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The presence of a calmodulin-like protein in feline leukemia virus and the effect of calmodulin antagonists on feline leukemia virus infection

Chang, Jason Yeh-Cheng, Ph.D.

The Ohio State University, 1988
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THE PRESENCE OF A CALMODULIN-LIKE PROTEIN IN FELINE
LEUKEMIA VIRUS AND THE EFFECT OF CALMODULIN
ANTAGONISTS ON FELINE LEUKEMIA VIRUS INFECTION

DISSERTATION
Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of the Ohio State University

By
Jason Yeh-Cheng Chang, B.S.

The Ohio State University
1988

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To My Parents
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To my wife, Angie, I give her my deepest appreciation for her understanding and constant support during my academic career.
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PUBLICATIONS


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INTRODUCTION

Viruses are among the most primitive forms of life. They basically consist of nucleic acids wrapped in a protein shell. Some viruses also have an outer membrane or envelope around the protein shell. In general, genetic material in the virus serves as a template for viral proteins, while the lipid components found in the membrane are derived from the host cell.

A virus is an obligatory cellular parasite requiring a host for its reproduction and replication. There are two major ways that the viruses are transmitted from one host to another. In "horizontal" infection, the virus passes from one host to another by some medium, such as air, water, saliva or blood. In "vertical infection" the virus is directly transmitted to the cells of the offspring through the genomes of the parents.

Although the structures, the life cycles and the infectious patterns of a variety of viruses have been extensively studied, viral infection is still one of the most difficult diseases to deal with. The major difficulty arises from the fact that viruses usually utilize the host's cellular machinery for their own reproduction.
Thus, whatever interferes with the viral life cycle may also hamper the normal physiology of the host cells. This accounts for the low therapeutic index seen with antiviral agents.

The ultimate cure for viral infection will result from basic research. The more which is known about the details of viral structure, biochemistry and life cycle, the more likely it is that an effective antiviral therapy will be developed.

**Life cycle of a typical virus**

The life cycle of a typical virus starts with the binding of a viral protein to a site on the host cells (14,31,44,54,75,89). Some viral binding sites on the surface of the host cell membrane have been identified. For example, Human Immunodeficiency Virus (HIV) binds to CD 4 molecules on helper T lymphocytes, Semliki Forest virus binds to the major histocompatibility proteins, H-2K and H-2D, on BHK cells, and reovirus binds to beta-adrenergic receptors (44). On the other hand, the binding sites for other viruses have not been identified. For example, Sendai virus can bind to sialic acid residues on some cells, and vesicular stomatitis virus binds to phospholipid or glycolipid residues on the host cells (44).
The binding of the virus is followed by its internalization into the host cells. There are at least two pathways by which viruses can get into the host cells: receptor-mediated endocytosis and direct fusion between the viral membrane and the plasma membrane of the host cells.

Receptor-mediated endocytosis is a pathway by which cells normally take up macromolecules from the extracellular medium (24). Macromolecules taken up by this pathway include transport proteins (low-density lipoprotein, transferrin), growth factors (epidermal growth factor), hormones (insulin) or others (maternal immunoglobulins). The process of receptor-mediated endocytosis starts with the binding of the macromolecule (ligand) to a receptor site on the cell membrane. The receptor-ligand complex then translocates to a special region of the cell surface, known as the coated pits, where the internalization of the complex occurs. The membrane in this region invaginates to form intracellular coated vesicles. The coated vesicles then fuse with a specific intracellular vacuole, known as the endosome (32). In endosomes, the receptor-ligand complex dissociates, the receptor is recycled to the surface and the ligand passes to still another kind of intracellular vesicle (lysosome) where enzymatic digestion of the macromolecules occurs.

Many viruses enter the host cells by receptor-mediated endocytosis. The best studied example is the
entry of the Semliki Forest virus into BHK cells (30,75). At a high multiplicity of infection (90,000 viruses per cell), the cell takes up 3,000 viruses per minute at 37°C. The viruses appear in the endosomes 10 minutes after the endocytosis begins. After 20 minutes the viral components are found in the lysosomes. It is believed that the low pH in the endosomes and lysosomes can initiate rapid fusion between the viral membrane and the plasma membrane. This leads to the release of viral nucleic acids into the host cell cytoplasm. Agents which increase the pH of the endosome and lysosome, such as NH₄Cl and chloroquine, inhibit the fusion process, and thereby inhibit viral infectivity.

Sendai virus is the best example of virus which enters the cell by the direct fusion pathway (89). This virus contains two kinds of glycoprotein (HN & F) on the surface of its outer membrane. The HN protein binds to sialic acid residues on the host cell membrane. After this binding, the F protein initiates a fusion process between the viral membrane and the plasma membrane. Unlike the fusion between the virus membrane and the endosome/lysosome membrane described in the previous section, the fusion initiated by the Sendai virus F protein is not pH dependent. The optimal temperature for the fusion is between 30-40°C.
After entering the host cell, the virus takes control of the cellular machinery and starts to reproduce viral nucleic acids and viral proteins. The mechanism by which this occurs depends on the virus type. For example, a single-stranded positive RNA virus uses this RNA as the messenger RNA for its own proteins. On the other hand, a single-stranded negative RNA virus has to make a messenger RNA out of its own RNA before viral protein synthesis can be initiated.

The final stage of the viral life cycle occurs when the viral nucleic acid and protein are assembled into a mature virus. This is followed by the release of the virus from the host cell by budding. The virus may bud either from the plasma membrane of the host cell, as in the case of the HIV (20), or the budding may occur from an intracellular membrane, as in the case of rotavirus which buds from the endoplasmic reticulum. After intracellular budding, the mature virus is then transported to the plasma membrane by vesicles, and is released by exocytosis (14).

**Life cycle of a retrovirus**

As a result of recent intensive studies, there is accumulating information about the life cycle of human immunodeficiency virus (20). These studies provide
information about the retroviruses in general. The life cycle of a retrovirus will be described in this section, with HIV as a model.

The life cycle of the HIV starts with the binding of the viral glycoprotein, gpl20, onto the CD 4 antigen of the host cells (68). Although HIV primarily infects a subgroup of lymphocytes, called T-helper cells, macrophages, which also contain the CD 4 antigen, can also be infected by HIV. Binding sites have been proposed for other retroviruses, including the Rauscher murine leukemia virus and Friend murine leukemia virus (37,66). However, neither site has been well characterized.

Following the initial binding, the virus is internalized. Despite intensive study, the actual route of entry of the HIV is not clear. Maddon et al.(51) demonstrated that infection by HIV could be inhibited by agents that increase the pH of the endosome and lysosome. Based on this observation, they suggested that the virus entered the host cells by the process of receptor-mediated endocytosis. However, Stein, et al.(76) found that fusion between the viral envelope protein and the host cell membrane was required for viral entry. They demonstrated that fusion was pH independent, a characteristic similar to the entry of Sendai virus into the host cells. Accordingly,
they suggested that HIV entered the host cell by fusion of the viral membrane with the host cell plasma membrane. Further studies are needed to resolve this question.

After the virus enters the host cell, the viral RNA is transcribed into complementary DNA by the viral enzyme, reverse transcriptase. The newly formed DNA is replicated into a double-stranded DNA (called proviral DNA), which is then circularized and integrated into the host genome. The proviral DNA usually remains latent until the host cell is activated by some stimulus such as infection. The activation initiates the transcription of the viral genomic RNA and messenger RNA which results in viral protein synthesis and processing.

