilibrated metal block and subsequently thawed at 37°C, a total 3 times). The cell debri was spun away and a Bio-Rad protein assay was performed to determine lysate protein concentrations. After incubating the lysate at 37°C for 10 minutes, 5 ml of

CAT cocktail (16,2 mg/ml of Acetyl CoA [Sigma, Mississauga, Ontario]; 67 μCi/ml [C^{14}] Chloramphenical [Amersham, Oakville, Ontario] in water) was added to 35μl of lysate, vortexed, quickly spun and incubated at 37°C for 30-60 minutes. The reaction was stopped with 500μl ethyl acetate, vortexed vigorously and allowed to settle for 5 minutes. This was repeated twice more. 400 μl of the upper organic phase was transferred to a new tube which was spun under vaccum and heat in a heatovac (Heto, Scandinavia) for 15-20 minutes. The pellet was then resuspended in 20μls ethyl acetate and dotted onto a thin layer chromatography plate. The end closest to the dotted samples was then immersed in a shallow solution of 95 volumes of chloroform to 5 volumes of methanol in a pre-equilibrated humidity chamber. The solution was allowed to be drawn up the plate for 30-45 minutes. The plate was then air dried and placed into a phosphorimaging cassette overnight. Radioactivity was measured with a Phosphorimager (Molecular Dynamics, Sunnyvale, California) using ImageQuant software.

Cell Permeabilization and Cell Staining

To analyze cellular antigens, cells were washed at least twice with cold D'PBS. 0.5 million cells/ stain were used when staining cell surface antigens while 2 million cells /stain were used when staining intracellular antigens. The primary antibodies used include CT3 (anti-chicken CD3), llC6 (anti-Ig light chain) and HY23 (anti-p19, a protein encoded by the ALV GAG region). Secondary antibodies were fluorescinated isotype specific antibodies (Southern Biotechnology Associates, Birmingham, AL). If the cells had to be permeabilized before staining, they were incubated with either ice cold 70% ethanol for 30 minutes at 0°C or ice cold 100% methanol for 15 minutes at 0°C, after which they were washed three times with D'PBS, once with D'PBS supplemented with 2.5% FCS and then blocked for

15 minutes at room temperature with D'PBS supplemented with 2.5% FCS. Cells were incubated for either15 minutes on ice for live cell staining or 30 minutes at room temperature in the case of fixed cell staining. Cells were then washed twice with D'PBS supplemented with 2.5% FCS and incubated in secondary antibody for either 15 minutes on ice for live cell staining followed by two washes or 15 minutes at room temperature in the case of fixed cell staining followed by two washes. Cells were then resuspended in D'PBS supplemented with 2.5% FCS and cells were analyzed on a FACScan. (Becton Dickinson, Mountain View, CA)

DNA Extraction

Total cellular DNA was extracted from $5x10^6$ eukaryotic cells by first lysing cells in RBS buffer (10mM Tris pH 7.4, 10mM Nacl, 5mM $MgCl_2$, 0.5% NP40) for 30 minutes on ice. The nuclei were then pelleted for 10 minutes at 2000g and solubilized in 0.5% SDS, 300mM NaCl, 10mM Tris pH 7.4, 5mM EDTA supplemented with proteinase K (250µg/ml) overnight at 37°C. An equal volume of TE-buffered phenol was used to extract proteins from the solution. The mixture was spun at 5000 g for 5 minutes. The upper layer was transferred into a new tube and subjected to 3, 5 minute ether extractions using an equal volume of water saturated ether. The ether was boiled away at 56°C for approximately 20 minutes. Genomic DNA was precipitated with 2M ammonium acetate and an equal volume of isopropanol for at least 20 minutes at -30°C. DNA was washed twice with 70% ethanol, 56mM NaCl, dried, and resuspended in TE pH 8.

Southern Blotting

DNA to be blotted was either digested as in the case of plasmid DNA or not as in the case of PCR amplified fragments. The DNA was run on 1%

agarose 14.5 X 14.5 cm gels containing 0.5 µg/ml ethidium bromide for 1-1.5 hours in 1X TBE (0.89 M Tris, 0.89 M boric acid, 0.02 M EDTA pH 8) containing ethidium bromide (0.5µg/ml) at 110 Volts, after which it was photographed. The gel was then denatured with 0.4M NaOH, and 0.1M NaCl for 45 minutes with agitation and neutralized with 0.7M Tris pH 7.4, 2M NaCl for 15 minutes followed by an additional 30 minutes. Blotting onto nytran (Shleicher and Shuell, Keene, NH) was carried out by constructing a pyramid consisting of the gel, nytran, 1 wet (20X SSC) 3MM Whatman sheet, 7 3MM Whatman sheets, papertowels and a heavy book. The transfer occured overnight in 20X SSC, after which the DNA was crosslinked onto the nytran filter by UV irradiation (120 000 mJ/cm^2 nytran) in a UV Stratalinker (Stratagene, La Jolla, CA)

RNA Extraction

Total RNA was isloated from approxiamtely $2\text{-}5x10^6$ cells. The cells were incubated for 15-30 minutes at 37°C in D'PBS supplemented with 2.5% FCS and 50 µg/ml cycloheximide. The cells were then pelleted and solubilized into a 350 ml solution of 50 mM trs pH 6.8, 2mM EDTA, 50 µg/ml cycloheximide, by vortexing. Cells were disrupted by the addition of 400 ml TE buffered phenol. After vortexing for 1 minute, sodium acetate and SDS was added to a final concentration of 0.1M and 0.33% respectively. This was then microfuged for 10 minutes at 15 000g and the top layer was transferred to a new 1.5 ml tube. The RNA was precipitated by adding 1/20 volume of 5M NaCl and 3 volumes of 95% ethanol and incubating the mixture at -30°C for at least 1 hour. The pelleted RNA was then washed twice with 95% ethanol, dried under vaccum dessication and resuspended in dH_2O.

Northern Blotting

The RNA samples were prepared by adding 1X MOPS (0.04 M MOPS pH 7.0, 10mM sodium acetate, 1mM EDTA), 37% formaldehyde, 50% formamide and incubating at 55°C for 15 minutes prior to the addition of 10% loading buffer (50% glycerol, 1mM EDTA, 0.4% bromophenol blue, 0.4% xylene cyanol, 100µg/ml ethidium bromide). Samples were then separated by running on a 1% agarose gel containing 1X MOPS and 18% formaldehyde at 120 Volts for 1-1.5 hours in 1X MOPS and 18% formaldehyde.

Prior to being photographed, the gel was soaked in 20X SSC (3M NaCl, 0.3M trisodium citrate pH 7) for 20 minutes. Passive transfer onto a nytran filter was performed by the following method. A pyramid was constructed by first placing the gel onto 3 wicks of 3MM Whatmanpaper (presoaked in 10X SSC for 10-20 minutes), placing the nytran onto the gel, followed by 12 sheets of 3MM Whatman, paper towels, and a suitably large book. Transfer occured overnight in 10X SSC at room temperature. RNA was then crosslinked onto the filter by using the UV Stratalinker with 120 000µJ/cm^2 nytran.

Polymerase Chain Reaction

Typical PCR reactions consisted of 0.1-0.2 µg DNA template, 25pmol of each primer, 0.2mM dNTPs, 10mM Tris pH 9, 5mM KCL, 0.1% Triton X-100, 1.5 mM $MgCl_2$, and the equivalent of about 2 U Taq DNA polymerase. Reactions were performed in a Hypercell Biological (Chalk River, Ontario) thermal cycler for 30 cycles, followed by a 20 minute extension reaction at 72°C. Each cycle typically consisted of a 1 minute amplification step at 72°C, 1.5 minute annealing step at 60°C and 1.5 minutes of strand separation at

93°C. PCR amplified products were electrophoresed and visualized on 1-2% agarose gels containing 0.5mg/ml of ethidium bromide.

Sequencing

Dideoxynucleotide sequening reactions were performed using 3µg of double stranded plasmid DNA or 6-7µg of double stranded proviral DNA with a Sequenase kit according to the supplier's protocal (US Biochemical Corp., Cleveland, Ohio). Labelling of the elongating strand was carried out using S^{35}-labelled deoxyadenosine triphosphate (NEN/Dupont, Mississauga, Ontario). The reaction products were then separated on 35cm X 43cm 5% Long Ranger (J.T. Baker Inc., Phillipsburg, NJ) sequencing gels in 0.6X TBE running buffer, transferred to 3MM Whatman filter paper, dried and exposed on a Kodak Diagnostic film X-OMAR for 3-5 days.

Westerns Blots

Approximately 1.5-3 million cells per well were prepared for separation through SDS-PAGE. For When determining c-rel or v-rel protein concentration by western blot, subsaturating levels of cellular equivalents were loaded per well (0.75 million cells in the case of CEFs and 1.5 million cells per well for lymphocytes) Cells were pelleted in individual 1.5 ml tubes and broken open by adding 35µl of boiling 1X protein sample buffer. The DNA was sheared by pippetting up and down 60 times with a cut-off 100 ml pippette tip. 2-ME was added to a final concentration of 5%, before samples were boiled for 4 minutes and spun at 10 000g for 1.5 minutes in order to pellet large debri away.

Protein lysates were then fractionated by SDS PAGE (7.5% polyacrylamide) for 16 hours at approximately 40 volts then 2 hours at 100

volts using a SE 600 Vertical Slab Gel Unit (Hoefer Scientific Instruments, San Francisco). Proteins were then transferred to a nytran membrane using a semi-dry gel blotter (Tyler Research Instruments, Edmonton, Alberta) for 1,5 hours at 300mA. Protein transfer efficiency was assessed by Coomassie staining the polyacrylamide gel. The nytran containing proteins was then blocked for 1 hour at room temperature with 1% milk, stained with primary antibody (for c-rel and v-rel, neutralized undiluted HY87 supernatant was used) and washed four times in 1X saline 50mM Tris pH7.5 and 0.05% Tween 20. Stained proteins were revealed by using an anti-mouse antibody coupled to horse radish peroxidase and developed by chemilluminescence ECL system (Amersham, Oakville, Ontario) . Protein concentrations were determined by scanning an undersaturated chemilluminescense produced film. The resulting file (undersaturated) was transferred to ImageQuant software (Molecular Dynamics) for band intensity analyses (volumetric analyses).

Radioimmunoprecipitations

Approximately 5 million subconfluent cells in log phase were harvested, washed twice in D'PBS and incubated in 300 µl of IMDM + 5% dialyzed FCS containing 300 µCi of S^{35} methionine. Cells were metabolically labelled for a total of 3 hours, harvested and washed once with D'PBS. Cells resuspended in 500 µl hypotonic solution (20mM Tris pH 7.8, 20mM KCL, 1mM $MgCl_2$ with 0.1 mM PMSF and 1 µg/ml Leupeptin) and swelled on ice for 15 minutes were ruptured by dounce homogenization (approximately 100 strokes). Nuclear and cytosolic fractions were separated by spinning at 2000 Xg in a microcentrifuge for 10 minutes. Nuclear fractions were lysed in 1X RIPA buffer (25mM Tris pH 7.8, 150mM NaCL, 2mM EDTA, 1% NP40, 0.5% Deoxycholate, 0.1% SDS).

Fractions were clarified by microcentrifuging at top speed (4°C) and precleared for 30 minutes at 4°C with 200µl (10%) prewashed Pansorbin cells (Formalin fixed Staphylococcus A, Calbiochem). Antisera was then added (about 5 µl), incubated with rotation for 1 hour at 4°C and then incubated with 20 µl of protein A and G beads (Santa Cruz Biotechnology, Santa Cruz, California) for 1 hour at 4°C in order to precipitate target proteins. The pellets were washed once with 500µl of each Opperman wash buffer (OP1: 20 mM Tris pH7.8, 1M NaCL, 0.1% NP40, OP2: 20mM Tris pH 7.8, 200mM NaCl, 1% NP40, 0.1% SDS, 2 mM EDTA, OP3: 20mM Tris pH 6.8, 200mM NaCl, 2 mM EDTA, 0.1% NP40) by vortexing the pellets for 30 seconds. Pellets were then resuspended in protein sample buffer. Proteins were fractionated by SDS-PAGE, the gel stained with Coomassie Blue to confirm antibody position and gel dried and exposed overnight to X-OMAR Kodak film or phophorimager screen.

RESULTS SECTION

Introduction

The REV-T retrovirus mediates avian cell transformation through the v-rel oncogene

The acutely transforming retrovirus REV-T, is the most virulent of all known retroviruses. Evolution of the parental REV-A virus, incorporated the turkey c-rel proto-oncogene into the ENV region of REV-A, creating the defective REV-T virus. Oncogenic viruses, such as REV-T, cause the transformation of target cells through the action of their incorporated oncogene. V-rel, the corrupted form of the turkey c-rel, mediates the transformation of avian cells by the REV-T virus.

V-rel mediated transformation

V-rel mediates the transformation of cells , partly through the deregulation of individual REL/NFκB complexes. This viral oncogene forms part of a growing family of transcriptional regulators called the individual REL/NFκB family. This group of transcription factors play key roles in lymphoid cell development, differentiation and proliferation. individual REL/NFκB transcription factors are regulated by their association to inhibitors such as IκBalpha. While, their specificity and activity is controlled by the individual REL/NFκB members making up the complex, each contributing to the overall function of the unit. V-rel, clearly, also associates with other members of the REL/NFκB family. Association of REL/NFκB members to this corrupted form of c-rel is thought to deregulate normal REL/NFκB function effectively leading to a

transformation event. Given the perturbation of REL/NFκB function, deregulated gene expression manifests itself in the altered phenotype of the transformed cell.

C-rel and cell proliferation

C-rel's capacity to regulate cell growth was partly inferred by v-rel's ability to provoke uncontrolled lymphocyte proliferation. C-rel, also a member of the REL/NFκB family, associates with the same family members as v-rel. Furthermore, c-rel transcripts are quickly upregulated by growth factor stimulation in cells of hematopoetic origin, classifying it as a member of the imediate early gene family, implicated in cell growth regulation. Indeed, the c-rel null mouse contains immune B and T cells incapable of proliferating in response to receptor stimulation. Therefore, to further understand v-rel mediated transformation, the functional contribution of other REL/NFκB members to lymphocyte growth regulation requires investigation.

Antisense Technology for the disruption of REL/NFkB gene expression

Phosphorothioate oligonucleotides have been used to disrupt the specific expression of a number of genes. The sequence of the oligonucleotide allows the specific hybridization of the oligonucleotide with the transcript in question. The RNA-DNA duplex is then cleaved by the host cell and specific gene expression is limited. Phosphorothioate linkages provide the oligonucleotides with greater protection against cellular nucleases, therefore allowing a more efficient disruption of gene expression as compared to normal oligonucleotides.

REL/NFκB gene disruption by phosphorothioate antisense allows one to investigate the role of individual REL/NFκB members. By revealing the consequences of eliminating a specific REL/NFκB member, one may define the functional contribution of individual members to the various REL/NFκB functions which will lead to a better understanding of v-rel mediated cell transformation.

RESULTS

Detection of v-rel and c-rel *in vivo* and *in vitro*

REV-T virus produces a potent transforming protein called v-rel. Immunoblots using the HY 87 antibody (which recognizes both c-rel and v-rel) detected $p58^{v\text{-}rel}$ protein expression in cells transformed by REV-T(CSV) transformed B cells, but was not detected in B cells (TLT-1) transformed by the avian leukosis virus (Figure 3).

v-rel protein and bursal cell transformation

A high level of v-rel protein expression was detected in REV-T transformed cells, consistant with the role of v-rel in the transformation process. We therefore, attempted to inhibit v-rel induced transformation with anitsense directed against v-rel. The outgrowth of *ex vivo* bursal cells transformed by REV-T(CSV) harbouring the v-rel oncogene is readily seen (Figure 4) while *ex vivo* bursal cells incubated in the absence of the virus die very quickly by apoptosis (Jacobsen et al 1996). The inhibtion of v-rel expression was attempted using phosphorothioate oligonucleotides. In bulk cultures, bursal cells incubated in the presence of 10μM of v-rel antisense and REV-T(CSV) did not lead to any growth inhibition as compared to cells incubated with REV-T(CSV) alone (Figure 4). The transformation frequency, under limiting dilution conditions, of bursal cells by REV-T(CSV) in the presence of the antisense oligonucleotide was also not altered (data not shown).

We considered the possibility that the amount of v-rel expression inhibited by the antisense oligonucleotide was not sufficient to inhibit growth, but enough to alter the phenotype of cells transformed with REV-

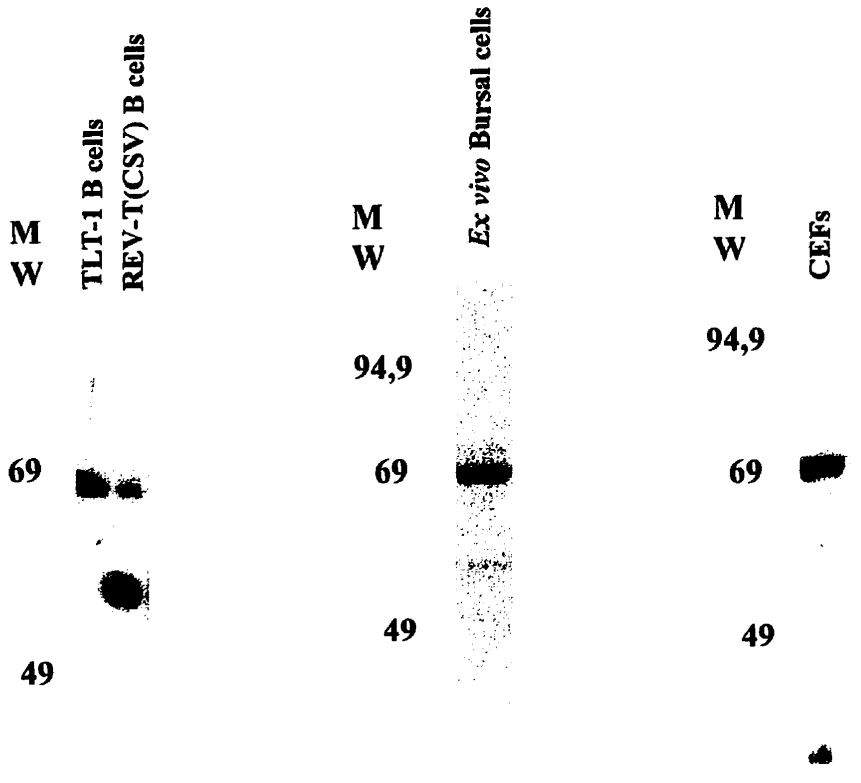


Figure 3. Westerns performed with HY 87 anti-rel antibody. HY87undiluted supernatant was used to stain 1.5 million cellular (only 1 million CEFs) equivalents per lane blotted. C-rel protein migrates at about 68 kDa, while v-rel migrates at 59 kDa. Only REV-T(CSV) transformed cells contain v-rel protein.

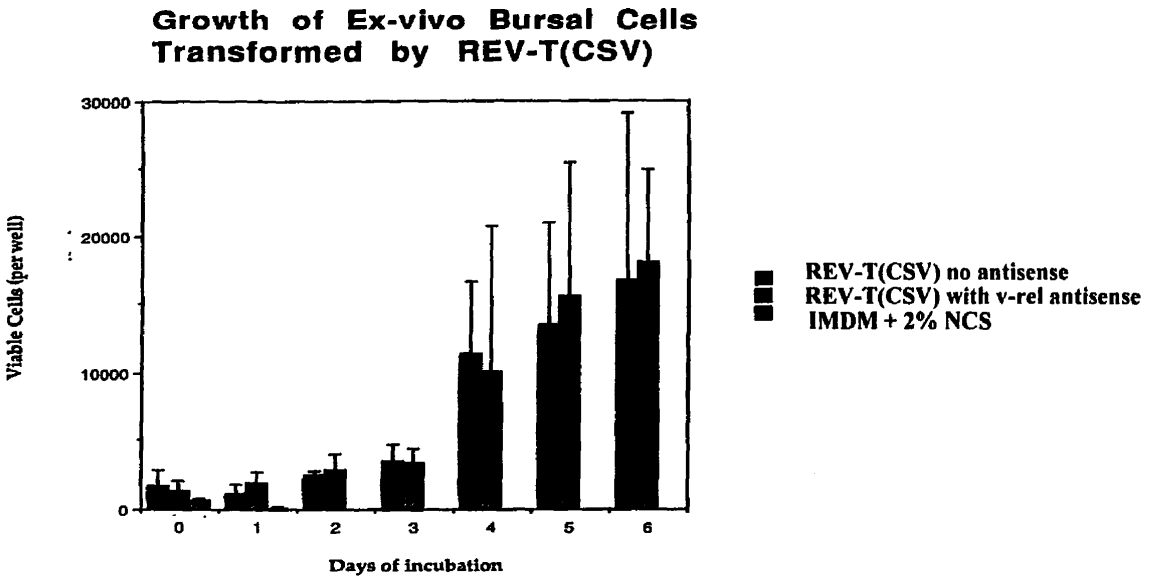


Figure 4. Transformation and Growth of *ex vivo* Bursal Cells exposed to REV-T(CSV). Approximately 1000 *ex vivo* bursal cells were incubated in IMDM + 2% NCS in the presence or absence of REV-T(CSV) containing supernatant (75%). Outgrowth of transformed bursal cells is readily apparent even in the presence of v-rel antisense. *Ex vivo* bursal cultured *in vitro* quickly die by apoptosis (90% death in 12 hours).

T(CSV). Cell surface molecules expressed on bursal cells are altered by REV-T(CSV) transformation. Characteristic of cells transformed with REV-T(CSV) is the downregulation of Bu-1 (a pan B cell marker) expression, not seen in the ALV transformed TLT-1 B cells (Figure 5). However, flow cytometric analysis of a number of cell surface antigens failed to reveal differences in REV-T(CSV) transformed cells cultured in the presence or absence of antisense (data not shown).

REV-T(CSV) transformed bursal cells were incubated with v-rel phosphorothioate antisense. Western blots performed on the antisense treated cells also indicated that v-rel protein levels had not changed (Figure 6). Altering the amounts (over a range of 1-20μM) and duration of antisense (2 hours up to 3 days) treatment as well as cell concentration failed to alter high level v-rel expression (data not shown).

C-rel protein is involved in the cellular growth response

The comparitively low levels of c-rel (Figure 3,6), a protein implicated in growth regulation, suggested the possibility that manipulation of c-rel by antisense treatment might be easier than that of v-rel. We show that c-rel protein is maximally induced in serum deprived CEFs 2 hours after serum induction (Figure 7). Total C-rel protein then levels out at 24 hours post-serum stimulation. Also, of possible importance, was the observation that there was a small difference in the amount of c-rel protein found in cellular equivalents of non-confluent CEFs grown in the presence or absence of serum. In other words there was a difference in the levels of c-rel protein found between proliferating and growth arrested (Figure 8) CEFs.

REL protein and the cell cycle

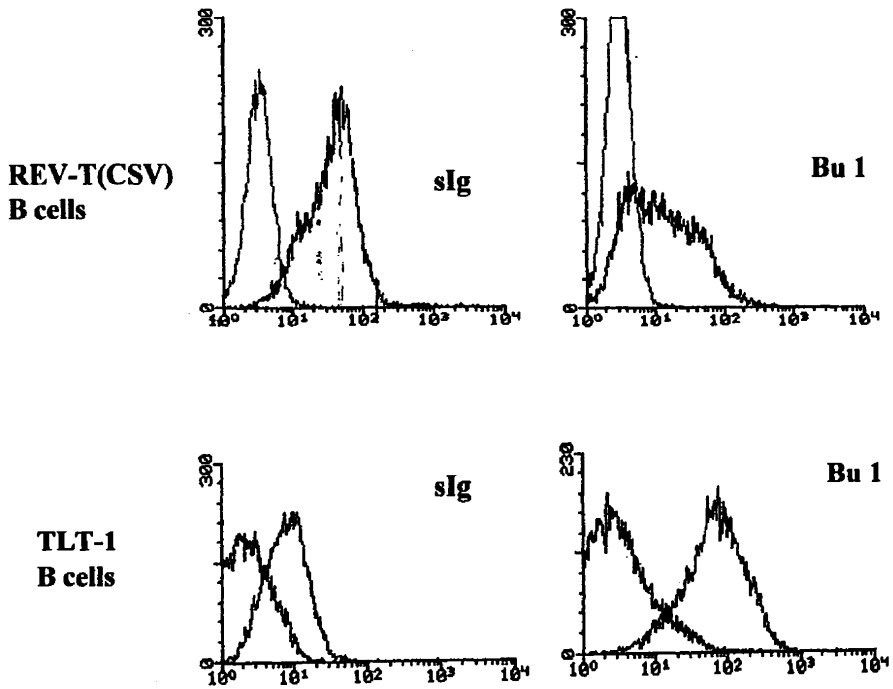


Figure 5. REV-T(CSV) cells display low levels of Bu 1 antigen. REV-T(CSV) transformed bursal cells and TlT-1 were stained with llC6 (anti-LC of Ig, 10 micrograms/ml) and with Bu-1 supernatant. REV-T(CSV) cells demonstrate lower levels of Bu 1 antigen than TLT-1 cells. Staining was controlled by using isotype matched flourescent antibodies.

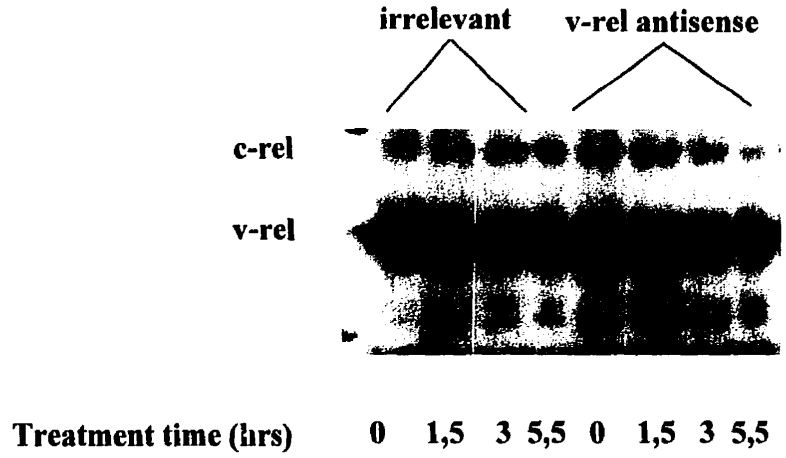


Figure 6. Antisense to v-rel does not dimish v-rel levels in REV-T(CSV) transformed Bursal cells. REV-T(CSV) transformed bursal cells were transfected for 4 hours and then incubated an additional amount of time ranging from 0-5,5 hours. V-rel expression as assayed by Western blot using HY 87 anti-rel antibody was not diminished. Irrelevant antisense is anti-human c-rel (5'-GAGGCCATGGCTCCGCTCCCCG) and anti-vrel is (5'-GTTGGTGAGAAA GTCCAT). Underlined are the translational start sites.

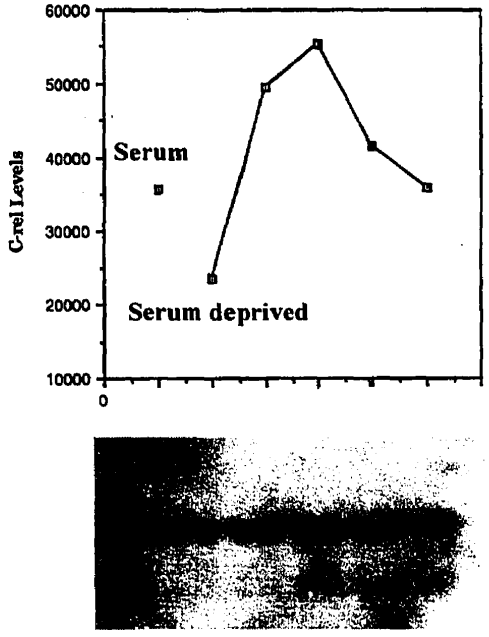


Figure 7. C-rel protein uregulation in serum stimulated CEFs. Non- confluent serum deprived CEFs were stimulated with 10 %NCS for the times indicated above. Sub-saturating amounts (0.75 million cellular equivalents) of CEF protein lysate were separated by (7.5%) SDS PAGE and Western blotted using HY87 antibody. The above example is representative of at least two more independant experiments. Points marked Serum and Serum deprived indicate c-rel levels in CEFs grown in the presence of 1% NCS and no serum, respectively. Protein levels were analyzed with ImageQuant software using adjacent areas for subtracted background.

Given the correlation between growth stimulation and REL protein production, a role in cell-cycle progression was investigated. Rel protein levels were analyzed in permeabilized *ex vivo* PBLs by flourecence activated cell sorting using two different antibodies (C31 and HY 87). C31 had been previously used in immunoflourescence sorting to determine the average c-rel and v-rel proteins levels in permeabilized cells (Hrdlickova et al 1994b). Although, the cell cycle data presented was acquired using the C31 antibody, both antibodies yielded similar results. C-rel protein levels were observed to steadily increase from the more resting populations (G0 and G1 phases) to the more actively dividing populations (S and M phases) (Figure 7).

Testing the efficiency of the antisense oligonucleotides

Various antisense oligonucleotides were tested for the efficiency with which they could eliminate the production of c-rel protein. Antisense oligonucleotides with phosphate and phosphorothioate backbones as well as chimeric oligonucleotides with both types of backbones were constructed.

Antisense nucleotides were used to disrupt the production of c-rel protein. Various attempts to eliminate c-rel protein production by simply "salting" in the antisense oligonucleotides into the supernatant of the cells in question without the use of cationic lipids to enhance the cellular uptake of the antisense nucleotides, failed to result in reduced v-rel expression (data not shown).

In order to increase the efficiency with which the antisense oligonucleotides were taken up by the target cell, cationic lipids were used to surround the oligonucleotides and fuse with the target membranes. The

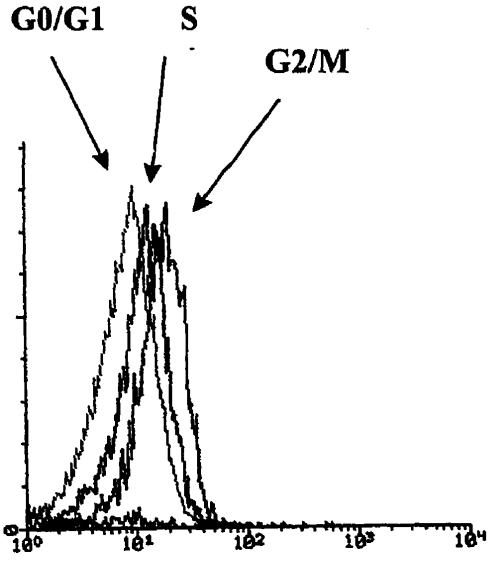


Figure 8. C-rel protein levels in different stages of the cell cycle in *ex vivo* chicken PBLs. A double stain was performed on permeabilized *ex vivo* chicken PBLs using propidium iodide to stain the DNA and C31 antibody to stain c-rel. Upon analyses of the DNA content of the PBLs, gating was performed on populations in different stages of the cell cycle in order to determine the mean expression levels of the c-rel protein within those populations.

kinetics under which c-rel protein was produced following serum stimulation was known, and provided us with a system in which we could test the ability of the oligonucleotides to prevent c-rel protein production.

Interestingly, depending on the antisense oligonucleotide tested, partial or complete blocks in c-rel protein upregulation by serum could be observed (Figure 9). The nuclease sensitive oligonucleotide was unable to mediate any blocking in c-rel upregulation (data not shown), while the chimeric phosphate-phosphorothioate oligonucleotide had partial results (Figure 9). Complete blocking of c-rel protein upregulation was seen only with the phosphorothioate oligonucleotides. The longer antisense species was slightly more efficient at blocking the serum mediated c-rel upregulation. However, for reasons which are unknown, the amount of c-rel being produced before serum stimulation, was unaltered by any of the antisense oligonucleotides. Many attempts were made with various antisense-cationic lipid incubation times on Tlt-1 and CEFs to eliminate uninduced c-rel protein. In all cases, the amount of c-rel protein, as revealed by western blots was indistinguishable from the controls (data not shown). The possible implications of this discovery are discussed in the next section. In any case we had been able to eliminate the upregulation of c-rel protein in response to serum stimulation.

Preliminary experiment eliminating c-rel protein induction by serum in CEFs

The possible role of c-rel upregulation in the growth response of CEFs to serum stimulation was investigated (Figure 10). Serum-deprived CEFs were treated with control and antisense oligonucleotides. Serum was then added to the treated CEF cultures. 3 hours after serum stimulation CEFs were harvested, permeasbilized and DNA stained with propidium iodide. Serum deprived CEFs had very little cells in the growth phase of the cell

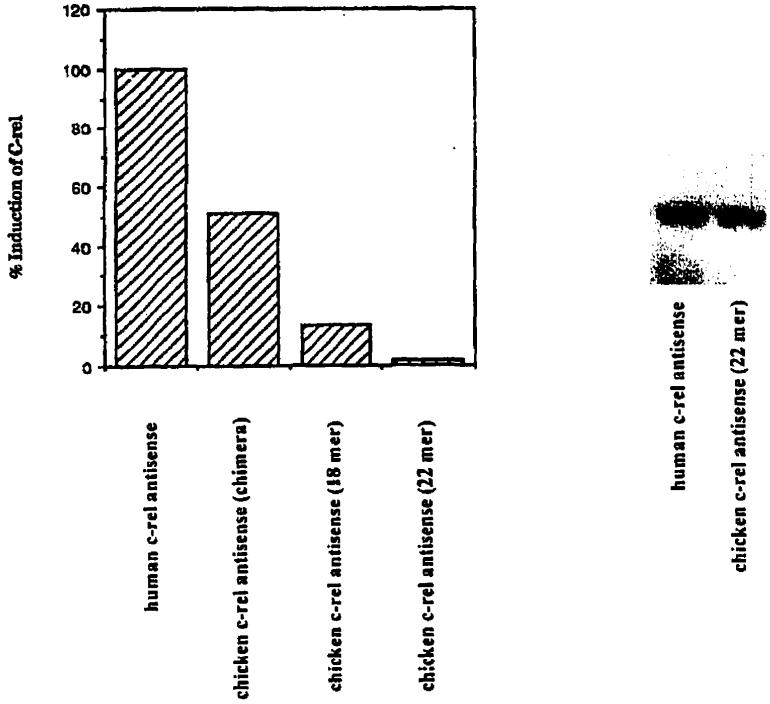


Figure 9. Efficiency of different antisense phosphorothioate oligonucleotides to inhibit c-rel upregulation in CEFs. Non-confluent serum deprived CEFs, transfected with a variety of c-rel antisense oligonucleotides were stimulated with 10 %NCS for 2 hours and harvested to determine c-rel protein levels by Western blot. The upregulation of C-rel protein levels in the antisense treated CEFs is expressed as a percentage of the amount of c-rel upregulated in non-treated serum stimulated controls. Nucleotide sequence of c-rel chicken antisense (18 mer), same as chimera 5'- CGCCATGCTGACAGCTCC, (22 mer) 5' - CG CCATGCTGACAGCTCCGCGT

cycle (S phase), and growth arrested cells may be seen in the n and 2n DNA phases (G0, G1 and M,G2) with the majority of cells arrested in the G0, G1 phase. The cell cycle profiles of serum treated CEFs demonstrated a concerted initiation of DNA production by the majority of cells arrested in G0, G1. However, significant differences in the cell cycle profiles between antisense and control treated cells was not seen at 3 hours post-serum induction. Whether c-rel protein induction is required for the serum growth response to serum is still unclear. Further time points in the cell cycle of treated CEFs are required, in order to be able to see a more pronounced concerted entry of CEFs into the S phase of the cell cycle after serum addition.