There are three major gene products encoded by the retroviruses (13,20). The first is from the gag gene, which encodes for structural proteins of the retrovirus. The second is from the pol gene, which is responsible for the production of the retroviral specific enzyme, reverse transcriptase. The third is from the env gene, which encodes for the envelope proteins. The proteins encoded by the gag and pol genes are made in the free ribosomes. On the other hand, the env gene products are made in the rough endoplasmic reticulum. All of them are transported to the plasma membrane for final processing.

The final stage in the life cycle of a retrovirus is viral assembly and budding. The envelope proteins are
inserted into the host cell plasma membrane. In close contact with the envelope proteins is the nucleocapsid, which is a complex of the structural proteins, reverse transcriptase and the genomic RNA. The association of the envelope proteins and the nucleocapsid results in a distortion of a region of the plasma membrane, which is followed by the budding and release of the mature virus (13).

Feline leukemia virus as a model system
The nature of FeLV:

FeLV is a retrovirus which can cause both neoplastic and non-neoplastic diseases in cats (29,58). FeLV infection can lead to an immunodeficiency syndrome which is very similar to that of AIDS in human beings. Death following FeLV infection is usually due to opportunistic infections which may lead to pneumonia, upper respiratory infection, stomatitis and chronic skin ulcers. About 80% of infected cats die within 3 years after diagnosis. The similarities between FeLV infection and AIDS, and the extensive information available about FeLV, makes FeLV an attractive model to work with.

Pathogenesis of FeLV infection:

Saliva is the major medium of infection for FeLV among cats (29,58). The virus first reaches the oral, nasal or
ocular membrane, then starts to multiply in the lymph nodes of the head or neck. The infection proceeds to the bone marrow and attacks the stem cells. The infected cells then get into the circulation and finally reach the salivary gland, where they serve as a source for another round of infection.

The life cycle of FeLV is essentially parallel to that of HIV (29,58). The major target for FeLV is the T cells. However, the viral binding site on the host cell membrane has not been identified and the exact route of entry by FeLV has not been established. FeLV infection can result in a dysfunction of the T cells as well as a decrease in the number of total T cells. It is believed that the viral envelope protein, p15E, is responsible for the T-cell malfunctions. FeLV infection appears to impair the functions of other immune cells as well. There is evidence that B cells, macrophages and neutrophils do not function properly when the cat is infected (29,58).

In vitro model for FeLV infection:

The study of FeLV infection can be carried out in vitro. The virus can be grown in large quantities using the feline lymphocyte cell line N-FL-74 (79). There are a number of cell lines that can serve as the host cells for the viral infection. The degree of infection can be determined by several assays, such as plaque assay (22),
reverse transcriptase assay (64,65) and ELISA (48). These factors make feline leukemia virus a convenient model to work with.

The role of calcium in the viral life cycle

There is ample evidence indicating that calcium plays an important role in the events of the viral life cycle, including binding, entry, uncoating, maturation, assembly and budding. In addition, calcium appears to play a role in maintaining viral integrity.

The plasma membrane of the murine fibroblast cell line, KA31, has specific binding sites for gp70, the envelope glycoprotein of Rauscher murine leukemia virus. The binding of the viral glycoprotein to the receptor is calcium dependent. Virtually no binding takes place if the calcium is removed by the calcium chelator, EGTA (37).

Many viruses enter the host cell by receptor-mediated endocytosis, a process which is dependent on calcium and calmodulin. Accordingly, calmodulin antagonists have been shown to inhibit viral infection. For example, Nemerow, et al. (59) demonstrated that infection of human B lymphocytes by the Epstein-Barr virus was inhibited by a variety of calmodulin antagonists. The concentration needed to inhibit viral infectivity correlated well with the concentration needed to inhibit calmodulin activity.
There is evidence that calcium chelators have the ability to disrupt viral particles. Cohen et al. (10) demonstrated that treatment of rotavirus with EGTA leads to the disruption of the outer capsid layer. As a result of this treatment, the viral polymerase is exposed and fully functional. They speculated that the removal of calcium is essential in the process of viral uncoating. They argued that the low calcium concentration inside the host cells was similar to the condition created by EGTA. When the virus entered the host cell, the outer capsid might disrupt in response to the low intracellular calcium concentration. This in turn could lead to the release of viral enzymes and nucleic acids into the cytoplasm, initiating the viral life cycle. This idea was supported by the work of Ludert, et al. (46), who demonstrated that agents which increased intracellular calcium concentration, such as the calcium ionophore A23571, inhibited the intracellular uncoating of the rotavirus.

Calcium also appears to play an important role in maintaining the integrity of polyoma virus. Brady, et al. (6) demonstrated that the removal of calcium from the viral particle is a prerequisite for viral disintegration. Based on this observation, they proposed that the removal of viral-associated calcium within the host cell may be a necessary event for viral uncoating.
Additional studies concerning the relationship between calcium and viral integrity were conducted by Wunderlich and Sydow (92), who demonstrated that EGTA and EDTA had the ability to disrupt retroviruses. For example, incubation of Rauscher murine leukemia virus with EGTA or EDTA led to viral disintegration, as evidenced by the release of the viral enzyme, reverse transcriptase. Removal of calcium decreased the virulence of these leukemia viruses as judged by the plasma RT level and the survival time of mice infected with the calcium-chelator treated viruses.

The role of calcium in viral uncoating was also studied extensively in plant viruses. Durham et al. (16-18,62) observed that a number of plant viruses, such as tobacco mosaic, tobacco rattle, brome mosaic, turnip crinkle and turnip yellow mosaic virus, contain calcium binding site(s) on the viral particles. They demonstrated that calcium had the ability to prevent the viral particles from disintegrating, and proposed that the low intracellular calcium was a favorable environment for viral disassembly.

Studies by Shahrabadi and Lee (71) indicated that calcium is necessary for the formation and stabilization of the outer shell of rotavirus. To demonstrate this, they examined the maturation of the virus in the presence and absence of extracellular calcium. Their results indicated that rotavirus grown in calcium-deprived cells did not have
an intact outer shell. Virus formed under these conditions has very low infectivity. Calcium deprivation between 6 to 12 hours post-infection, a period corresponding to the log phase of viral growth, was most detrimental to the virus maturation. On the other hand, calcium deprivation before or after this period had very little effect. Further studies (70) indicated that calcium can prevent the degradation of a specific outer capsid protein, VP7, thus ensuring that assembly and maturation of the virus could proceed normally.

Based on these studies, it is clear that calcium can participate in virtually every stage of the viral life cycle. In fact, after extensive observation of virus-calcium interactions, Durham proposed that calcium may play a key role in viral disassembly, takeover and transformation of the host cells (15).

**Calcium binding sites in viruses**

Although the importance of calcium in viral function has been demonstrated, the nature of calcium binding sites in viruses is not clear. Durham proposed that the phosphate group of the viral RNA and carboxyl groups on the viral protein together made up the calcium binding sites (18). Gallagher and Lauffer demonstrated that the outer coat of tobacco mosaic virus had the ability to bind calcium (23). Working with $^{45}\text{Ca}$, Shahrabadi et al. (70)
demonstrated that calcium was incorporated into the mature, double-shelled rotavirus but not into the immature, single-shelled viral particle. Based on the observation that calmodulin antagonists had the ability to lyse retroviruses, Wunderlich and Sydow speculated on the presence of a calmodulin-like protein in the retroviruses (92). The only clear identification of a viral calcium binding site came from work with the polyoma virus. After demonstrating that calcium was involved in the disassembly and reassembly of polyoma virus, Ludlow and Consigli demonstrated that VP1, the major capsid protein of polyoma virus, was a calcium-binding protein (47).