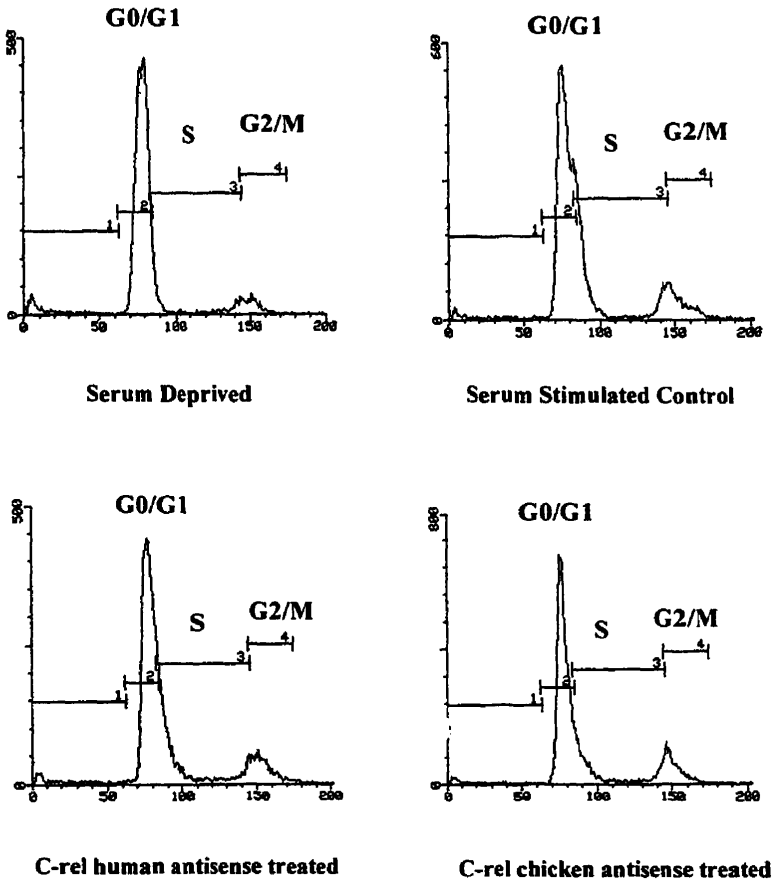


Figure 10. Cell cycle analysis of serum stimulated CEFs in the presence of c-rel antisense. Serum deprived antisense and non-antisense (Serum Stimulated Control) transfected CEFs were serum stimulated with 10 %NCS for 3 hours, harvested, and DNA content stained with propidium iodide. 24 hour serum deprived CEFs contained 18% of cells in S phase. 32% of Serum Stimulated Controls were in S phase, control c-rel human antisense treated CEFs had 32% of cells in S phase while 26% of chicken c-rel antisense treated cells were in S phase.

Discussion

Lymphoid cells are regulated by members of the REL/NFκB family

Expression of c-rel protein

REL/NFκB members play a role in tissue development, cell differentiation and cell function. The c-rel member of the NFκB family, for example, plays a vital part in the differentiation and growth regulation of lymphoid cells such as pre B and mature B cells. We demonstrate that the c-rel protein is expressed in all B cells looked at, including transformed and *ex vivo* B cells and also in CEFs (Figure 3). C-rel is expressed, as other members of the REL/NFκB family, within cells of hematopoetic origin allowing them to participate in the cell processes of activation, differentiation and growth.

The possible function of the c-rel protein in CEF growth when stimulated by serum

C-rel is the prototype REL/NFkB member to study the mechanisms of growth regulation. C-rel transcripts are upregulated upon growth factor and mitogen stimulation. Maximal levels of c-rel transcript are seen approximately 1 hour after serum and mitogen stimulation in murine fibroblasts and T cells. The upregulation of c-rel protein (maximal at 2 hours) in serum deprived CEFs (Figure 7) correlates with what has been documented for the mitogen induced expression of c-rel transcripts. There is evidence suggesting that the activated NFκB (induced by serum) binds the κB site in the c-rel promoter and transactivates c-rel in response to

serum stimulation. This upregulation, places c-rel within an important regulatory family termed the immediate early genes implicated in the mechanism of the proliferative response to growth stimulation. Indeed, the c-rel null mouse where T and B cell proliferative responses are defective confirms this association between c-rel and proliferation.

The increasing c-rel protein levels (Figure 8) observed during the cell cycle were most probably just a reflection of the increasing cell size during cell cycle progression. However, there appears to be a role for a c-rel-like protein in the cell cycle (Evans et al 1993) as well as other REL/NFκB related proteins (Xu and Gelinas 1995) whose appearances in the cell are cell cycle phase dependant. Further characterization of these new REL/NFκB members remains to be done.

C-rel protein levels increase two fold upon serum stimulation in serum deprived CEFs (Figure 7). The levels of those proteins involved in growth regulation are carefully maintained and do not need to fluctuate greatly to have a strong influence in the growth regulation of a cell. Good examples may be found among some of the cell cycle proteins (Sherr 1994) whose levels never change that profoundly but considerably influence cell cycle progression and arrest. Also of importance, was the amount of active c-rel which was not determined. It would be important to possibly determine the amounts of c-rel protein in the nucleus as compared to the cytoplasm. This would allow a better understanding of the state of the c-rel protein. Antisense oligonucleotides were used to alter the protein production of c-rel upon stimulation by serum in CEFs.

Phosphorothioate oligonucleotides and the antisense affect

Phosphorothioate oligonucleotides have been used to inhibit the production of certain proteins *in vitro*. There exist many examples in the literature where the phosphorothioate oligonucleotides were added directly into the supernatant of the target cell and were deemed to inhibit the expression of the gene in question. However, without the help of the cationic lipids which enhance oligonucleotide take up we could see no affect when adding the oligonucleotides directly to the cell media (data not shown) . Antisense review articles (Stein and Cheng 1993, Milligan et al 1993, Nellen and Lichtenstein 1993) plainly discuss the problems of cellular uptake not revealed in the literature and recommend transfections to enhance the uptake of antisense oligonucleotides. The antisense phosphorothioate oligonucleotides may not have been readily taken up by the cells either because A) the cells were not permissive or B) the antisense phosphorothioate oligonucleatides did not lend themselves to be readily taken up by the cell. Lastly, C) although the possibility is remote, serum nucleases may have brocken down the oligonucleotides.

The structure of the antisense oligonucleotides influenced its ability to inhibit c-rel gene upregulation (Figure 9). The parameter which influenced the antisense oligonucleotides ability to disrupt c-rel production the most was the type of linkages between the individual nucleotides (Figure 9). The normal antisense nucleotide which had the same sequence as the phosphorothioate oligonucleotide was unable to inhibit the upregulation of c-rel protein (data not shown). This was presumably due to the sensitivity of the normal oligonucleotides to cleavage by endogenous nucleases. The chimeric oligonucleotides were not as effective as the complete phosphorothioate oligonucleotides in inhibiting the upregulation of c-rel protein after serum stimulation. This would argue that the protection afforded by the phosphorothioate linkages outweighed the relative hybridization ineffeciency of phosphorothioate oligonucleotides as compared to regular oligonucleotides.

The antisense oligonucleotides were unable to decrease the levels of c-rel protein prior to serum stimulation of the CEFs. The half life or REL/NFκB members has been estimated to be about 8 hours (Bose Jr. 1992). It is possible that there was not a sufficient amount of antisense oligonucleotides against c-rel within the cells for the length required to mediate the abrogation of c-rel gene expression.

The initial levels of c-rel were not diminished yet the upregulation of c-rel was sensitive to the effects of the antisense nucleotides. Perhaps the serum response of the cell lead to c-rel transcripts which were more accessible to the antisense oligonucleotides, but this would then argue that c-rel gene expression should have been abolished in its entirety, which is not the case. The possibility remains open because there is a small chance that an equilibrium may have been set up where not all the c-rel transcripts could have been inhibited, but only some, which would allow the production of some c-rel at levels that are seen prior to serum stimulation.

The c-rel gene has not been fully studied. Spliced variants of c-rel have been observed, but not fully characterized (Brownell et al 1988). Though the chances are remote, a spliced variant of c-rel (intronic splice variant) upregulated under serum stimulation and sensitive to antisense mediated disruption could account for the observed results.

Antisense oligonucleotides are thought to exhibit their effects by inducing the destruction of the RNA to which they bind. However, other methods of inhibiting gene expression are known to occur through the use of antisense oligonucleotides. For example, there is a possibility of triplex formation with DNA which may inhibit transcript production. There are also examples in the literature where antisense oligonucleotides have been used to mask splice sites which lead to the disruption of that splice variant

(Dominski et al 1993). The mechanism by which antisense oligonucleotides exert their effect is unclear and remains in some cases an empirical observation. This argument is sometimes used to explain why western blots as oppossed to northern blots are used to assay the antisense's ability to disrupt the target gene expression. It has also been shown in the literature (Chiang et al 1991) that disruption of gene expression by antisense phosphorothioate oligonucleotides was apparent only when a western blot was performed, while the northen blot demonstrated normal transcript levels, for reasons which were unclear.

Nevertheless, c-rel protein upregulation may be inhibited by the use of antisense phosphorothioate oligonucleotides. This allows one to study the importance of c-rel upregulation in the growth response of CEFs to serum stimulation. As was realized from the c-rel null mouse c-rel activity is believed important in the proliferative response of cells. Although fibroblast growth was not looked, it is obvious that c-rel function is not essential for the proliferative response of all hematopoetic cells. It could be that c-rel function is redundant or taken over by other mechanisms in other types of cells. This would still not eliminate a possible role for c-rel in the speed with which cells may respond to growth factor stimulation. In this respect CEFs entry into the cell cycle may be studied under conditions where c-rel upregulation is inhibited. The preliminary results obtained with respect to CEFs entry into the cell cycle after serum stimulation (Fig. 10) still need to be clarified. 3 hours after serum stimulation seemed to be too early a time point to look at the effects of c-rel inhibition. Therefore more experiments using later time points are required to determine if cell cycle entry of CEFs is slowed by the lower c-rel levels.

Understanding the mechanism of v-rel induced transformation

By developing our understanding of how c-rel functions in a cell one is able to better understand the mechanism of v-rel induced transfromation. It is very simple to transform *ex vivo* bursal cells with supernatant containing REV-T(CSV) virus. It is known that c-rel is involved as well as other members of the REL/NFκB family in the differentiation and gene regulation of B cells. Indeed, the differentiation of B cells revolves around the regulation of the B cell receptor which is partly regulated by the κB enhancer sequences which first lead to the discovery of the NFκB transcription factor.

The relationship between growth factor stimulation and immunoglobulin receptor levels remains to be determined. It is clear that serum stimulation leads to the production of c-rel protein in lymphoid and fibroblast cells. LPS stimulation of the preB cell line 70Z/3 leads to the expression of the immunoglobulin receptor, which is thought to be regulated by the NFκB transcription factor (Singh 1994). We know that Tlt-1 cells, an avian B cell line not transformed by REV-T virus expresses the immunoglobulin receptor only in the presence of serum (data not shown). The link between B cell receptor expression, growth regulation and REL/NFκB members is unclear. We do know that REV-T(CSV) transformed bursal cells express different BCR levels for reasons that are unknown (data not shown). Also, *ex vivo* bursal cells doubly stained with anti-immunoglobulin and c-rel demonstrate that only those cells which express high levels of surface Ig express high levels of c-rel (data not shown). It is obviously a complicated, but intriguing question to determine the link between growth factor regulation, B cell receptor levels and REL/NFκB members.

Characterizing the role of REL/NFκB members in B cell differentiation may lead to a better understanding of v-rel transformation and the relationship between B cell receptor levels and growth regulation. For example, while the major REL/NFκB transcriptional complex in mature murine B cells seems to be composed of c-rel containing complexes, the immature murine pre B cell REL/NFκB transcriptional regulator is the NFκB factor. In the pre B cell line 70Z/3, the immunoglobulin receptor is not expressed unless the cells are mitogen stimulated. Since mitogen stimulation readily activates REL/NFκB transcription factors, which bind κB sites in the Ig enhancer, a direct link may be made between proliferation, REL/NFκB members and Ig receptor levels.

It is interesting to note at this point that the cells which were transformed by the v-rel oncogene expressed under the control of the lck promoter in transgenic mice had an immature phenotype. Therefore, the cells vulnerable to the transformation effects of v-rel seemed to reside among the immature murine T lymphoid cell population. Also of interest were experiments using v-relER, a chimeric protein inducible by the addition of exogenous estrogen. Bone marrow cells were transformed by this chimeric protein leading to a cell type which expressed B cell determinants. The removal of v-rel function from these cells allowed their differentiation into neutrophil-like and dendritic-like cells mimicking more closely the type of cell growth seen in young chickens exposed to REV-T virus.

The levels of NFκB in the immature population as compared to the adult popultation do not change. It is the levels of the c-rel protein which change, resulting in an increase in the levels of c-rel protein in the mature murine B cell population. The continual expression of the B cell receptor

in the mature B cell population is thought to be partly due to the transcriptional regulator to which c-rel is associated. Also pertinant, the Ig kB site whose regulation is involved in both B cell and receptor level regulation binds p50-c-rel complexes with a twenty fold higher affinity than p50-p65 complexes (Myamoto et al 1994). Lastly, the NFkB transcription factor seen in immature murine B cells is mostly cytoplasmic, presumably held there by IκB while in mature murine cells the c-rel containing complexes are both nuclear and cytoplasmic.

Introduction

RCAS vectors for antisense expression

The RCAS system of viruses have been used in the past to infect cells wherein they express inserted genes from the cloning site (Hughes et al 1987). The fact that these viruses use a strong promoter in the avian leukosis virus long terminal repeat to drive the expression of the cloned genes allows the exploration of using these viruses for antisense purposes. Although the use of RCAS viruses as antisense producing vectors has never been tested, past precedents in the literature using retroviruses and the fact that RCAS viruses feature strong promoters provided enough assurance that these viral vectors could potentially play such a role. These vectors also have the added ability of disseminating throughout infected individuals therefore potentially allowing a more general application of antisense technology.

Concatomerization of transcript

Antisense technology is relatively new not well understood. For example, when an RNA containing duplex is recognized by the cell and

targetted for cleavage we do not know if only those areas creating the duplex are degraded leaving the single stranded unhybridized areas intact. One can imagine that the cleavage of the hybridized areas would be enough to destabilize or render the transcript useless therebye disrupting gene expression while only cleaving the areas of duplex formation. It is not unreasonable to assume then that concatomerization of the antisense transcript may increase the efficiency of gene disruption by allowing one concatomerized antisense transcript to disrupt many sense transcripts.

RAG genes

The recombination activating genes 1 and 2 (RAG-1 and RAG-2) were discovered on the basis of their functional importance in the recombination processes required for generating the diversity of the immune B and T cell receptors. (RAG-1, Schatz et al 1989; RAG-2, Oettinger et al 1990) Expression of the RAG genes is restricted to the lymphoid lineages where it correlates with the recombinase activity needed to rearrange receptor DNA. Indeed, this recombinase activity may be induced in cells normally unable to recombine DNA such as NIH 3T3 fibroblasts by transfecting in just these two genes. (Oettinger et al 1990)

RAG Function in Chicken

Shortly after their discovery, the RAG-1 and RAG-2 genes, originally cloned in mouse and man, were identified and/or cloned in other species such as rabbit (Fuschiotti et al 1993), shark, zebra fish (RAG-1, Greenhalgh and Steiner 1995) and chicken (Carlson et al 1991). Examination of these cloned genes reveals a high degree of sequence homology between RAG genes from different species including chicken (Fuschiotti et al 1993) implying that the recombinase function required by the immune systems of all these species has been conserved.

The two major cell types in the avian immune system, B cells and T cells require a recombinase function in order to rearrange their DNA elements. This recombination process is carried out in the thymus for T cells (Pickel et al 1993) , while B cell specific recombination takes place in more than one organ in the developing embryo (Reynaud et al 1992). Consistent with the well characterized functions of the RAG genes in the murine system, avian RAG gene expression correlates with cells undergoing DNA rearrangements. For example, in the avian thymus, RAG-2 protein is expressed primarily in the nucleus of immature CD4- CD8- and CD4+ CD8+ thymocytes but is absent in the single positive CD4+ CD8- and CD4- CD8+ populations (Fergusen et al 1994). While in the avian embryo spleen, an organ where B cells are undergoing Ig rearrangement, splenic B cell precursors express both RAG-1 and RAG-2.

The correlation between RAG gene expression and immune receptor recombination in chicken is not perfect. RAG-1 gene expression is seen in late embryonic bursa where evidence has suggested that cells are no longer undergoing Ig rearrangement (Reynaud et al, 1992). It is also known that RAG-2 protein is expressed by bursal cells which are not in the process of recombining their DNA. However, given the abundant corollary evidence for the role of RAG proteins in the recombination event it is more than probable that the observed RAG expression in chicken in the absence of rearrangement may be a peculiarity of chickens themselves and/or may participate in the diversification of B cells by gene conversion. The role of the RAG-2 gene in gene conversion has been hypothesized but never clearly addressed *in vivo*.

Production of phenotype

An animal with a RAG protein "knock out" would present a simple phenotype which could be easily assayed for. Both RAG-1 (Mombaerts et al 1992) and RAG-2 (Shinkai et al 1992) deficient mice have been produced and both lack mature lymphocytes "owing to inability to initiate V(D)J rearrangement". This lack of lymphocyte phenotype provides a simple and quick method to determine whether the avian RAG-2 gene has been successfully "knocked out". Thus, it allows an easy way for testing the efficacity of using the RCAS system to somatically knock out RAG-2 from a chicken using a concatomerized antisense RAG-2 transcript.

Results

Cloning of the RAG-2 fragment concatomers into the RCAS vector

Cloning the RAG-2 fragment

A fragment of the first exon of the RAG-2 gene was chosen for the purpose of creating an antisense construct. Precedents in the literature have established the ends of genes as the favoured sites to direct antisense nucleic acids. Evidence would suggest that sites close to the translational start and the 3' untranslated region lead to a more efficient disruption of gene expression. This efficiency occurs because both the translational 5' and 3' ends are thought to be more accessible for the formation of RNA-DNA or RNA-RNA duplexes (Stein and Cheng 1993) therefore leading to the eventual disruption of translation.

Cloning of a fragment of the RAG-2 gene was carried out by amplifying the first 128 nucleotides of the RAG-2 open reading frame by performing polymerase chain reactions on total bursal DNA. The PCR fragment was cloned directly into the *In vitro*gen PCR II vector where its authenticity was

confirmed by southern blotting and hybridization to a radioactive RAG-5' primer (data not shown). The integrity of the whole fragment was confirmed by sequencing from both sides of the PCR II vector containing one copy of RAG-2 (POM-1).

Strategy for Concatomer production

Our next objective was to build exclusively "head to toe" concatomers of the RAG-2 fragment. The strategy developed for this purpose exploited the use of two restriction sites on either side of the RAG-2 fragment within the POM-1 polylinker. These two restriction sites yield compatible ends which may or may not reproduce the original restriction site. "Head to head" and "tail to tail" concatomers reproduced the Spe-1 or Xba-1 restriction sites enzymes rendering the DNA vulnerable to redigestion by these enzymes.

Enough Pom-1 was digested with a combination of Xba-1 and Spe-1 so as to isolate, after electrophoresis through 1% agarose, the small RAG-2 fragment. This became the source of DNA for 3 consecutive sets of reactions consisting of a 2 hour ligation, heat inactivation and 2 hour redigestion with a combination of Xba-1 and Spe-1. The final products were then electrophoresed across a 1% agarose gel yielding the ladder of DNA fragments which represent multiples in length of the original 128 nucletide fragment. The 1mer, 3mer and 9mer were then isolated from the gel and were cloned into the Xba 1 site of the Cla 12 adaptor plasmid polylinker. The Cla 12 adaptor was designed by hughes's lab to provide a polylinker flanked by Cla 1 sites allowing easier cloning of genes first in the adaptor and then into the unique Cla 1 site of the RCAS group of retroviral vectors.

Cloning of Concatomers into adaptor plasmid

The 1mer, 3mer and 9mer fragments were cloned into the Xba-1 sites of Cla 12 and confirmed through colony hybridizations (data not shown). A Cla1 diagnostic digest was used to confirm the insert length and that only one concatomer fragment had been cloned into the Xba-1 site. The Cla 1 fragments were then isolated and cloned into the unique Cla 1 site of RCAS(B).

Cloning of Concatomers into RCAS vector

Concatomer insert within RCAS was confirmed through colony hybridizations (data not shown) and hybridizations to the amplified insert. PCR primers homologous to either side of the cloning site of the RCAS-concatomers were used to amplify inserts which were electrophoresed, blotted and probed with RAG-2 5' primer. This confirmed, not only, the insert's size, but that only one copy of the insert had been cloned within the RCAS vector. Furthermore, orientation of the inserts were confirmed by two main diagnostic digests. The existence of a unique Sal 1 site approximately 800 nucleotides away from the Cla 1 site within RCAS and another Sal 1 site within the Cla 12 polylinker allowed for the determination of insert orientation, since the source of the insert (Cla12-concatomer) was clonal (Figure 11). Another restriction digest used to determine orientation was Pst 1 naturally found at the 5' end of the 128 nucleotide RAG-2 fragment. Since the source of the Cla 1 insert was clonal and a Pst 1 site was located 2000 nucleotides away from the Cla 1 cloning site within RCAS, the 5' end of all the RAG-2 concatomers could be oriented according to the RCAS Pst 1 site (Figure 11)

Viral Production *in vitro*

Sense and Antisense Producing RCAS vectors

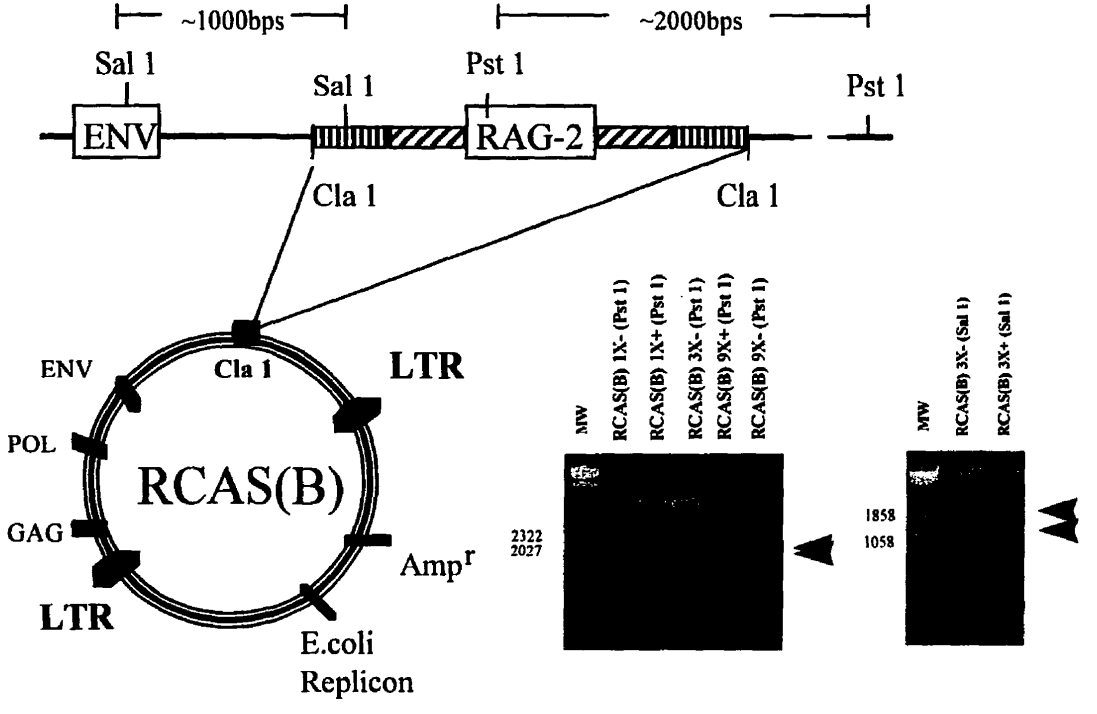


Figure 11. Diagnostic Sal 1 and Pst 1 digests determine RAG-2 Orientation Diagnostic digests using Pst 1 enzyme which cuts once in the five prime end of the RAG-2 gene and once downstream of the Cla 1 cloning site of RCAS(B) was used to determine orientation as shown in the first gel. Sal-1 digests were also used to determine orientation as depicted in the second gel. The arrows point to the DNA species released by the digests and schematically represented in the diagram above for the 1 mer RAG-2 gene cloned in the sense orientation relative to RCAS(B).

Production of virus from CEFs

The proviral constructs were then tested for their ability to permutate as viable virus from eukaryotic cells *in vitro*. RCAS-RAG DNA was transfected into CEFs grown in 100mm plates via the calcium-phosphate method. Production of virus was measured indirectly by assaying the supernatant for reverse transcriptase activity (Figure 12). As presented in the figure, reverse transcriptase activity increased from a value of 0 on the day of transfection (day 0) and reached saturation at about day 8 increasing minimally in most cases up to day 15. The ability of the virus produced to infect and spread *in vitro* was confirmed by collecting supernatant (devoid of cells) from transfected cells positive for reverse transcriptase activity in the supernatant and infecting other CEFs with the supernatant and checking for reverse transcriptase activity and viral protein production (data not shown).

Viral Dissemination and gene expression *in vivo*

Gene expression *in vivo*

RCAS-CAT producing chickens were produced in parallel with the RAG-2 transgenics and were used as a control for the expression of the CAT protein from the RCAS virus *in vivo*. CAT assays revealed CAT activity from lysates of both red blood cells and leukocytes isolated from CAT transgenics, but not from blood cells isolated from normal age-matched chickens (data not shown). Lymphoid tissues isolated from sacrificed RCAS-CAT transgenics were also assayed for CAT activity (figure 13). Levels of CAT activity were standardized according to protein sample concentrations and levels of CAT enzyme activity. The amount of CAT activity found within each organ varied according to the organ, however all organs tested displayed at least some CAT activity. This data confirms

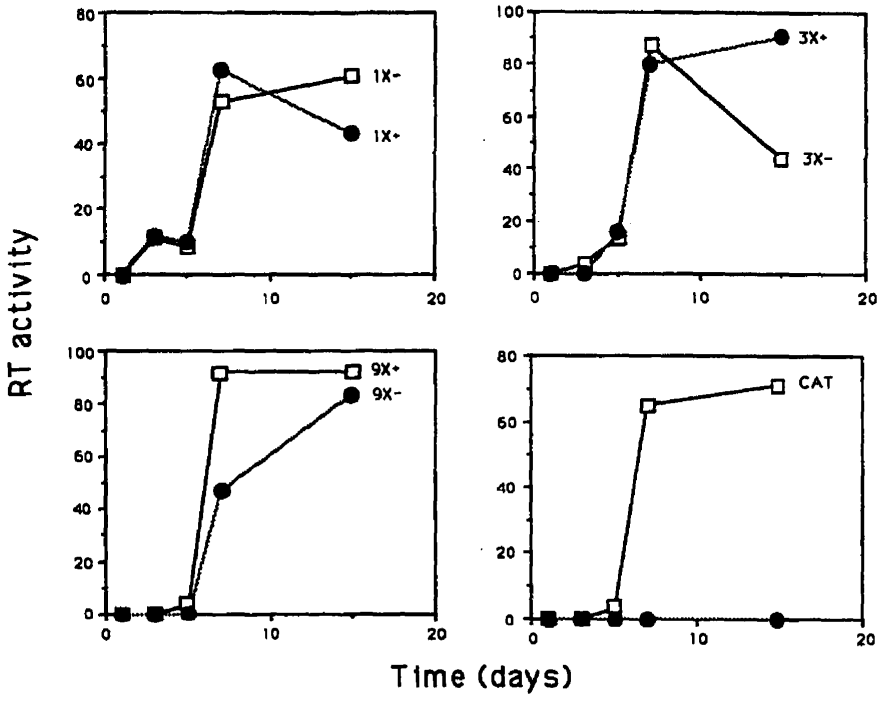


Fig.12 Reverse Transcriptase assays. CEFs were transfected with RCAS vectors containing RAG-2 concatomers (1X designates RCAS containing 1 mer RAG-2 fragment and the - or + sign represents antisense or sense insert orientations respectively). Supernatant from the transfected CEFs was taken from days 1, 3, 5, 8, 15 and then assayed for reverse transcriptase activity.

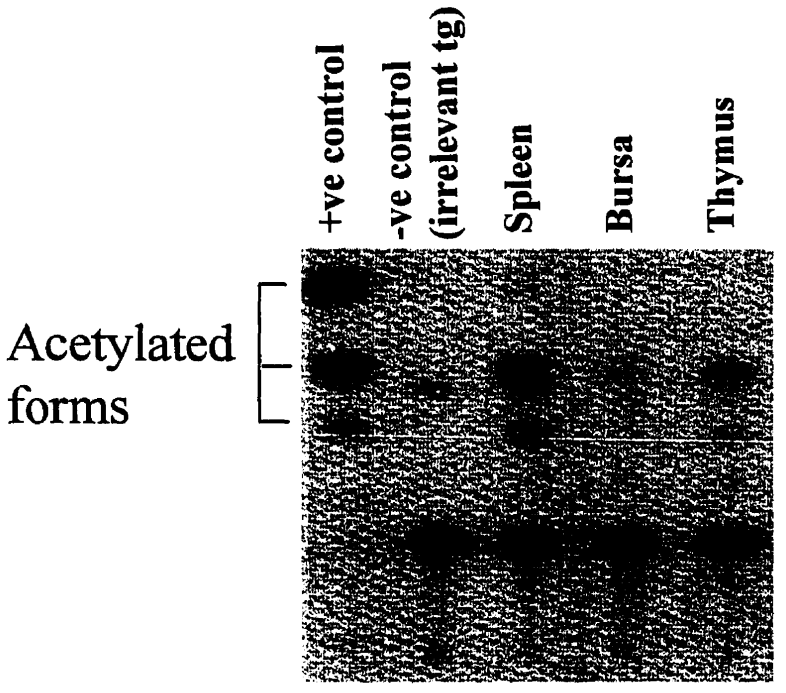


Fig13. CAT assays performed on lymphoid tissue of CAT transgenic. Lymphoid tissue was harvested from the RCAS-CAT transgenic (tg) as the source for several CAT assays demonstrated in the above phosphorimage. Source of the negative control was an RCAS-RAG-2 transgenic. Acetylated forms of chloramphenical reveal the presence of the CAT enzyme.

that the transgenisis protocal utilized, produced somatically transgenic chickens that were capable of widespread expression of a cloned gene from the unique cloning site of the RCAS vector.

Viral dissemination in chicken blood

Somatically transgenic chickens produced through the dissemination of RCAS virus following injection into day 3 embryos were characteristically different in several respects to normal age-matched chickens. Transgenics were typically 82% the body weight of normal age-matched chickens (data not shown). Although lymphoid organ size displayed heterogeneity, no real distinction between transgenic and normal chickens was apparent. Approximately, one third of transgenics eventually succumbed to complications from having the replication competent RCAS virus, a derivative of avian leukosis virus within their system. The major complications as assessed from a cursory observation of the internal organs after termination following malidity, seemed to stem from the production of both solid and leukemia type cancers. The majority of complications arose about 2 months post-hatch coincidentally the time required for a retrovirus with strong LTRs and without an viral oncogene to produce a neoplasia.