Although the nature of calcium binding sites in the virus is not clear, much is known about the calcium binding proteins in the host cells. Among the cellular proteins which can bind calcium, calmodulin has been recognized as a unique protein which participates in a number of important cellular events.

**Calmodulin**

Calmodulin is a ubiquitous protein present in all eukaryotic cells examined to date (9,35,56). This protein is either free in the cytosol or membrane-bound, and constitutes 0.1-1% of total proteins in the cells. It is a highly conserved protein. This can be seen from the fact that calmodulin isolated from one species is usually active
in stimulating the enzymes from another species. Furthermore, the antibody against the calmodulin from one species can cross-react with the calmodulin from another species.

Properties of calmodulin:

Calmodulin is an acidic, heat stable protein with a molecular weight of about 17,000. A close examination of the 148 amino acids which constitute calmodulin reveals that about 30% are either glutamic acid or aspartic acid. This results in the acidic nature of calmodulin. The $\text{pI}$ for this protein is approximately 4.3. Another unusual feature is that calmodulin does not contain tryptophan, cysteine or hydroxyproline. It is believed that this unique structure allows great flexibility of the calmodulin molecule and explains why it can participate in many biological process.

One important feature of calmodulin is its internal homology. The primary structure of calmodulin can be subdivided into four functional domains with homologous sequences. The amino acid sequence of the first domain is almost identical to the third, and that of the second is essentially the same as the fourth. There is one calcium binding site in each of the four domains. The conformation of the protein in each of these regions is such that six to eight oxygen atoms forms a binding site for the calcium. These calcium binding sites are referred to as the E-F
hands. The dissociation constant for these calcium binding sites is approximately 4-18 uM. When calcium is bound, there is a conformational change in the molecule, which is reflected by an alteration in the absorption spectrum, a difference in mobility in SDS-PAGE and the pattern of cleavage by trypsin.

Function of calmodulin:

Calmodulin is involved in a number of crucial cellular events, including DNA replication and repair, progression of the cell through the cell cycle, cell differentiation, secretion, endocytosis of extracellular ligands, exocytosis of cellular contents, contraction of smooth muscles and glycogen metabolism.

The metabolic events controlled by calmodulin are highly coordinated. This is best exemplified by its role in the control of glycogen metabolism. Calmodulin is able to activate phosphorylase kinase. This in turn can activate phosphorylase, hence the catabolism of glycogen. At the same time, calmodulin is able to activate glycogen synthase kinase. This can lead to the phosphorylation of glycogen synthase and inactivation of this enzyme. The formation of glycogen from glucose is thus inhibited. The net result is an increase of glycogen breakdown and an inhibition of glycogen synthesis.
Inhibitors of calmodulin:

The activity of calmodulin can be inhibited by a number of structurally different compounds. The best characterized examples are the phenothiazines, such as trifluoperazine and chlorpromazine (86,87). In the presence of calcium, these drugs are able to bind calmodulin with very high (approximately 1 uM) affinity. This binding can inhibit the activity of calmodulin. A number of recently developed calmodulin antagonists have been shown to have higher selectivity and sensitivity than the phenothiazines. Examples of these new compounds include calmidazolium (81) and W-7 (77). In addition to the synthetic inhibitors, there are also naturally-occurring calmodulin inhibitors. For example, melittin, a peptide from bee venom, is able to inhibit calmodulin activity at nanomolar concentrations (3). Finally, since the activity of calmodulin is calcium dependent, agents which can remove calcium from the system, such as EDTA and EGTA, can inhibit the calmodulin activity in an indirect way.

Assays for calmodulin:

There are two major ways by which the presence of calmodulin can be detected. The first is an enzymatic assay, which takes advantage of the fact that calmodulin is able to activate a variety of enzymes. Among the enzymes
that can be activated by calmodulin, cyclic nucleotide phosphodiesterase is most commonly used (83). Calmodulin has a very high affinity for this enzyme. The dissociation constant is approximately 1-10 nM. This makes the enzyme a very good indicator for the presence of calmodulin.

The other common way to detect the presence of calmodulin is by radioimmunoassay (8). Since calmodulin is a highly conserved protein, antibody raised against the calmodulin from one species can usually recognize the calmodulin from another species. This makes the production of calmodulin antibody and the development of radioimmunoassay very useful. The sensitivity of radioimmunoassay is usually able to detect the presence of nanograms of calmodulin. Recently, an ELISA for calmodulin has been described, which has similar sensitivity to the RIA (88).

Initial observations:

Initial observations carried out in our laboratory indicated that the mitogen concanavalin A can stimulate the production of cAMP in cat lymphocytes, and co-incubation of inactivated feline leukemia virus (FeLV) decreases the cAMP level in these mitogen-stimulated lymphocytes. In order to explain this effect on cAMP, we tested the possibility that FeLV could alter the activity of cAMP phosphodiesterase. We found that the lysate of sucrose-gradient purified FeLV
does, in fact, stimulate cAMP phosphodiesterase activity in vitro. The extent of PDE stimulation was similar to that seen with authentic calmodulin. This led us to consider the possibility that FeLV might be associated with calmodulin or a calmodulin-like protein.

Hypotheses

The following observations were used in formulating our working hypotheses:
1. Calcium plays a crucial role in the life cycle of a virus;
2. Virus has the ability to bind calcium although the nature of the binding sites is not clear;
3. Calmodulin antagonists can disrupt retroviruses;
4. Calmodulin is the most important calcium binding protein in eukaryotic cells. It is possible the virus can take advantage of this protein for its own purpose;
5. The lysate from purified FeLV increases cAMP phosphodiesterase activity to a same extent as authentic calmodulin.

These observations led us to formulate and test the following hypotheses:
1. Calmodulin is the calcium binding protein in FeLV;
2. Calmodulin is responsible for the structural integrity of the FeLV;
3. Calmodulin antagonists can disrupt FeLV and may alter its infectivity.
METHODS

Cell culture:

81 C cell

The 81 C cell line was used in the infectivity experiments. This cell line was originally cloned by Fishinger, et al.(22). A subclone of this cell line was obtained from Dr. Mark Lewis of the Department of Veterinary Pathology and Microbiology, the Ohio State University.

1. Culture media

The medium used was McCoy's 5A (Gibco, #430-1500) supplemented with 15% Fetal Calf Serum (Armour Pharmaceutical Co. #0265-01, heated at 55°C for 30 minutes before use), Glutamine (Gibco, #320-5030, a 200 mM solution, use 2 ml in a total of 100 ml medium) and NaHCO$_3$ (Gibco, #895-1810, a 8.8% solution, use 2 ml in a total of 100 ml medium), pH 7.2-7.4. This was designated as the "complete medium". For the infectivity experiment the medium was modified either by omitting the serum or adding DEAE-dextran (0.03 mg DEAE-dextran per ml medium). Any chemicals added to the culture medium were sterilized by filtering the solution through a 0.2 um syringe filter.
2. Maintaining the 81 C cell line

The cells were grown in 150 cm\(^2\) culture flasks (Corning), and kept in an incubator at 37°C and 5% CO\(_2\). The cells were split every week to 1/40 of the original cell number by the following procedure: The medium of the cell culture was transferred to a waste beaker by a 10-ml sterilized plastic pipet. The culture was washed twice with 10 ml of phosphate-buffered saline (PBS), then rinsed with 2 ml of trypsin (Gibco, #610-5300). After removal of the trypsin, another 2 ml of trypsin was added and incubated with the culture for 2-3 minutes or until the cells started to detach from the bottom, as observed under the microscope. When most of the cells detached from the bottom, 18 ml of the complete medium was added to the flask to stop the trypsinization. The cells in the medium were mixed well by triturring the medium several times with a 10 ml pipet. 0.5 ml of this cell suspension was then transferred to a new 150 cm\(^2\) flask, and 25 ml of fresh medium was added. This flask was then incubated in the 37°C incubator until the next split. If the cells were grown in 75 cm\(^2\) flask, 1 ml of trypsin was used for the splitting. If the cells were grown in 25 cm\(^2\) flask, 0.5 ml of trypsin was used. The trypsin was then inactivated by 9 x volume of the complete medium.
3. Infectivity studies using the 81 C cell line:

A confluent cell culture was split to 1/3 of the initial cell density and incubated overnight. The cells from this culture were collected the next day and the cell number was adjusted to 50,000 cells per ml of medium. One ml of this cell suspension was put into each well of the 24-well plate. We routinely run 4 replicates per sample. This culture was used after 24 hours.

To initiate the experiment, the culture medium from the wells was removed and 0.5 ml of the drug to be tested was added to the wells and incubated for one hour. At the end of this drug preincubation period, 0.2 ml of FeLV was added to each well and incubated for one hour. Free virus and drug were then washed away with PBS (2 x 2 ml), and 1 ml of fresh medium without drug was added to each well. The cells were incubated for another 7 days without any medium change. At the end of the 7th day, the medium was collected and analyzed for the concentration of p27, as described in the ELISA section.

The drug for each experiment was prepared on the day of use. A stock solution of the drug (usually 1 mM) was prepared in distilled water then sterilized by filtering through a 0.2 um syringe filter. This filtered solution was then diluted in the medium to the appropriate concentration.
It is worth noting that the infectivity experiments were routinely carried out for 7 days. There was an uneven evaporation of the fluid from the wells during this 7-day incubation. The outer wells appeared to evaporate more than the inner wells. In order to correct this problem, only the inner 8 wells of the 24-well plate were used for growing the cells. The surrounding 16 wells were filled with 1 ml of PBS during the 7-day incubation.

The virus used in the infectivity experiments was pre-titrated before the experiments were conducted, as described in the ELISA section.

3201 cell

The 3201 cell line was used for the initial comparison between the ELISA and reverse transcriptase assay. This is a feline T-cell line originated from a thymic lymphoma, cloned by Dr. J.L. Rojko et al. in the Department of Veterinary Pathology and Microbiology, the Ohio State University. A subclone of this cell line was obtained from Dr. Mark Lewis of that department.

1. Culture medium

The cells were grown in a 1:1 mixture of RPMI-1640 medium and L-15 medium containing 15% fetal calf serum, 1% sodium pyruvate (from 100 mM stock) and 2% glutamine (from 200 mM stock).
2. Maintaining the 3201 cell line:

The cells were grown in 150 cm$^2$ flasks (Corning) and kept in an incubator at 37°C and 5% CO$_2$. The cells were split to 1/60 of the original density every week. To split the cells, the cell suspension in the culture was mixed well, then 0.5 ml of the cells was transferred to a new flask and 29.5 ml of fresh medium was added.

3. Infectivity studies using the 3201 cell line:

A 3-5 day old cell culture was split to 1/3 of the initial density on day 1. On day 2, the cells were counted, then centrifuged at 400 x g for 10 minutes. The pelleted cells were incubated with 5 ml of DEAE-dextran medium (0.03 mg DEAE-dextran per ml medium) for 30 minutes at 37°C. After the incubation, the cells were concentrated by centrifugation and used for the viral infection. Typically, the cells prepared above were incubated with the challenge virus (see the ELISA section) for 2 hours in the 37°C incubator. After this infection period, the cells were centrifuged and separated from the free virus. The cells were resuspended in the fresh medium at a density of 2 x 10$^6$ per ml, put into the flask and kept in the incubator.
Biochemical Assays

Phosphodiesterase (PDE) assay

(I) Luciferin-luciferase method

1. Principle

This assay was conducted according to the method of Fertel, et al. (21). PDE converts cyclic AMP to 5'AMP. In the presence of myokinase and pyruvate kinase, the 5'AMP formed is converted to ATP. The ATP, in the presence of luciferin and luciferase, generates light which was measured by a photometer. The overall reaction is outlined below:

(1) \( \text{cAMP} \xrightarrow{\text{PDE}} \text{5'AMP} \)
(2) \( \text{5'AMP} + \text{ATP} \xleftarrow{\text{MK}} \text{2ADP} \)
(3) \( \text{2ADP} + 2\text{phospho(enol)pyruvate} \xleftarrow{\text{PK}} \text{2ATP} + 2\text{Pyruvate} \)
(4) \( \text{ATP} + \text{luciferin} + \text{oxygen} \xrightarrow{\text{LF}} \text{Light} \)

\[ + \text{AMP} + \text{PPi} \]
\[ + \text{oxyluciferin} \]
\[ + \text{CO}_2 \]

2. Material

All material was purchased from Sigma unless otherwise stated. Two reagents were prepared for this assay. Reagent A contained 150 mM glycylglycine buffer (pH 7.6),
75 mM ammonium acetate, 9 mM MgCl$_2$, 0.03 mM CaCl$_2$, 0.78 mM phospho(enol)pyruvate, 15 mM dithiothreitol and 3 mM ATP. A stock solution was prepared and stored at -4°C. Reagent B contained 9800 ul reagent A, 100 ul of 3% BSA, 150 units myokinase, 75 units pyruvate kinase and 30 ul of 10 mM CaCl$_2$. This solution was prepared before each assay.

myokinase (Sigma, # M-3003) : The stock solution from Sigma was 10,000 units per 2.2 ml in 3.2 M ammonium sulfate. An activity equivalent to 1,000 units (220 ul) was centrifuged at 1,000 x g for 10 minutes. The pellet was resuspended into 1,000 ul of reagent A to get 1 unit/ul. 150 ul of this solution was used to prepare reagent B.

pyruvate kinase (Sigma, # P-1506) : The stock solution from Sigma was 5,000 units per 2.8 ml in 2.2 M ammonium sulfate. An activity equivalent to 500 units (280 ul) was centrifuged at 1,000 x g for 10 minutes. The pellet was resuspended into 1,000 ul of reagent A to get 0.5 unit/ul. 150 ul of this solution was used to prepare reagent B.

Standards: 1 mM 5'AMP standard (Sigma, # A-1752) and 3 mM ATP standard (Sigma, # A-2383) were prepared in deionized water and stored at -4°C.
Calmodulin-deficient PDE from bovine heart (Sigma, # P 0520): A stock solution of 1 mg/ml was prepared in reagent A, and stored in 100 ul aliquot at -4°C.

Calmodulin from bovine heart (Sigma, # P 0270): A stock solution of 50 units/ml was prepared in reagent A and stored at -4°C.

MOPS (Morpholinopropane sulfonic acid) buffer: 10 mM MOPS, (pH 7.8), 10 mM MgSO₄ and 1 mM dithiothreitol.