The dissemination of virus was revealed by processing blood taken from the normal, control and 9X transgenic chickens. PBLs were isolated, permeabilized and stained for the production of the retroviral GAG protein p19 (Figure 14). Levels of p19 protein were at levels detectable by flow cytometry only when PBLs from transgenics and not when PBLs of normal age/strain-matched chickens were analyzed. Not only were the majority of PBLs from transgenics positive for the production of this protein, but the protein itself was expressed at high levels. Reverse transcriptase assays

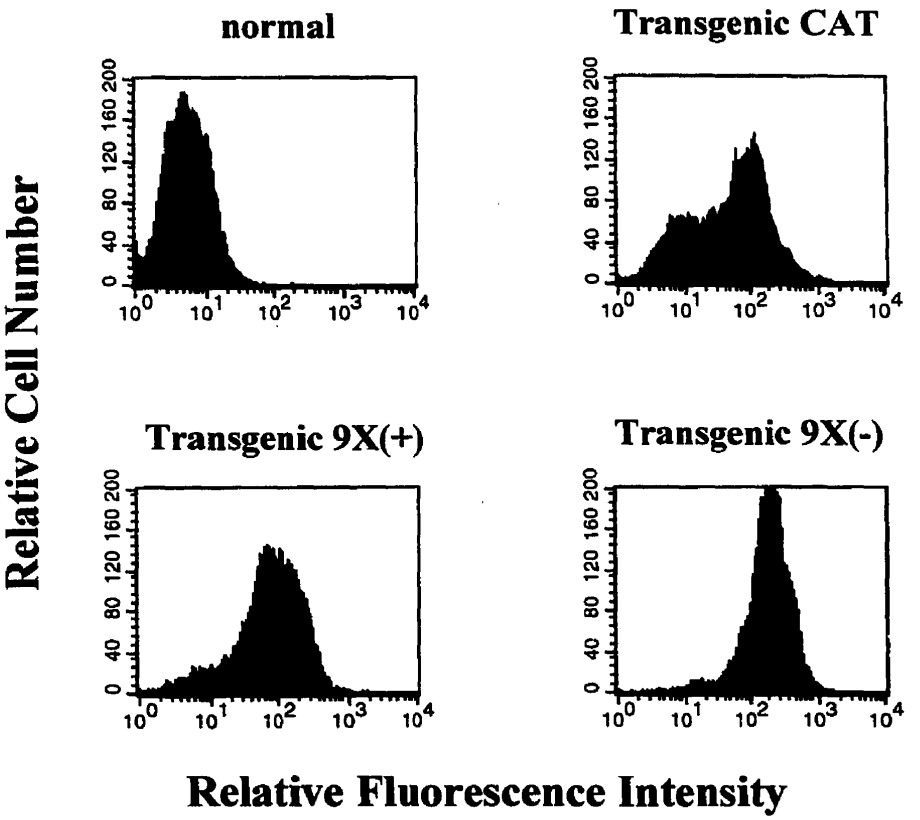


Fig.14 FACs profiles of PBLs stained for the ALV GAG p19 nucleocapsid protein. PBLs were isolated from age-matched normal control chickens, RCAS-CAT transgenics, RCAS-9X(+) and RCAS-9X(+) transgenics. The permeabilized cells were assayed for the presence of viral capsid protein indicative of ALV production.

from *ex vivo* samples were attempted using either serum or cell extracts. However, all assays developed negative results.

Lymphocyte levels

Tissue lymphocyte levels were analyzed by flow cytometry. Transgenics were sacrificed, tissues harvested, lymphocytes isolated and T and B cells stained with anti-CD3 and anti-immunoglobulin light chain antibodies respectively. The relative numbers and proportions of the different lymphocytes were found to be no different when comparing B and T cell levels from lymphoid tissue isolated from sense (Figure 15) and anti-sense (Figure 16) producing RAG-2 transgenics. This observation was also extended to peripheral blood lymphocytes when sense and anti-sense RAG-2 transgenic blood T and B populations were compared (Figure 17). Thus, a general distinction between the two different sense and antisense producing transgenic populations could not be found.

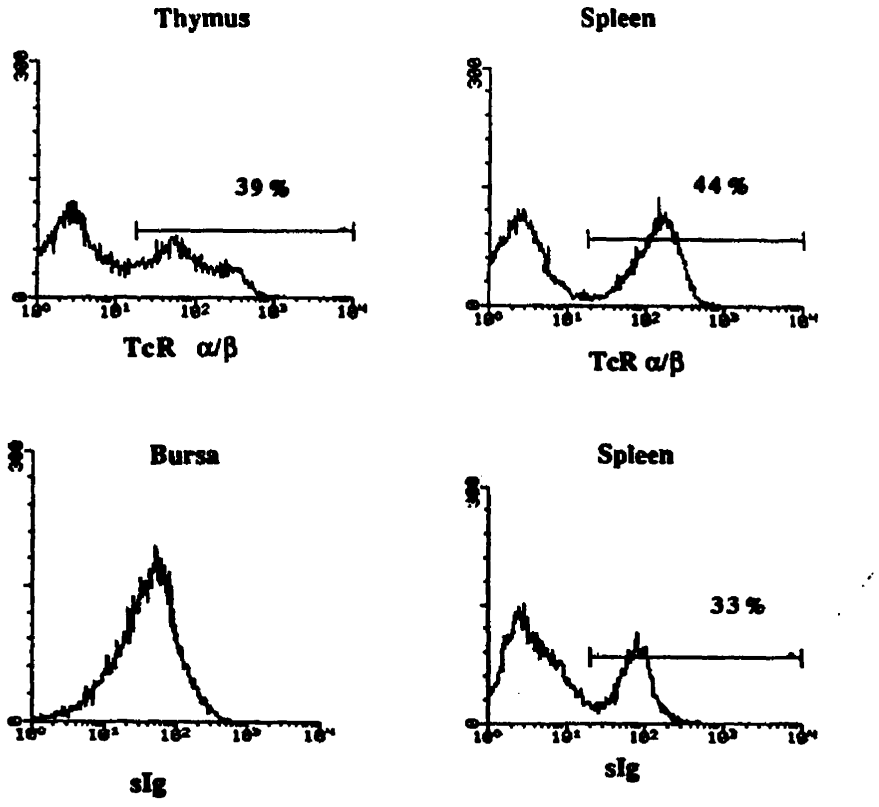


Fig.15 B and T cell percentages in various organs of a representative transgenic control. Leukocytes isolated from various tissues were stained for the presence of T cells (anti-T cell receptor) and B cells (anti-surface immunoglobulin). The samples were then acquired and analyzed on a FACscan from which percentages of T and B cells were determined.

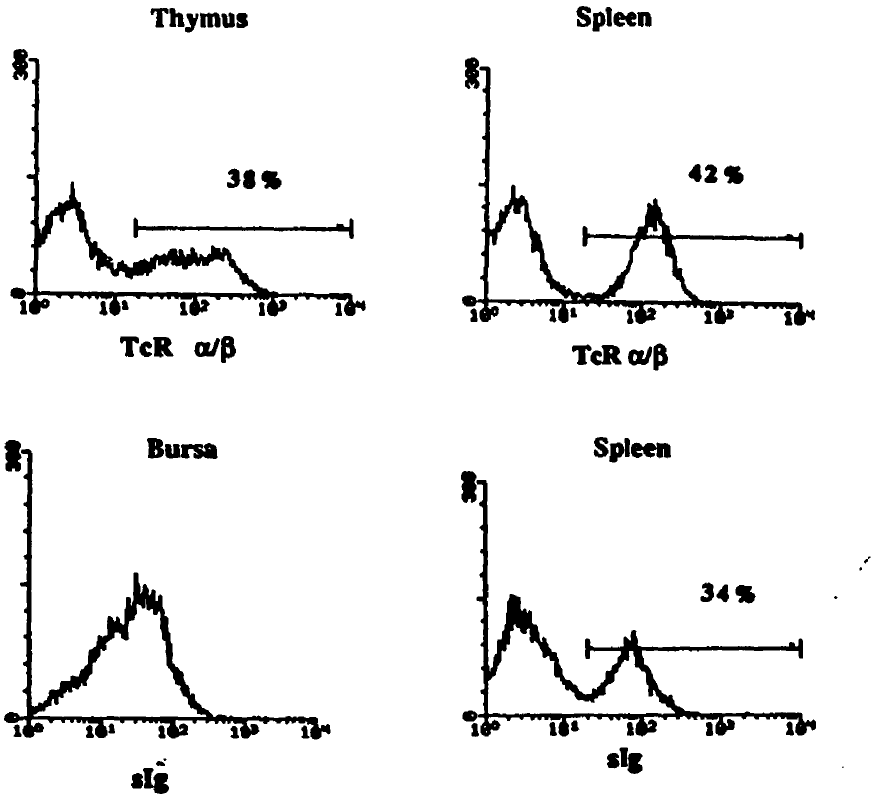


Fig.16 B and T cell percentages in various organs of a representative antisense transgenic. Leukocytes isolated from various tissues were stained for the presence of T cells (anti-T cell receptor) and B cells (anti-surface immunoglobulin). The samples were then acquired and analyzed on a FACscan from which percentages of T and B cells were determined.

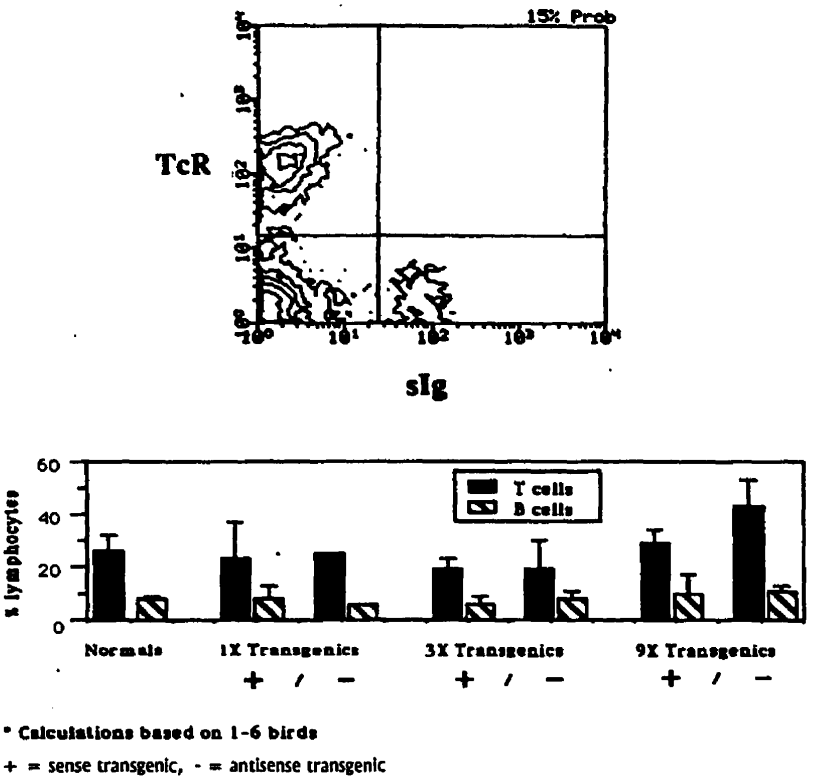


Fig.17 Percentage of blood T and B cells in transgenic chickens. Leukocytes were isolated from the blood of various transgenic chickens. Percentages of blood T and B cells were determined by staining leukocytes for the T-cell rceptor and surface immunoglobulin respectively and then analyzing on a FACscan.

Discussion

Cloned genes within the RCAS system of vectors are expressed both *in vivo* and *in vitro*

CAT activity *in vivo*

The RCAS system of viral vectors have been used to express genes of interest in a wide variety of avian cells. We have used this system of replication competent retroviruses in an *in vivo* situation. Avian embryos infected with these vectors disseminate throughout the infected individual, producing chimeric chickens in which the infected cells express the gene of interest. In order to test the system, a reporter gene (CAT) was cloned into the RCAS vector. Avian embryos infected with RCAS-CAT vector were later (after hatching) sacrificed and tissue lysates tested for the presence of CAT activity.

The RCAS-CAT viral vector released into the day 3 developing embryo spread throughout the infected individual. CAT activity was evident in the lymphoid organs and PBLs tested from a sacrificed individual infected with RCAS-CAT (Figure 13) while no CAT activity was seen from organs originating from normal and other transgenic chickens (data not shown). This foreign genetic material cloned into the cla 1 cloning site was expressed *in vivo,* and in those organs which were to be targeted for the disruption of RAG-2 gene expression (lymphoid organs).

The heterogeneity of gene expression *in vivo*

There is some heterogeneity in the observed protein levels of viral p19 GAG expressed by PBLs of infected chickens (Figure 14). The PBLs displaying this heterogeneity are comprised of population of cells with different phenotypes. These phenotypes include 1) cells not expressing the viral protein (presumably uninfected cells) and 2) cells expressing varying levels of the protein. This cellular heterogeneity may reflect the state of infection of that cell (for example early infection with low viral production capacity), and/or may reflect tissue-specific efficiencies with respect to viral protein production.

The mechanism of *in vivo* gene transfer by the RCAS system

The CAT activity present in lysates from various dispersed tissues of the RCAS-CAT infected individual is most probably a reflection of viral dissemination into the organs themselves through both the constant release of virus leading to the infection of as of yet uninfected cells and the proliferation of infected cells rather than just the expansion of the initially infected cells. Evidence for this method of transmission would have been given by the presence of reverse transcriptase activity from the serum of the infected individual, which was not observed (data not shown). However, it remains to be determined whether the lack of reverse transcriptase in infected chicken serum and cellular lysates is due to a lack of viral production, viral neutralization by antibodies or because of assay insensitivity. The reverse transcriptase assay used was not the method of choice for sensitivity. This protocol was chosen for processing large numbers of samples quickly and easily from the supernatant of infected cells *in vitro* where virus is allowed to accumulate. It is possible that virus levels in the blood stream and within the heterogeneically infected PBL population would be too low to be detected.

Assumptions with respect to the mechanism of RCAS-mediated gene transfer

The mechanism of gene transfer by the RCAS system in an infected individual would lead to the proposition of several assumptions: 1) Not all cells will express the gene of interest early in their life. This is supported by the fact that there appears to be some heterogeneity in the numbers of viral protein p19 expressing PBLs (Fig. 14), and the observed heterogeneity of bcl-2(human) positive bursal cells from young RCAS-BCL-2(human) infected chickens (Jacobsen et al 1996). 2) The numbers of virally infected cells in an individual increases with age. This would be a logical assumption because A) some infected hematopoeitic cells will continue to divide thereby spreading the virus and B) the viral vectors are replication competent. 3) Later in the infected chickens life, the amount of virally infectable cells will decrease as the amount of cells infected will plateau. This means that the numbers of cells presumably expressing the gene of interest will reach maximal levels at some point in the infected chicken's life. However, it is important to note that although points 2 and 3 are logical assumptions, it is very likely that they depend on the transgene in question.

RAG-2 sense and antisense message expression *in vivo*

Also tested, was the ability of these vectors to be used as vehicles for the purpose of disrupting the expression of the RAG-2 gene through the use of vector derived antisense transcripts.

Although there is no direct data demonstrating transcript production from the antisense and sense constructs, it is likely that these transcripts would have been produced since 1) CAT activity was present in different tissue preparations of RCAS-CAT infected chickens (S/C Hyline, the same

chickens used for infection by the RAG-2 sense and antisense producing virus), 2) The RCAS-CAT vector which was shown to produce CAT protein from various tissue was prepared in the same manner and the same time as the RAG-2 fragment producing vectors and 3) RAG-2 sense and antisense vectors produced viable virus *in vitro* and *in vivo* as demonstrated by A) reverse transcriptase activity in the supernatant of infected fibroblasts (Fig. 12) and B) viral envelope protein production in the PBLs of infected chickens (Fig 14). Therefore, it is highly likely, given the expression of viral vector proteins, *in vivo* that the sense and antisense RAG-2 messages were transcribed.

The parental ALV from which the RCAS system was constructed is known to generate a certain ratio of spliced and full length viral transcripts. The RCAS vectors produce full length virus as well as two other spliced products. The first spliced product is responsible for the transcription of the genes downstream of the GAG region which includes the ENV region. The second splice product encodes the gene of interest.

Embryonic expression of RAG-1 and RAG-2 proteins

The recombination activating genes 1 and 2 are required for the productive rearrangement of the B cell and T cell immune receptors. Mice deficient in these proteins are unable to generate mature T and B cells. Chickens also express RAG-1 and RAG-2 in locations known to be conducive for the rearrangement of the T and B cell receptors, T cell receptor rearrangement takes place in the thymus among the immature double positive and double negative populations (Pickel et al 1993). B cell receptor rearrangement takes place as early as day 5-6 of embryogenisis within the yolk sac (Reynaud et al 1992) and continues for only a limited period of time prior to hatching.

Immune receptor expression in RAG-2 sense and antisense somatic transgenic chickens

Fluorescence sorting was used to determine the levels of B and T cells from the somatically transgenic chickens generated. The antibodies that were used to separate immune T and B cells were those recognizing the T and B cell receptors respectively. Thus, the FACS analysis performed assayed directly for the presence of the T and B cell receptors. The presence of immune receptors on the cell surface of immune cells strongly suggest that the receptors had been productively rearranged. The presence of normal levels of immune cells with productive receptors (Fig. 15,16,17) in the RAG-2 sense and antisense producing transgenic chickens suggest that the expression of the RAG-2 gene was not fully disrupted. There remain too many variables to establish the exact reason for why RAG-2 expression was not fully disrupted.

Immune receptor rearrangement begins as early as day 5-6 of embryogenisis for B cells and although eggs were injected at day 3 it is possible, even if the virally infected antisense producing cells had RAG-2 expression disrupted, that some of the B cell progenitors escaped viral infection. This pool of progenitor B cells would then be able to expand and seed the bursa of fabricius leading to further differentiation of the B cells. The same argument may also be made for pre-T cells which express the RAG genes as early as day 14 of embryogenisis. It may also be argued that RAG-2 gene expression was not disturbed or that the amount of transcripts disrupted by the antisense RNA did not lead to the decrease in the levels of RAG-2 protein required for interfering with the rearrangement process. The levels of antisense transcript is crucial for the efficiency of transcript disrupted. If the levels of antisense RNA was not sufficient this may be a reflection of a weaker than necessary promoter or inefficiencies of

promoter activity because of the tissue infected. It may be that the levels or types of transactivating factors required for good promoter transactivation were inadequate in the lymphocyte populations targeted. Indeed the heterogeneity observed in the levels of viral protein within the PBL population provides evidence but not proof that cell specific factors were contributing to the efficiency of viral production.