Luciferin-luciferase (Sigma, # L0633): prepared fresh each time.

3. Procedure

The 5'AMP standard curve was prepared by adding 240 ul of stock 5'AMP (1mM) into 1760 ul of reagent A. 1 ml of this solution was diluted with 1 ml of reagent A to get the second point. This serial dilution was repeated 5 more times to get a total of 7 concentrations. The reaction mixture was composed of 5'AMP (50 ul), reagent B (50 ul) and reagent A (100 ul). This reaction mixture was prepared on ice in triplicate 6 x 50 mm tubes. The concentration of 5'AMP in this standard curve ranged from 94 pmole/200 ul to 6,000 pmole/200 ul.
The ATP standard was prepared by adding 80 ul of stock ATP solution (3mM) into 1920 ul of reagent A. This was then serially diluted with reagent A as described in previous section to provide a total of 7 concentrations. The reaction mixture in the standards (triplicates) contained ATP (50 ul), reagent B (50 ul) and reagent A (100 ul). The concentration of ATP in this standard curve ranged from 94 pmole/200 ul to 6,000 pmole/200 ul.

The reaction mixture in the samples (triplicates) contained PDE (30 ul), cAMP (30 ul), reagent B (50 ul) and reagent A (90 ul). To prepare the PDE, a stock solution of 100 ug/100 ul was mixed with 4,900 ul of reagent A to get a concentration of 20 ug/1,000 ul before each experiment. 30 ul of stock cAMP (1.5 mM prepared in deionized water) was used in this reaction mixture. The final cAMP concentration was 225 uM. The volumes of PDE (30 ul), cAMP (30 ul) and reagent B (50 ul) were constant in each experiment. The activator or inhibitor tested were prepared in reagent A and added in a volume which brought the final volume to 200 ul.

Both the standards and the samples were prepared on ice. The reaction was initiated by incubating the reaction mixture at 37°C for one hour, and stopped by immersing the tubes in a boiling water bath for 5 minutes. The tubes were then cooled to room temperature and the reaction product (ATP) was measured by luciferin-luciferase.
As described in the Principle, the cAMP added in the reaction mixture was converted first to 5'AMP by PDE and then to ATP by myokinase and pyruvate kinase. The ATP formed was determined by injecting luciferin-luciferase into the reaction mixture and measuring the light emitted. The luciferin-luciferase reagent was composed of 1 ml MOPS buffer, 7.5 mg luciferin-luciferase and 10 mg BSA. This solution was prepared in an aluminum foil-wrapped plastic bottle no more than one hour before use and kept at 4°C all the time. Twenty ul of this solution was injected into each sample tube, and the light emitted was measured by an integrating photometer (SAI Technology Co., Model 3000) with a setting of Sensitivity = 3.5, Zero = 2. Sample activities were calculated from a computer-generated curve of best fit for the standards.

30 ul of this PDE solution (0.6 ug) could convert 1 nmole of cAMP into 1 nmole of 5'AMP in one hour. Since the activity was slightly different among different batches of Sigma PDE, it was necessary to do a titration of PDE activity when a new batch was received. The basal activity of PDE appeared to increase after prolonged storage in the freezer. This might be a result of limited proteolysis of the enzyme. This resulted in an increase of basal PDE activity and a decrease of degree of activation by calmodulin. One way to solve this problem is to prepare fresh PDE from lyophilized powder for each experiment.
Under the experimental conditions described in the Methods, the maximal PDE activation we obtained was about 300% of basal PDE activity. This agrees with the manufacturer's result. This PDE activation was obtained by incubating the PDE with calmodulin at 37°C for 5 minutes before the substrate was added.

The photometer has a reading range from 0 to 85,000. Using the 5'AMP standard, we found when the reading was 1,000 and above, the photometer reading correlated very well with the concentration of 5'AMP in the sample. When the reading was lower than 1,000, there was no good correlation.

In order to see the effect of the calmodulin antagonists, it is necessary to add the components of the reaction mixture in the right order. The agents to be tested must be incubated first with calmodulin prior to the addition of PDE. Once calmodulin is bound to PDE, the binding of inhibitors to calmodulin is decreased.

(II) Fluorescent cGMP method

1. Principle

This PDE assay was performed according to the method of Johnson, et al.(36). The fluorescent 2'-methylantraniloyl derivative of cGMP (Mant-cGMP) undergoes a 45% decrease in fluorescence when it is cleaved by
phosphodiesterase. When calmodulin is added to this system, the rate of decrease of fluorescence is greatly increased. This assay is usually done by a continuous recording of the fluorescence level. However, a fixed time comparison between the control and the samples can also be done.

2. Material

Assay buffer: The buffer for this assay consists of 10 mM MOPS, pH 7.0, 90 mM KCl, 1 mM CaCl2 and 5 mM MgCl2. This buffer is stored at 4°C and is warmed to room temperature prior to the assay. One ml of this buffer is used for each sample in a routine assay.

Phosphodiesterase: The bovine heart PDE used in this assay was purchased from Boehringer Mannheim (#709883). It was prepared in the assay buffer at a concentration of 4 mg/ml, and 25 ul of this solution was used per sample. Brain PDE was prepared according to the method of Sharma, et al.(72), and provided by Dr. David Johnson, the Department of Physiological Chemistry, the Ohio State University.

Mant-cGMP: The Mant-cGMP was obtained from Molecular Probes (Eugene, OR). A stock solution of 2.5 mM was prepared in
distilled water and stored in the freezer. 3.2 ul of this
stock solution was diluted in 1 ml of assay buffer to get a
final concentration of 8 uM.

Calmodulin: The calmodulin used in this assay was prepared
by the methods of Gopalakrishna and Anderson (25), and was
provided by Dr. David Johnson in the Department of
Physiological Chemistry, the Ohio State University.

3. Procedure

The heart PDE assay is described in this section. The
procedure for brain PDE assay is essentially the same. The
fluorescence study was conducted on a Perkin-Elmer LS-5
fluorescence spectrometer. The excitation wave length was
280 nm and the emission wave length was 450 nm. The slit
widths were 10 nm. The results were recorded on a Perkin-
Elmer R100A recorder at a speed of 1 cm per 10 minutes.
The assay was conducted at room temperature. Depending on
the purpose of the experiment and the size of the sample
number, this assay could be carried out either by a
continuous recording or by a fixed-time measurement.

Continuous recording: One ml of the assay buffer and 3.2
ul of the stock Mant-cGMP were pipetted into a quartz
cuvette and monitored on the spectrometer. The
fluorescence intensity was set at 90.0% full scale, then 25
ul of the stock heart PDE was added to the cuvette and the basal PDE activity was monitored for 10 minutes. At this time, the activator to be tested was added to the cuvette and the reaction was monitored for about 15 minutes to determine the rate of cGMP hydrolysis. The PDE activation was calculated by a comparison between the rate of fluorescence decrease with or without the activator.