Crucial to the development of a vector which may be used for antisense purposes is the ability of the promoter to express high levels of antisense DNA. However, the RCAS system has the disadvantage of dividing the transcriptional power of the promoter between the whole virus and two other splice accepted areas. The division of promoter power between the transcripts leads to an overall decrease in the levels of functional antisense transcripts and lessens the efficiency of the reaction.

Introduction

Regulated Gene Expression

A protein's function may be partially elucidated by understanding the regulation of its expression. For example, proteins that play a role in embryogenesis are expressed, although not always exclusively, during embryo development. By the same token, the role of a protein may be better understood by observing the consequences of its inappropriate expression under carefully controlled circumstances. To continue from the example above, one can imagine that when a regulatory protein, thought to be involved in lung development is expressed, at an inappropriately early time in embryogenisis, could lead to the premature development of this organ. The forced early expression of the regulatory protein, if correlated with the inappropriately early development of lung features would confirm its involvement in this organ's development. Thus, artificially controlling the expression of certain genes at times or under circumstances where it would not usually be expressed may further clarify the role of that protein in such complex systems.

The Tetracycline Responsive System

Protein expression of genes cloned adjacent to a set of tetracycline operator sequences may be controlled by the tTA transactivator protein. Constitutive tTA protein expression in the absence of tetracycline allows tTA binding of the tetracycline operators. Once bound, the tTA protein mediates the transcriptional activation of genes cloned downstream of a minimal promoter. The transactivation of the tTA is provided by the strong transactivation domain of the herpes VP16 gene. However, in the

presence of tetracycline, gene expression is inhibited. Exogenous tetracycline binds to and inhibits the action of the tTA protein. In this fashion, gene expression may be controlled by the tetracycline system.

Merging Tetracycline responsiveness with the RCAS system

Various promoters, successfully introduced into the RCAS system of viruses, have been used to manipulate the expression of inserted genes at the cloning site. Both the mouse metallothionein 1 and chicken b-actin promoters linked to the CAT gene have been inserted into RCAN vectors (RCAS vectors that lack an upstream splice acceptor from the cloning site). The expression of the CAT gene was observed when the chicken β-actin promoter-CAT constructs were inserted into the RCAN viruses in either the sense or antisense orientation arguing that at least some expression was being driven by the inserted promoter (Petropoulos et al 1991). Expression of the CAT protein could also be induced upon the addition of exogenous $ZnSO_4$ to CEFs infected with the mouse metallothionein 1 promoter CAT constructs, proving that exogenous promoters may be introduced into the RCAS system of viruses in order to more finely control the expression of cloned genes.

Transactivation in the tetracycline responsive system is carried out by a specific transacting DNA element (tTA) which then acts on a specific set of tetracycline operators, therefore the system depends on the actions of two separate and distinct DNA elements. These two DNA elements must therefore be introduced into the same cell in order for tetracycline sensitive transactivation to occur. The RCAS system of viruses allow the superinfection of cells with viruses which harbour different ENV regions so long as the target cell expresses both receptors. The ability of infecting a common cell allows the introduction of more than one DNA element per cell. One may therefore envisage a cell being infected by one virus of

envelope A constitutively expressing tTA and by another virus of envelope B harbouring the DNA elements necessary for tTA controlled expression of a cloned gene (Figure 18). As illustrated, the addition of tetracycline will silence transcription from the tetracycline promoter complex contained within the viral vector. Transcription of the cloned gene will cease because genes cloned in the reverse orientation relative to the vector's first LTR, responsible for the bulk of vector mediated gene transcription, are poorly transcribed and the RCAN virus does not contain a splice acceptor immediately upstream of the cloning site. Consequently, inserted genes (in either orientation) are not expressed from the promoter found in the viral 5' LTR. The insertion of the promoter-CAT complex was inserted into the viral vector in the reverse orientation to avoid the potential of any interference being caused by the cloned poly A signal on the transcription of the full length virus.

Control Plasmids

A set of control plasmids were included in many of the transactivation assays performed. These include pREP4tTA, pCMVtCAT, Cla 12-CAT, and *RCAN(B)CAT. The first two plasmids (provided by Allan Cochrane) have been used in COS-7 cells to demonstrate tetracycline regulated CAT expression. pREP4tTA uses the RSV LTR to drive the transcription of the tTA protein, while pCMVtCAT (a variation of pRCMVtH6REV, which has the HIV REV protein under the control of the tetracycline promoter element instead of CAT) has CAT expression under the control of the tetracycline promoter element. At the end of each insert of both pREP4tTA and pCMVtCAT plasmids there exists a poly A signal sequence. *RCAN(B)CAT and Cla 12-CAT both contain a tetracycline promoter element-CAT gene-β-globin poly A signal construct inserted at the cloning site of RCAN(B) in the sense orientation and within the polylinker of the Cla 12 adapter plasmid respectively. Both vectors were constructed as

Tetracycline-Responsive System

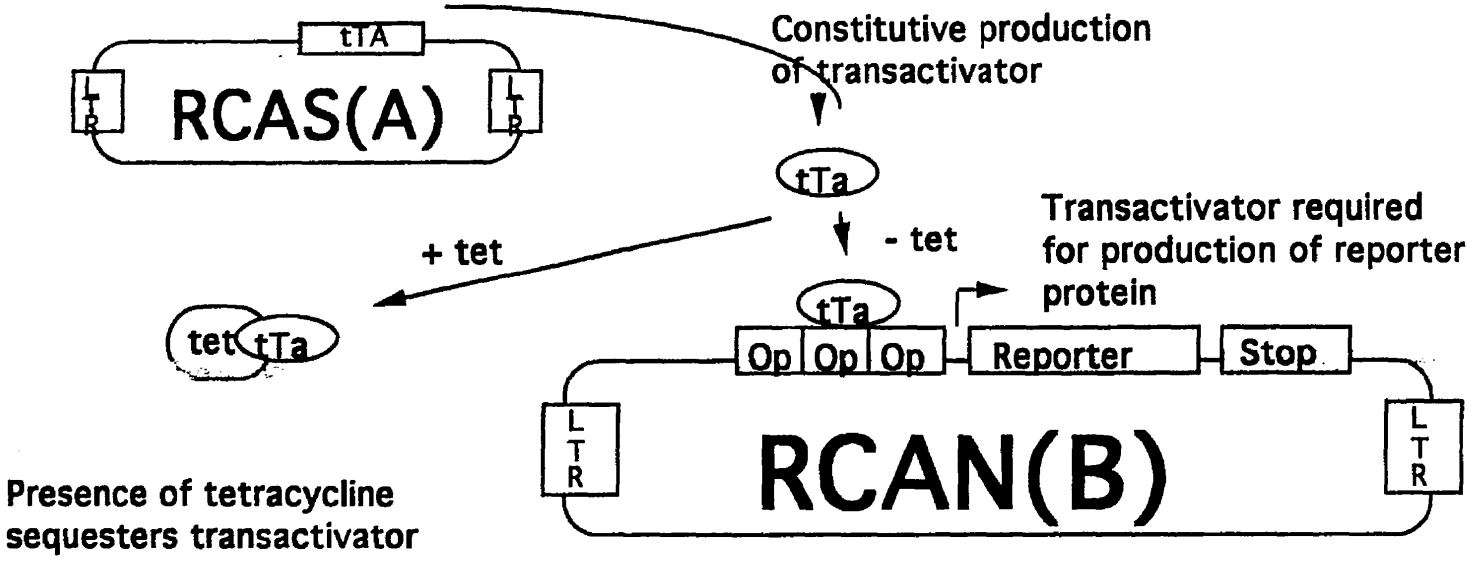


Figure 18 Merging of the Tetracycline Responsive System and the RCAS Retroviral Group The tTA transactivator cloned into the RCAS(A) vector is produced constitutively and mediates the transactivation of the reporter gene from the tetracycline operator sites. Addition of exogenous tetracycline, sequesters tTA effectively inhibiting gene trans-activation.

intermediates in the development of RCAN(B)CAT which contains the same tetracycline promoter element-CAT gene-b-globin poly A signal inserted within the RCAN(B) cloning site in the antisense orientation relative to the 5′ LTR.

Results

Cloning the tetracycline-responsive transactivator into RCAS(A)

The tetracycline responsive transactivator (tTA) was first isolated by digesting a Bluescript (Stratagene) plasmid containing the tTA (kindly provided by Allan Cochrane) with Eco R1 and Bam H1, isolating the 1 kb insert and subsequently cloning it into the Eco R1 and Bam H1 site of the Cla 12 adaptor plasmid. Positive colonies were identified, plasmids extracted, Cla 1 fragments were isolated and cloned into the RCAS(A) provirus (data not shown) . Transformed colonies were "lifted" onto membranes and subsequent probing with radioactive tTA fragments allowed identification of colonies harbouring provirus with the appropriate insert while digestion of the provirus revealed appropriate orientation and its single insertion. (Figure 19). Sequence analyses from the proviral 5' end of the unique Cla 1 site confirmed the orientation of the insert. (Figure 19)

Cloning the Responsive elements and Reporter gene into RCAN(B)

Cloning of the tetracycline-responsive elements into Cla 12

The tetracycline responsive element was isolated from the pUHC-13-3 plasmid (kindly given by Allan Cochrane, Gossen and Bujard, 1992) using an Eco R1 digest. The Eco R1 fragment was then cloned into the Eco R1 site of the Cla 12 polylinker. (Figure 20) Sal 1 digests demonstrated nonconcatomerized inserts that were revealed by Cla 1 digests. (Figure 20)

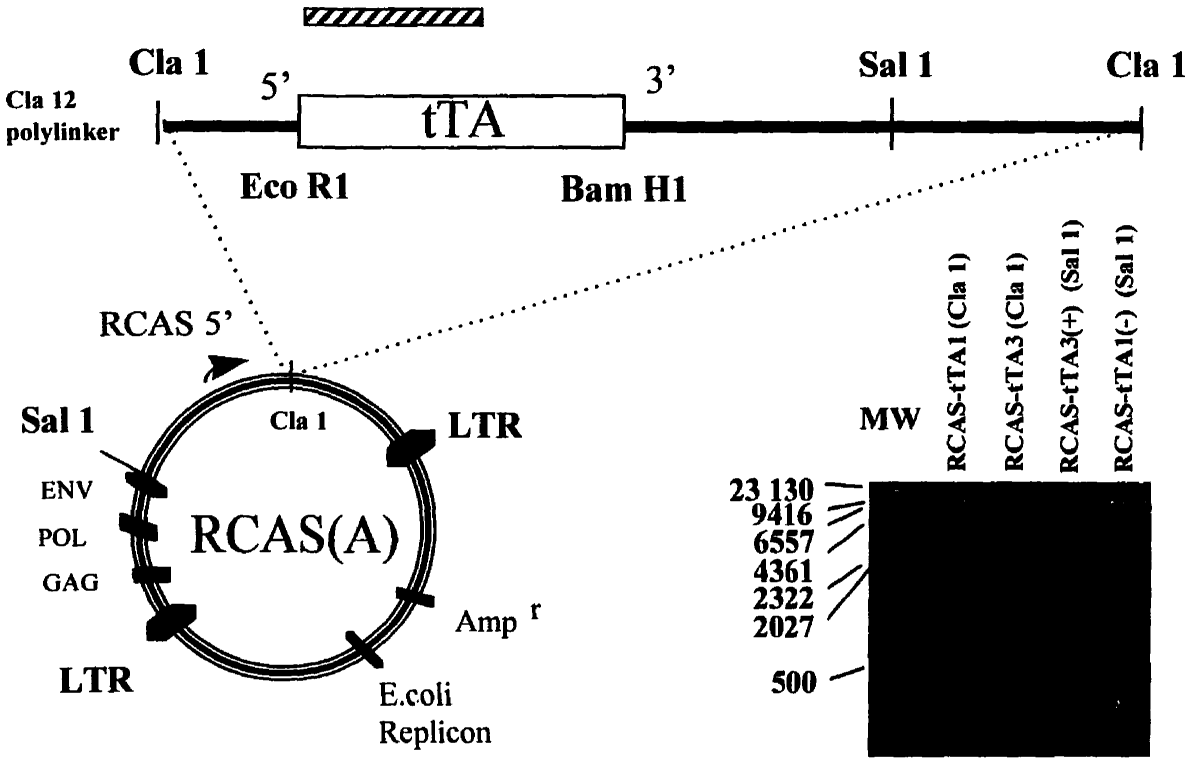


Fig. 19 Cloning the Tetracycline responsive transactivator into RCAS(A). The transactivator cloned into the Eco R1, Bam H1 site of the Cla 12 plasmid was isolated as a Cla 1 fragment that was subsequently cloned into RCAS(A). Cla 1 digestion (see lanes 1,2 on gel) released the ~1000 nucleotide tTA fragment from two positive clones identified through clony hybridizations. A Sal 1 diagnostic digest revealed that one clone had the tTA fragment in the wrong direction (RCAS-tTA1(-) (Sal 1) and that both clones contained only one copy of the tTA fragment. The hatched bar above the schematic of the Cla 12 polylinker reveals that part of the provirus sequenced using the RCAS 5' primer.

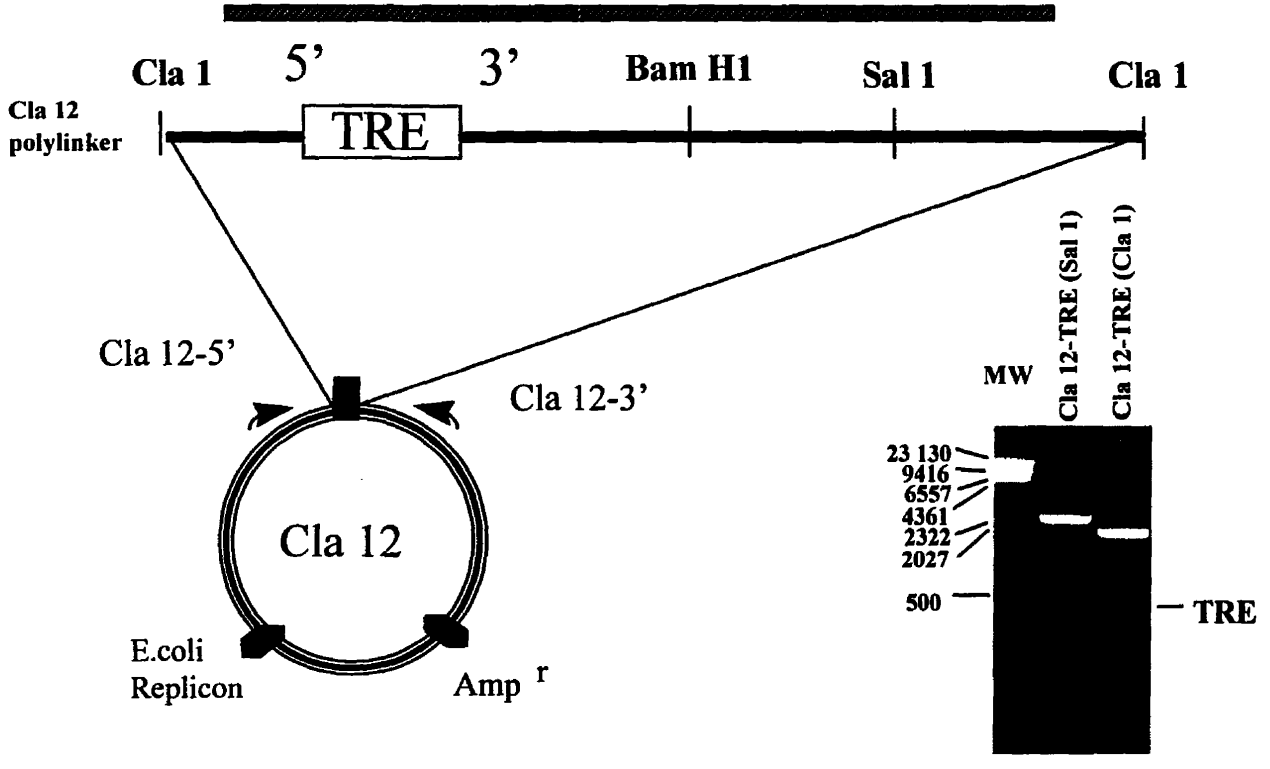


Fig.20 Cloning of the TRE element into Cla 12 adaptor plasmid. The TRE element, isolated from the pUHC-13-3 plasmid as an Eco R1 fragment, was cloned into the Eco R1 site of the Cla 12 polylinker. The gel on the right demonstrates Cla 12-TRE digested either (lane 1) with Sal 1 (cutting it once) or (lane 2) with Cla 1 releasing the ~480 base pair TRE fragment. The hatched bar above the schematic of the Cla 12 polylinker depicts the area of the plasmid that was fully sequenced using the Cla 12-5' and Cla 12-3' sequencing primers.

Sequencing confirmed the orientation as well as the integrity of the whole tetracycline responsive element within Cla 12.