**Fixed-time measurement:** The activator to be tested was added to the 12 x 75 mm test tubes containing 1 ml of the assay buffer with 8 uM Mant-cGMP. The reaction was initiated by the addition of 25 ul stock PDE solution into the tubes. This reaction was carried out at room temperature for 30 minutes. At the end of the incubation, 50 ul of 0.4 M EDTA was added to each tube to stop the reaction. The fluorescence level in each tube was then measured by the spectrometer, and compared to that from the controls. Two sets of controls were prepared for this experiment. The first set measured the basal PDE activity. It contained PDE and Mant-cGMP only. The second set measured the basal fluorescence level at time zero. It contained Mant-cGMP, PDE and 50 ul of 0.4 M EDTA. To minimize the handling time during the initialization and termination of the reaction, the PDE and the EDTA were added by a automatic pipetter (Eppendorf Repeater).
The relative rate of cGMP hydrolysis was determined by the following calculation:

\[ Y = \text{the fluorescence level of the PDE with activator} \]
\[ Y' = \text{the fluorescence level of the PDE alone} \]
\[ Y_0 = \text{the fluorescence level of the PDE with EDTA at time zero} \]

Activation by the activator as compared to the basal PDE activity:

\[ \frac{Y - Y_0}{Y' - Y_0} \]

The absolute hydrolysis of cGMP was calculated by:

total cGMP in the one ml reaction mixture: 8 nanomoles
the fluorescence change after total hydrolysis: 40.5

\[ \frac{8}{40.5} = \frac{X}{Y - Y_0} \]

or \[ X \text{ (nanomoles hydrolyzed)} = \frac{8 \text{(nanomoles) } \times (Y - Y_0)}{40.5} \]

Calcineurin assay

1. Principle:

The calcineurin assay was conducted according to the methods of Anthony, et al. (1). Calcineurin is a phosphatase which is able to hydrolyze the phosphate group from a protein substrate or an artificial substrate. This assay uses a chemical compound, methyl umbelliferone phosphate (MUP), as the substrate for calcineurin. There is an increase in fluorescence when the phosphate group on
this compound is removed by the enzyme. Calmodulin can increase the activity of this enzyme in a dose-dependent manner and enhance the fluorescence accordingly.

2. Material:
Assay buffer: 50 mM boiled Tris-HCl (pH 8.6), 0.5 mM CaCl$_2$, 5 mM MgCl$_2$ and 0.2 mM EGTA.
Substrate: Methyl umbelliferone phosphate (MUP), 10 mM prepared in Tris-HCl (pH 8.6).
Calcineurin: This enzyme was prepared according to the methods of Sharma, et al.(72). A stock solution of 0.51 mg/ml was provided by Dr. David Johnson, the Department of Physiological Chemistry, the Ohio State University.
Calmodulin: see the Fluorescence PDE Assay section.

3. Procedure:
A fluorescence spectrophotometer (Perkin-Elmer LS-5) was used for this assay. The excitation wave length was 365 nm and the emission was 450 nm. The results were recorded on a chart recorder (Perkin-Elmer R100A), with a speed of 1 cm/min. To start the assay, 1 ml of assay buffer and 10 ul of MUP were added to a quartz cuvette. When the fluorescence level was steady, 5 ul of calcineurin was added to the cuvette. The reaction was run for 3-5 minutes to get a basal enzymatic activity. The activator to be tested was then added to the cuvette and the results
were recorded. The activation of calcineurin was determined by a comparison between the rate of fluorescence change with and without the activator.

**ATPase assay**

1. Principle

The membrane of human red blood cells (RBC) contains a calcium-magnesium ATPase which can be activated by calmodulin, as described by Cox, et al. (11). When a fixed amount of ATP was incubated with this ATPase derived from RBC membrane, we were able to measure the disappearance of ATP by the luciferin-luciferase assay described in the PDE assay section.

2. Material

All material in this assay was purchased from Sigma unless otherwise stated.

Isotonic Tris buffer: 172 mM Tris buffer, pH 7.6 (310 mOsM)

Hypotonic Tris buffer: 11 mM Tris buffer, pH 7.6 (20 mOsM)

Reagent J: The reagent used initially for this assay contained the following components: 100 mM NaCl, 6 mM MgCl₂, 20 mM KCl, 0.1 mM ouabain, 0.5 mM EGTA, 30 mM imidazole-HCl and 0.8 mM CaCl₂ (pH 7).

Reagent X: The reagent used for most of these studies
contained 20 mOsm Tris buffer (11 mM Tris, pH 7.6), 3 mM MgCl₂ and 0.1 mM CaCl₂. The results obtained with reagent X was comparable to that obtained with reagent J.

3. Initial observations with the ATPase assay:
   
   Initial experiments indicated that the FeLV preparations contained intrinsic ATPase activity. It was able to hydrolyze ATP in a dose-dependent fashion. This ATPase activity was abolished by boiling (data not shown). The association of ATPase activity with virus has been reported before (2).

   RBC membrane was initially prepared by the method of Hanahan and Ekholm (28), which itself is a modification of the methods originally described by Dodge, et al. (12). This method employs hypotonic lysis of the red blood cells. The membrane prepared in this way was found to contain ATPase activity and hydrolyzed ATP in a time-dependent manner. However, the ATPase could not be activated by authentic calmodulin. Further experiments indicated that the membrane was associated with calmodulin. This can be seen from the fact that the RBC membrane, which did not have PDE activity of its own, could activate PDE. The activity was still intact even after the membrane was boiled for 15 minutes (data not shown). The association of
calmodulin with RBC was previously reported by Jarrett, et al. (33,34). This intrinsic calmodulin could be removed by EDTA, as described below.

4. Procedure

Thirty ml of heparinized human blood was put into a 50 ml centrifuge tube and centrifuged at 1,000 x g for 10 minutes at 4°C. The upper portion, including plasma and a white buffy coat layer, were removed by a Pasteur pipet. Forty ml of 172 mM Tris buffer (310 mOsM) was added to the tube and the remaining RBC were resuspended by inverting the tube several times. The RBC were centrifuged at 1,000 x g for 10 minutes at 4°C. The upper layer was then removed, the RBC were resuspended in 40 ml of Tris buffer, and centrifuged again. This washing was repeated 3 times.

The RBC pellet from the last centrifugation was resuspended in 10 ml of hypotonic Tris buffer (20 mOsM, or 11 mM Tris), containing 1 mM EDTA. After 5 minutes at 4°C, the RBC were lysed and the solution was bright red. This solution was then centrifuged at 20,000 x g for 20 minutes at 4°C. The pellet was resuspended in 40 ml hypotonic Tris buffer and centrifuged again at 20,000 x g. This washing was repeated 3 times. The final pellet was almost colorless.

The RBC membrane pellet was then incubated with hypotonic Tris containing 0.1 mM EDTA at 37°C for 30
minutes. This removed the endogenous calmodulin from the RBC membrane. After the incubation, the membrane was washed twice using 40 ml of hypotonic Tris buffer and 20,000 x g centrifugation. The resultant RBC membrane was either used immediately or stored at 4°C and used the next day. A titration of the basal ATPase activity was done before this membrane preparation was used. Satisfactory activity was obtained using 50 ul of a 100 ug protein/ml preparation.

To set up the standard curve, a stock solution of 1.2 mM ATP was used as the highest standard. 1 ml of this stock ATP was added to 1 ml of water to get the second standard. This was repeated 5 more times to get a total of 7 data points. The reaction mixture in the standards was prepared by mixing the following components: 50 ul ATP standard, 50 ul of 11 mM Tris buffer and 50 ul of reagent X. The concentration of the standards ranged from 60,000 pmole/150 ul to 940 pmole/150 ul.

The reaction mixture of the sample for a typical ATPase assay contained the following components: ATP (50 ul of 400 uM stock solution), RBC membrane (50 ul of 100 ug protein/ml solution), activator (calmodulin or boiled FeLV in 20 ul of reagent X) and reagent X (30 ul). The final concentration of ATP in the reaction mixture was 20,000 pmole/150 ul. This reaction mixture was incubated at 37°C for 10 minutes, then stopped by immersing the samples in
boiling water for 5 minutes. The samples were cooled in 15°C water bath, and the remaining ATP was measured by the luciferin-luciferase assay as described in the PDE assay section. The difference between ATP before and after the incubation in each sample was calculated and taken as the ATPase activity.

ATPase activity appeared to decrease gradually when stored at 4°C. Although a complete study of the enzymatic activity over time during storage was not done, it is advisable to perform all the experiments desired in a few days. The effect of freezing on the ATPase activity was not determined. It has been reported that special treatment of the RBC membrane is necessary to preserve the ATPase activity upon freezing (11,33).

There was some precipitation after the reaction mixture was put into the boiling water. The precipitate did not appear to interfere with the measurement of ATP by luciferin-luciferase assay.

Calmodulin radioimmunoassay

1. Principle

Radioimmunoassay (RIA) is a competitive protein binding assay in which a fixed amount of labeled antigen competes with unlabeled antigen for a fixed amount of antibody. When an equilibrium of these three components
is reached, the unbound antigens are separated from the antibody-bound antigens. The radioactivity associated with the antibody-antigen complex is then counted by a gamma counter or a scintillation counter, depending on the radioactive label used. Since there is only a fixed amount of antibody in the reaction mixture, when a higher amount of the unlabeled antigen is present in the sample, less of the labeled antigen can bind to the antibody, thus the radioactivity will be low in this particular sample. When this radioactivity is compared with that from a standard curve, the amount of unlabeled antigen in the reaction mixture can be determined.

2. Material

A calmodulin radioimmunoassay kit was purchased from New England Nuclear (NEK-018). The kit contained CaM assay buffer (0.125 M borate, pH 8.4, 0.075 M NaCl, 0.2% BSA, 1 mM EGTA and 0.1% sodium azide), a calmodulin antibody prepared in sheep and purified by affinity chromatography, a CaM second Ab, $^{125}$I-labeled CaM which contained approximately 2 uCi of radioactivity at the time of calibration and calmodulin standards (preheated or unheated) prepared from bovine brain. We found that the use of preheated standards optimized the assay. The company stated that the antibody in this RIA kit could bind to the preheated standards only. As a result, only the
preheated standards were used in this study. It is worth noting, however, that the results using unheated standards correlated better with the activity seen in the PDE assay.

3. Procedure

The CaM RIA was performed according to the instructions which came with the RIA kit. The major steps are summarized below:

The preheated calmodulin standard was used to prepare the standard curve. With serial dilutions, a set of standards ranging from 100 ng/0.1 ml to 0.31 ng/0.1 ml was prepared. The FeLV sample used in this study was prepared by Dr. Mark Lewis in the Department of Veterinary Pathology and Microbiology, the Ohio State University, according to the method described by Mathes et al. (55). The other viral preparations were prepared in a similar way and were provided by Dr. Mark Lewis. These samples were preheated at 90°C for 30 minutes prior to the assay. Our preliminary results indicated that this treatment could increase the sample reading to four times that of the unheated sample.

The reaction mixture was prepared in 12 x 75 mm plastic tubes as follows:

Total: 100 ul $^{125}\text{I}$-calmodulin (about 20,000 cpm per sample).

Blank: 100 ul $^{125}\text{I}$-calmodulin and 200 ul buffer.
"0" standard: 100 ul $^{125}$I-calmodulin, 100 ul Ab and 100 ul buffer.

Standards: 100 ul $^{125}$I-calmodulin, 100 ul standard and 100 ul Ab.

Samples: 100 ul $^{125}$I-calmodulin, 100 ul sample and 100 ul Ab.

The reaction mixtures were incubated overnight (16-24 hr) at 4°C. After the incubation, 500 ul of second Ab was put into each tube except the "Total" tubes. This was incubated on ice for at least 30 minutes. The tubes were then centrifuged at 5,000 x g for 15 minutes at 4°C. The supernatant of each tube was poured into a bucket for liquid radioactive waste. The residual fluid was wiped off with tissue paper and the tubes were counted in a gamma counter for one minute per sample. The results of the standards, expressed as % of Total, were plotted on Log-Logit paper. The concentration of calmodulin in each sample was determined from the standard curve.
Protein assay

1. Principle

The determination of protein was by the method of Lowry et al. (45).

2. Material

Reagent A: 2% NaCO₃ in 0.1 N NaOH
Reagent B: 0.5% CuSO₄·5H₂O; 1% Na/K tartrate
Reagent C: 50 parts of reagent A & 1 part of reagent B, prepared before use.
Reagent D: phenol reagent (from the Lab stores, the Ohio State University) This reagent was titrated against NaOH & adjusted to 2 N in the store already.
Standard: Bovine serum albumin, 1 mg/ml.

3. Procedure

To prepare the standard, 480 ul of stock BSA solution was added to 720 ul of 0.1 N NaOH to make the highest concentration of the standard (400 ug/ml). 0.5 ml of this solution was added to 0.5 ml of 0.1 N NaOH to get 200 ug/ml. This was repeated four more times to get a set of standard solutions ranging from 400 to 12.5 ug/ml. The samples for protein determination were dissolved in 0.1 N NaOH before the assay.
To initiate the assay, 200 ul of standards (or samples) was put into duplicate 12 x 75 mm glass tubes, and 2 ml of reagent C was added to the tubes. This was incubated for 10 minutes at room temperature. Then 100 ul of reagent D was added to the tubes and this was incubated for another 30 minutes at room temperature. The tubes were then read at 580 nm by a spectrophotometer. The standards were plotted by a curve fitter program on a computer and the protein concentration in each sample was determined from the standard curve.

Reverse transcriptase (RT) assay

1. Principle

Retrovirus, such as the feline leukemia virus, has a unique RNA-dependent DNA polymerase (reverse transcriptase) which can transcribe RNA into DNA (65). If we provide RT with its substrate (thymidine), a template (polymer of adenosine) and a primer (a short stretch of thymidine polymer), this enzyme can build a polymer of thymidine on the poly-adenosine template. This polymer can be precipitated by trichloroacetic acid (TCA), and separated from free thymidine by filtration. If we provide the enzyme with $^3$H labeled thymidine, we can count the activity
retained on the filter paper as an indication of RT activity. The measurement of RT activity described below is based on the method of Poiesz, et al. (64).

2. Material

Suspension buffer: 25 mM Tris-HCl (pH 7.5), 5 mM dithiothreitol, 50 mM KCl, 50% (v/v) glycerol, 0.025% Triton X-100.

Disruption reagent: 0.9% Triton X-100 in 1.5 M KCl.

Reaction buffer: 80 mM Tris-HCl (pH 7.8), 8 mM dithiothreitol, 90 mM KCl and 0.5 mM MnCl₂. Since 50 ul of this buffer is added to a total reaction mixture of 100 ul, the final concentration of each component is half of the concentration listed here.

Template-Primer complex: poly(A).dT₁₂₋₁₈ (Pharmacia, # 27-7878) A stock solution of 250 ug/ml was prepared in deionized water, and stored in freezer.