Cloning of the poly A stop signal into Cla 12-TRE

The β-globin poly A signal (kindly provided by Nicholas Acheson, Lanoix and Acheson, 1988) was digested out of pGEM 3z f(-) as a Sal 1 fragment. This was cloned into the Cla 12-TRE plasmid downstream of the TRE element into the Sal 1 site. After confirmation of insert positive colonies using a nick translated poly A radioactive probe, several extracted plasmids were subjected to sequencing confirming the orientation of a singly inserted poly A signal. (Figure 21)

Cloning of the CAT reporter gene into Cla 12-TRE-poly A

The CAT gene was isolated from RCAS(A)-CAT-3 (kindly provided by Stephen Hughes, Hughes et al 1987) as a Cla 1 fragment. This fragment was subsequently digested by Sau IIIA (Figure 22) and cloned into the compatible Bam H1 site of the Cla 12-TRE-Poly A polylinker. Colonies possessing Cla 12 with CAT insert were positively identified by hybridization to nick translated radioactive CAT probes (data not shown). A Cla 1 digest was diagnostic for concatomerization of the insert (Figure 22). Sequencing from the 3' end of the Cla 12 adapter was used to determine orientation of the insert.

Cloning the Cla 12-TRE-CAT-polyA element into RCAN(B)

The Cla 1 fragment from the Cla 12-TRE-CAT-PolyA plasmid was cloned into the unique Cla 1 site of RCAN(B). Colony lifts were positively identified by hybridizing to radioactive nick translated Cla 1 fragments from Cla 12-TRE-CAT-Poly A plasmid (data not shown). Positive clones

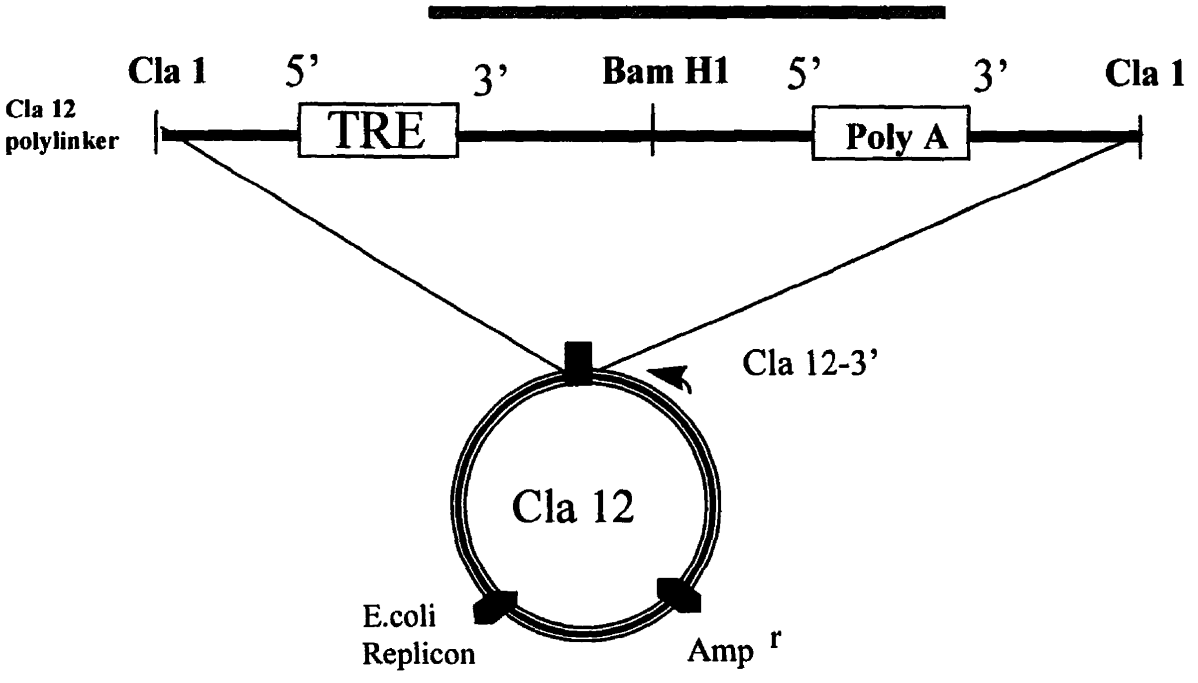


Fig.21 Cloning of the poly A stop signal into Cla 12- TRE plasmid. The rabbit β-globin poly A signal , isolated from the pGEM 3z f(-) as a ~100 nucleotide Sal-1 fragment, was cloned into the Sal 1 site of the Cla 12-TRE polylinker. Clones were positively identified through colony hybridizations and sequenced. The hatched bar above the schematic of the Cla 12-TRE polylinker depicts the area of the plasmid that was fully sequenced using the Cla 12-3' primer.

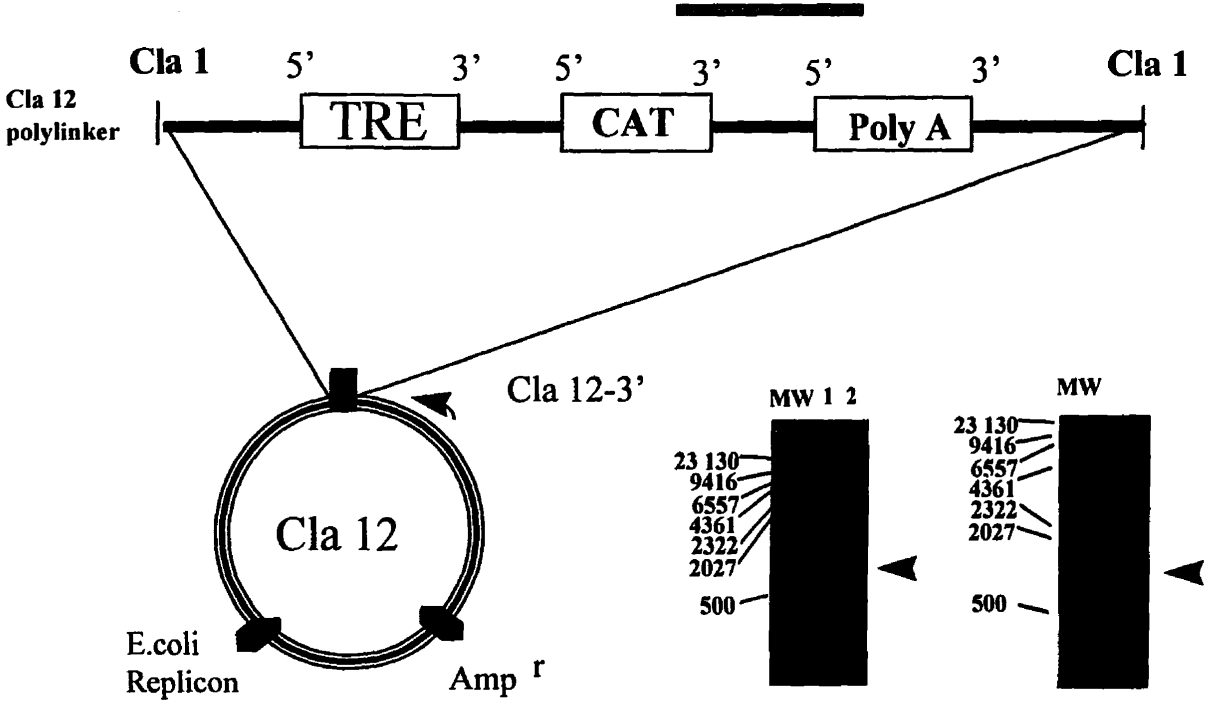


Fig.22 Cloning the CAT gene into Cla 12-TRE-Poly Aadaptor. RCAS(A)-CAT-3 was digested with Cla-1 (gel 1, lane 1) releasing the CAT insert (arrow). This Cla 1 fragment was further digested with Sau IIIA (lane 2) and cloned into the Bam H1 site of the Cla 12-TRE-Poly A plasmid. Two Cla 12-TRE-CAT-Poly A clones digested with Cla-1 (gel 2) released the ~1100 nucleotide TRE-CAT-Poly A fragment (arrow). The hatched bar above the schematic of the Cla 12-TRE-CAT-Poly A polylinker depicts the area of the plasmid that was fully sequenced using the Cla 12-3'.

were then subjected to restriction analyses (Figure 23). Cla 1 digests confirmed insert length and Sal 1 digests demonstrated no concatomerization of the antisense oriented insert. Further analyses by sequencing confirmed orientation of the insert (Figure 23).

CAT assays *in vitro*

CAT protein levels are readily seen in COS-7 and CEF cells

The tetracycline responsive system's ability to function in avian cells and within the context of the RCAS system was tested using a series of control experiments. A series of transient transactivation assays using the CAT enzyme as the reporter gene were performed (Figure 24) in avian CEFs as well as control cells (monkey COS-7 cells). In Panel A both CEFs and COS-7 cells are transfected while in Panel B extra control plasmids are transfected into only CEFs. The control plasmids pReptTA, which produces the transactivator and pCMVtCAT to which the transactivator binds to have already been shown to induce chloramphenical acetylation, an indirect measure of CAT protein in COS-7 cells (personal communication Sandra Iacampo). This was confirmed in Panel A where transfected COS-7 and CEF cell lysates contained CAT protein levels above negative control levels. The positive control vector in CEFs (RCAS-CAT) also yielded CAT protein levels above the negative control plasmids (Panel A and B). The plasmids RCAN(B)CAT, pCMVtCAT and Cla 12-CAT all require transactivation by tTA in order to produce a substantial amount of CAT protein therefore introduction of these plasmids by themselves into the appropriate cells produced very low or undetectable levels of CAT protein.

The tetracycline system is responsive in avian cells

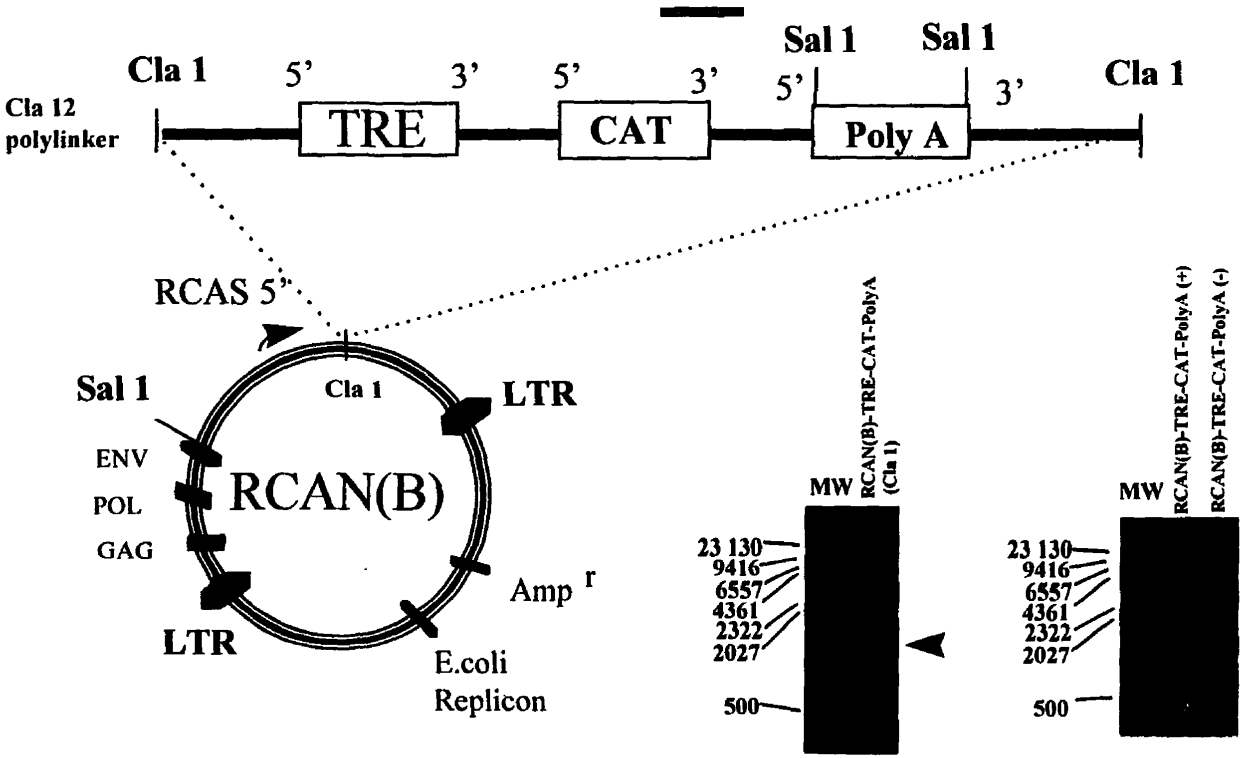


Fig.23 Cloning the TRE-CAT-PolyA construct into RCAN(B). The Cla 1 fragment from the Cla 12-TRE-CAT-Poly A plasmid was cloned into the Cla 1 site of the RCAN(B) provirus. Cla 1 digestion (gel 1) of RCAN(B)-TRE-CAT-PolyA released the ~1200 nucleotide long Cla 1 fragment (arrow) containing TRE (470 nts), CAT (640 nts) and poly A (107nts). Digestion with Sal 1 (gel 2) confirmed direction of positive clones and demonstrated no concatemerizations. Direction was also confirmed through sequencing (hatched bar) using the RCAS 5' primer.

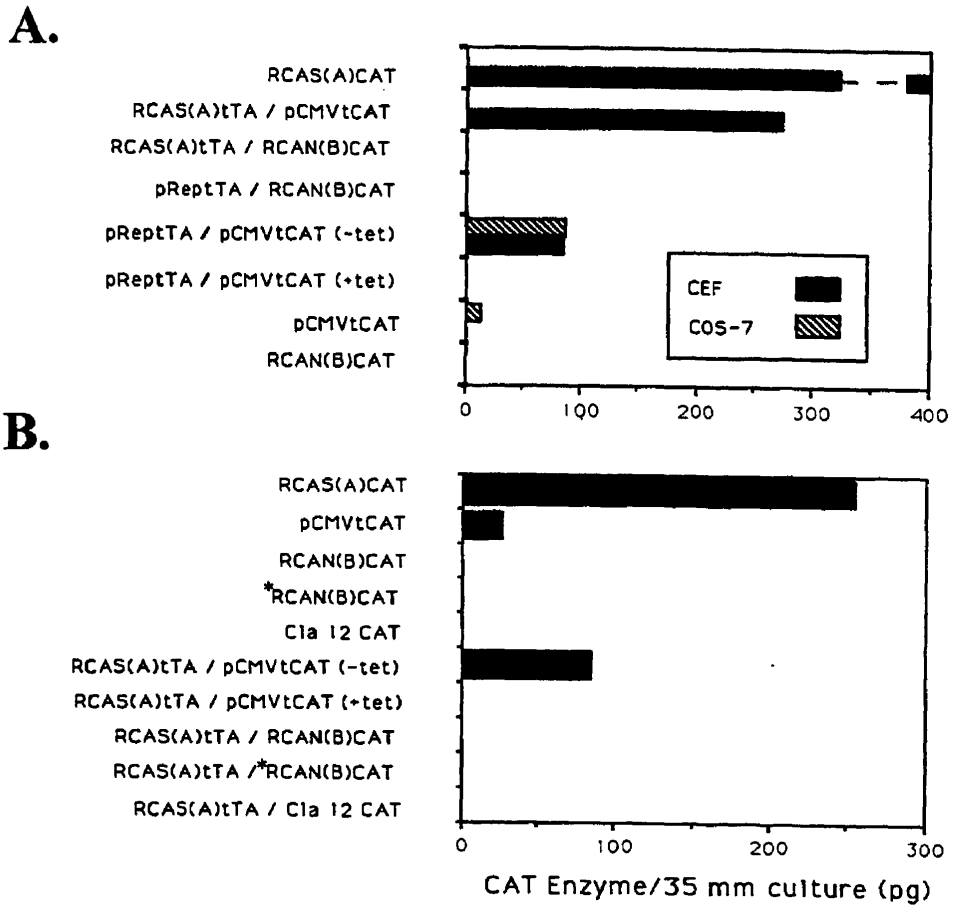


Figure 24. CAT Elisa assays. CAT elisa assays were performed on 35 mm plate cultures of either CEFs and COS-7 cells (A) or CEFs (B) using either 2.5micrograms (A) or 1.5micrograms (B) of total DNA per transfection per well. The column on the left hand side describes the combinations of plasmid and provirus transfected and the graphs depict the amount of CAT protein per well 48 hours post-lipofectamine-transfection with or without tetracycline treatment. (10 micrograms/ml for the last 24 hours)

Panel A demonstrates that the tTA transactivator produced by pReptTA is responsive to tetracycline levels in COS-7 cells. In the presence of tetracycline, CAT protein levels are equivalent to the negative control, confirming the sequestration of the transactivator by tetracycline. We can also presume that the tTA transactivator was being produced from the RCAS(A)tTA vector in CEFs (Panel A and B). The transactivator from RCAS(A)tTA was also functional in CEFs, since, in combination with the control pCMVtCAT plasmid (Panel A and B) CAT protein was expressed above the levels expressed by pCMVtCAT alone. CAT protein production was sensitive to the presence of tetracycline when using RCAS(A)tTA as the source of the transactivator. Addition of tetracycline 24 hours before harvesting CEFs reduced CAT protein production presumably by reducing transactivation by tTA. This control experiment demonstrated that the tetracyline responsive system functions in CEFs (Panel A and B). However, no amount of transactivation provided by pREPtTA or RCAS(A)tTA in either COS-7 or CEFS lead to the production of CAT protein from RCAN(B)CAT, *RCAN(B)CAT or Cla 12-CAT. The possible reason for the observed lack of CAT production from these constructs was unclear and is discussed in the next section.

Discussion

tTA-mediated transactivation readily occurs in avian cells

The tetracycline responsive system's ability to function in CEFs and within the context of the RCAS system of vectors was tested. A series of transient transactivation assays using the CAT enzyme as the reporter gene demonstrated that transactivation by the tTA transactivator readily occurs in CEFs (see Panel A). The combination of the control plasmids pReptTA (the source of the transactivator) and pCMVtCAT (containing upstream tTA binding elements to the CAT reporter gene) were able to produce CAT protein levels in CEFs and COS-7 cells above background levels (pCMVtCAT by itself). There was approximately a 10-fold induction of CAT protein above background in both avian and monkey cells. The similar induction levels caused by tTA-mediated transactivation in both cell types would suggest that all the components necessary for transactivation by the tTA transactivator in monkey cells are also available within these avian cells.

Previous experiments in monkey cells demonstrated that the tTA transactivator elicited the production of 5-30-fold levels of CAT activity above background (personal communication, Sandra Iacampo). In this thesis a series of transactivation assays (Panel A) induced protein levels (CAT) which fall within the induction levels of CAT activity seen previously in COS-7 cells. However, the levels of induction previously seen in Hela cells (up to 5 orders of magnitude, Gossen and Bujard 1992) were not reproduced. The lower levels of protein production in both monkey and avian cells as compared to the induction seen in Hela cells may just reflect species specific differences in the machinery required for efficient transactivation by tTA or may reflect a Hela specific enhancement of tetracycline mediated herpes virus VP16 transactivation.

tTA-mediated transactivation is sensitive to the presence of tetracycline

It has previously been demonstrated that the levels of transactivation as mediated by tTA are diminished by exogenously added tetracycline to the media of transfected COS-7 cells. Transactivation was clearly abrogated in the presence of 10μg/ml of tetracycline in transfected COS-7 and CEF cells as shown in Panel A. It was reported that 1μg/ml of tetracycline was more than sufficient to abrogate tTA-mediated transactivation in Hela cells (Gossen and Bujard 1992). However, it is known that 1μg/ml of tetracycline did not seem to be sufficient to diminish tTA mediated transactivation in transfected U937 cells. (Sandra Iacampo, personal communication) This, of course, illuminated the possibility that a similar case may exist for avian cells and so tetracycline levels were increased to the concentrations that were required for the abrogation of tTA transactivation in U937 cells (10μg/ml).

Lipofectamine mediated transfections in the presence of antibiotics may lead to cell death (Life Technologies (Gibco/BRL) lipofectamine protocol), and this could have explained the lack of transactivation. However this was unlikely because 1) lipofectamine-mediated death in the presence of antibiotic (tetracycline) was circumvented by adding the antibiotic 20 hours after the 4 hour lipofectamine-DNA treatment of the cells (see materials and methods). And, 2) dying adherent cells take on a characteristic phenotype whereupon numerous adherent cells "round up" and dislodge from the bottom of the culture plate and this was not observed 48 to 72 hours post-transfection with tetracycline treatment. (data not shown)

Further Experiments are required to clarify whether the tetracycline-sensitive system may be coupled to the RCAS system of vectors

The transactivation assays performed in CEFs clearly demonstrate that not only is this system potentially useful in mammalian cells but it is also potentially useful with respect to avian cells. It has also been demonstrated that the tTA protein produced from RCAS(A) is functional and sensitive to the presence of tetracycline. But, the transactivator was not able to mediate the production of CAT protein from the RCAS system vectors (CLA-12CAT, RCAN(B)CAT,*RCAN(B)CAT). There are several possible reasons why there was no observed production of CAT protein. 1) There was no transactivation by tTA or 2) CAT protein was not produced, or 3) CAT protein was produced but was not detected.

Transactivation by the tTA fusion protein is thought to occur by the binding of the transactivator to the tetracycline responsive elements upstream of the CMV IE minimal promoter. The binding of this transactivator would then drive the transcription of the downstream CAT gene and lead to the production of CAT protein. Although many possible reasons exist as to how a transactivation promoter located within a retrovirus may interfere with viral production and ultimately to the loss of protein production, this was avoided because the assays performed were transient. However, a well known characteristic of retroviruses is their ability to mediate, under certain circumstances, promoter occlusion. This is the method by which integrated retroviruses or provirus interfere with promoter activity downstream of their 5` LTR. This is the mechanism which is thought to mediate the silencing of the downstream 3` LTR. In cases of oncogenic transformation, the deregulated over expression of a proto-oncogene by an upstream integrated retrovirus, is thought to be partly due to the observed loss of sequences at the 5' end of the virus, thereby no longer silencing the promoter activity of the downstream 3`

LTR and thus leading to the overexpression of the host proto-oncogene (Boerkoel et al 1992). This phenomenon could have accounted for the observed lack of transactivation from the tetracycline elements located within the RCAN vector downstream of the 5` LTR. However, this does not seem to be the case because A) other promoters have been used within RCAS vectors. Both the mouse metallothionein 1 and chicken β-actin promoters linked to the CAT gene have previously been inserted into RCAN vectors. (Petropoulos et al 1991) Both of these promoters have been successfully used in the context of the RCAS system. B) the plasmid CLA-12CAT does not have viral components and contains all the elements required for transactivation induced CAT protein, yet it produces no CAT protein in the presence of either RCAS(A)tTA or pREPtTA.

The transactivation assays are based on the production of the reporter CAT protein. The CAT gene was cloned directly from the RCAS(A)CAT-3 vector into the CLA-12 adapter plasmid. Because there was no requirement for rounds of PCR amplifications in the cloning of the reporter gene, the CAT gene was not fully sequenced before it was used. Therefore, there exists a remote possibility that the CAT gene cloned may have obtained a mutation. The mutation would be such that there would be no productive protein being produced (for example by a frameshift mutation) or it would be defective and would not be recognized by the antibody in the CAT ELISA kit. In either case, CAT protein would not be seen in any of the assays. The lack of CAT protein production may also be due to mutations or errors in the tetracycline elements or poly A signal. This is highly unlikely since the TREs were completely sequenced (Figure 20) as well as most of the poly A sequence (Figure 21) within CLA-12.

There remain numerous reasons as to why the merged RCAS and tetracycline systems in the control plasmids tested did not lead to CAT production. Clearly, tetracycline sensitive transactivation is possible in

avian cells, but there still remains the question as to whether the two systems may be combined successfully. The obvious possibilities of merging the two systems merit further study in order to clarify the results obtained so far.

GENERAL DISCUSSION and CONCLUSIONS

REV-T virus is the most virulent of all the acutely transforming viruses. The v-rel oncogene, the first identified member of the REL/NFκB family, mediates the preferential transformation of avian lymphocytes by the REV-T virus. The mechanism of this transformation is unclear, however immunoprecipitations performed with anti-v-rel antibodies clearly reveal an assortment of associated proteins which include pp40 IκB alpha, p68 c-rel, hsc70 and the p124 and p115 precursors for the p50 and p52 subunits of the REL/NFκB family, respectively.

These proteins associate with the c-rel proto-oncogene in normal cells and suggested the possibility that v-rel functions by altering the normal function of these complexes. We have demonstrated that c-rel protein is upregulated upon serum stimulation of CEFs, consistent with earlier reports that demonstrated c-rel transcripts are increased upon mitogen or growth factor stimulation in murine fibroblasts and T cells. This places c-rel in the immediate early gene family implicated in the regulation of the cell growth response to growth factor stimulation.

Indeed, c-rel is involved in lymphocyte proliferation in response to receptor stimulation as demonstrated by the c-rel null mouse. However, the mechanism by which c-rel functions to stimulate cell cycle entry is unclear and creates a hole in our understanding of v-rel mediated transformation. Consistent with the findings of other groups, we have demonstrated that c-rel protein levels are not altered during the cell cycle progression of *ex vivo* bursal cells. But, the observation that REL-like proteins are expressed during certain phases of the cell cycle, including a c-

rel like protein merits the further investigation of REL/NFκB members relationship with cell cycle progression.

To further understand the role of c-rel in cell cycle progression, c-rel protein upregulation was inhibited using antisense phosphorothioate oligonucleotides against c-rel. We have determined that in avian cells, the phosphorothioate linkages of antisense oligonucleotides increases the efficiency with which gene expression is disrupted when compared to oligonucleotides without phosphorothioate oligonucleotides or chimeric oligonucleotides which feature normal and phophorothioate linkages. C-rel upregulation in CEFs was inhibited and cell cycle entry of the stimulated CEFs was investigated at 3 hours post serum stimulation. Although there was no apparent difference between the antisense and non-treated CEFs with respect to the entry to cell cycle, these preliminary results were not clear because the CEFs were just entering the cell cycle. Further experiments are required with longer serum stimulation times in order to clarify whether c-rel is required for the speed or efficiency with which cells re-enter the cell cycle.

Knock out mice have already been made for c-rel, relB, p65 and p50. The functions of some members in the transformation process have already begun to be looked at. For example, the v-rel transgenic mouse was crossed to the p50 knock out mouse with no change in the transforming activity of v-rel. The authors suggest that the transforming property in the v-rel transgenic mouse was composed of p50-vrel heterodimers, but changed to v-rel heterodimers in the p50 knock out/ v-rel crossed mice. Thus in this manner, one may step closer to understanding the mechanism of v-rel mediated transformation.

We have also tested the efficacity of using antisense transcripts as produced from the RCAS system of replication competent retroviruses ,*in*

vivo, against the RAG-2 gene. The control transgenic chicken infected with RCAS-CAT demonstrated CAT protein from the targetted lymphoid organs. For any number of possible reasons, the RCAS vectors expressing concatomerized and non-concatomerized antisense transcripts against the RAG-2 were unable to disrupt the expression of the RAG-2 gene at levels required to inhibit T and B cell receptor rearrangement. The possibility that the promoter activity, divided by the different transcripts produced by the replication competent virus, was not strong enough to produce a high level of antisense transcripts is very likely. Indeed, when a REV-T variant was constructed in which the promoter activity was divided between the v-rel oncogene and other viral genes, the transformation activity of the virus was abrogated by the presumably lower levels of v-rel expression. Another possibility that seemed equally likely was that a few B or T cells, which were able to rearrange their receptors, could have been enough to seed the peripheral lymphoid organs.

Our attention was then turned to an inducible promoter system, called the tetracycline responsive system which could induce a 4-log increase in reporter protein expression levels in Hela cells. The tetracycline system was inserted into two RCAS vectors, the transactivator was inserted into the RCAS(A) vector and the tetracycline operators (to which the transactivator binds) and reporter gene were cloned into the second RCAN(B) vector. In such a manner RCAS(A)tTA produced the tTA transactivator which would be able to transactivate, in a tetracycline-dependant manner, the expression of the reporter gene from RCAN(B).

We demonstrate, through the use of control plasmids, that the tetracycline-sensitive regulation of reporter gene expression by the tTA transactivator expressed from RCAS(A)tTA in CEFs was similar to tTA expressed from pREPtTA in the COS-7 control cells. Induction of CAT protein by the tetracycline system was less than 2 levels of magnitude, but

comparable in both avian and monkey cells. We show as well that CAT protein expression was inhibited in both avian and COS-7 cells by the addition of exogenous tetracycline. However, tetracycline-sensitive protein expression regulation was not observed from the RCAN(B) vector harbouring the tetracycline operators and reporter gene. This may be due to viral LTR regulatory interference preventing tTA mediated activation or perhaps a chance mutation had altered the CAT gene such that the antibody would no longer recognize the mutated reporter protein. Therefore, further study is required to establish whether the tetracycline system is able to function in the context of the RCAS vector.

Whether phosphorothioate oligonucleotides or antisense transcripts function to inhibit gene expression must be determined empirically. Until the mechanism of antisense mediated gene disruption is understood, the efficacity of this method for understanding gene function will be wanting in a number of systems where they have never been shown to perform well. This merits further study since gene disruption is hypothetically an exquisitely specific method. Another advantage of this technique is the speed with which results may be obtained as compared to the murine knock out protocal. Technology will also simplify the quest for a more efficient antisense method, including oligonucleotides which afford nuclease protection while keeping the hybridization of the antisense with the RNA efficient, ribozyme which cleave the specific RNA to which it binds and the creation of efficient transfection and transcript producing vehicles.

In conclusion, the investigation of v-rel mediated cellular transformation remains of fundamental relevance to our understanding of cancer. For example, REL/NFκB transcriptional regulators are known to regulate c-myc transcription from κB consensus sites located within the c-

myc promoter (La Rosa et al 1994). C-myc is clearly involved in cell cycle progression (possibly through transactivation of cyclin D and apoptosis). In recent years it has become obvious that cancer therapy induces tumor cell killing through the programmed cell death mechanism. Both the p53 and c-myc genes play key roles in regulating apoptosis of normal and transformed cells. In this november issue of journal Science, three groups have demonstrated how REL/NFκB members can block or enhance apoptosis involving c-myc and chemotherapeutic agents (Beg and Baltimore 1996, Wang et al 1996, Antwerp et al 1996). It is very possible there is a relationship between the mechanism of v-rel mediated transformation and apoptosis blocking, which has already been suggested (White et al 1995). Therefore, the link between REL/NFκB regulation and cancer therapy has become of central importance. Thus, the questions posed by v-rel mediated transformation become fundamental to the issues surrounding cancer and its therapy.

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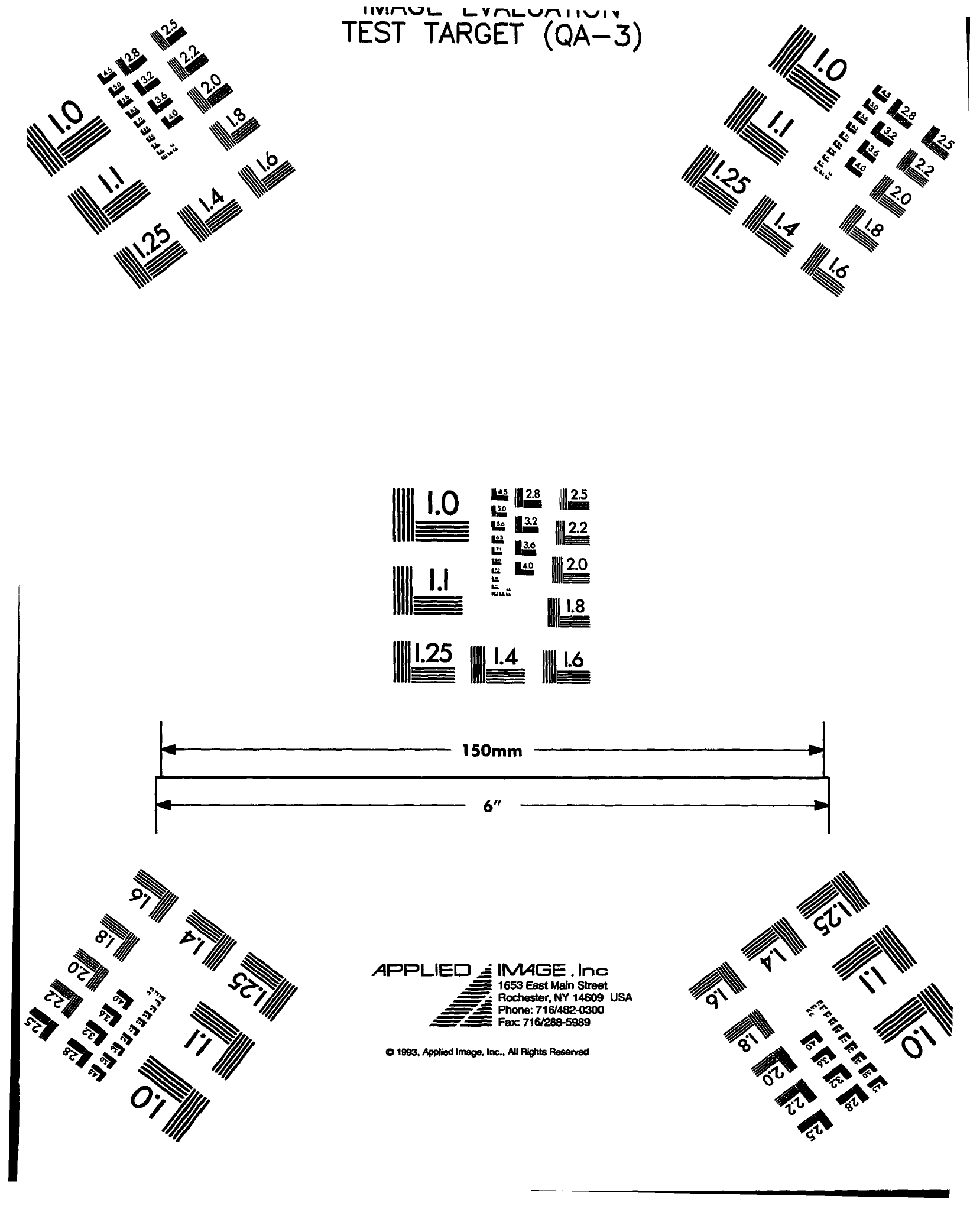
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