³H labeled thymidine & unlabeled thymidine:
(Methyl-³H)-thymidine 5'-triphosphate (Amersham, #TRK 424): The package was 5 mCi in 5 ml with a specific radioactivity of 47 Ci/mmol.
Thymidine 5'-triphosphate (Sigma, # T0251): The molecular weight is 482 g/mole.

The hot thymidine was diluted with the cold thymidine to get a specific radioactivity of 16.18 Ci/mmol, as described below:

The actual amount of thymidine in the 5 ml of $^3$H-thymidine:

$$5 \text{ mCi}/47 \text{ Ci/mmol} = 10.6 \times 10^{-8} \text{ mole}$$

Cold thymidine $20.3 \times 10^{-8} \text{ mole}$ (in 0.5 ml) was added to the hot thymidine to get a total thymidine of $30.9 \times 10^{-8} \text{ mole}$. (preparation of the cold thymidine: 9.785 mg/50 ml, use 0.5 ml)

The resultant mixture contained 5 mCi of radioactivity in $30.9 \times 10^{-8} \text{ mole}$ thymidine with a total volume of 5.5 ml. The specific radioactivity was 16.18 Ci/mmol at 9.1 uCi/10 ul.

3. Procedure

The samples for RT assay were either from a FeLV infected 3201 cell culture or from sucrose gradient purified virus. To test the RT activity in the infected cell culture, 7.5 ml of culture medium was collected and centrifuged at 1,000 x g for 10 minutes. The supernatant was mixed with 0.3 ml of 4 M NaCl and 3.6 ml of 30% polyethylene glycol. This was placed on ice for 2 hours followed by a centrifugation at 20,000 x g for 45 minutes.
The pellet, which contains the virus, was then resuspended in 1.5 ml of suspension buffer. To disrupt the viral particle, 100 ul of this viral preparation was mixed with 100 ul of disruption reagent at room temperature for 30 minutes. The RT activity in this solution was then measured, as described below. If the purpose of the experiment is to examine the ability of an agent to lyse purified virus, it is advisable to use FeLV sample which is freshly prepared. Freezing-and-thawing will disrupt the viral particle, causing release of the RT. This in turn will lead to a very high background RT activity.

Initial characterization of the RT assay:

Initial studies were designed to characterize the RT assay and determine its response to changes in the amount of substrate, the amount of enzyme and the length of incubation time.

The effect of viral lysis on the expression of RT activity: In order to determine the RT activity from a given amount of virus, the sucrose-gradient purified FeLV was treated with Triton X-100 (disruption reagent) and the RT activity released from the lysed virus was determined. As indicated in fig.1, there was a great difference of the RT activities between the Triton X-100 lysed sample and the intact virus.
Figure 1. Effect of viral lysis on the expression of RT activity.

The RT assay was performed as described in the Methods. 400 ul of unfrozen sucrose-gradient purified FeLV was centrifuged at 100,000 x g for 1 hour. The pellet was resuspended in the original volume of suspension buffer. Half of the viral suspension was lysed by incubating with the same volume of disruption reagent for 30 minutes at room temperature. The other half was mixed with the same volume of suspension buffer as control. 20 ul of the lysed FeLV solution was used in the assay, which contained 9.1 uCi of $^3$H-thymidine and 5 ug of poly A.dT$_{12-18}$. The total volume for each assay was 100 ul. The reaction was carried out at 37°C for 3 hours and terminated by adding 1 ml of 10% trichloroacetic acid solution. The precipitated nucleic acid polymer was collected on a filter paper by a cell harvester and read in a scintillation counter. Each bar represents the mean ± S.D. of two samples.
Figure 2. Time-response of RT assay.

Triton X-100 lysed FeLV was used in this experiment. The reaction was carried out for one, two and three hours then terminated by trichloroacetic acid precipitation. Each point represents the mean ± S.D. of two samples.
Figure 3. Titration of $^3$H-thymidine in RT assay.

The reaction conditions of this experiment were similar to fig.2, except that the $^3$H-thymidine incubated in each sample varied. The reaction time was three hours. There was a dose-dependent increase of nucleic acid polymer formed as a result of increasing $^3$H-thymidine present in the reaction mixture. Each point represents the mean ± S.D. of two samples.
Figure 4. Titration of FeLV by reverse transcriptase assay.

An aliquot of purified FeLV was serially diluted in normal saline, then lysed with Triton X-100. 20 μl of the lysed virus from each dilution was used in the RT assay. There was a dose-dependent relationship between the amount of nucleic acid formed and the amount of RT present in the reaction mixture. Each point represents the mean ± S.D. of two samples.
Time-response of RT assay: In order to determine the optimal time of incubation for the RT assay, several incubation times were used. The incorporation of $^3$H-thymidine was a function of the incubation time. This is illustrated in fig.2. We determined that a 3-hour incubation was suitable for our purpose although higher counts could be obtained if the incubation time was longer.

Titration of $^3$H-Thymidine: In order to determine the optimal amount of substrate needed for the RT assay, we incubated different amounts of substrate in the reaction mixture. The incorporation of thymidine onto the template is a function of the substrate present in the reaction mixture, as indicated in fig.3. We determined that 9.1 uCi/sample was suitable for our purpose.

Titration of FeLV by RT assay: We attempted to establish that there was a linear relationship between the amount of RT in the sample and the incorporation of substrate onto the template. Using sucrose-gradient purified FeLV as the source of RT, we demonstrated that the incorporation of $^3$H-thymidine onto the template is proportional to the amount of virus used. This is illustrated in fig.4.

On the basis of these experiments, the optimal conditions for RT assay were determined. In a typical RT
assay the reaction mixture contained 50 ul stock reaction buffer, 20 ul poly A.dT₁₂₋₁₈ (5 ug), 10 ul ³H-thymidine (9.1 uCi) and 20 ul viral preparation. Each treatment contained two samples, and two assay tubes were used to determine the RT activity for each sample.

The reaction mixture was incubated in a 37°C water bath for 3 hours. The reaction was terminated by adding 1 ml of 10% trichloroacetic acid solution to the reaction mixture. The precipitated nucleic acid polymer was collected by a cell harvester (Brandel, M-24R) on a Whatman filter paper (934-AH, 2.0" x 12"). The radioactivity on the filter paper was measured in the scintillation counter.

**Determination of viral infectivity:**

There are at least three ways to determine the infectivity of FeLV in vitro. First, when 81 c cells are infected with FeLV, there will be a group of infected cells (plaques) formed after the cells reach confluence. The number of plaques is proportional to the infectivity of the virus (22). Second, FeLV contains a viral specific enzyme, reverse transcriptase (RT). We can measure the level of this enzyme as an index of the amount of virus present (64,65). Third, the virus produces several specific proteins, including p27. This can be measured using an enzyme-linked immunosorbant assay (ELISA) (48).
We evaluated all three techniques in order to determine which one was best for our purpose. Initially the plaque assay was used to measure viral infectivity. The 81 c cells were plated into 24-well plates, infected with FeLV, and the number of plaques formed was determined after 7 days. Although plaque formation does provide useful information about infectivity, scoring of the number of the plaques was highly subjective. We therefore chose to use infectivity assays which were more objective. We next measured reverse transcriptase after 7-day viral infection. Our results indicated that the level of RT produced by FeLV was too low to be measured in our experimental setting. Although RT was measurable when larger quantities of infected cells were used, the time, expense and use of large quantities of radiolabelled material was not desirable. Finally, we measured the viral specific protein p27 by ELISA as an index of infectivity. Results from the ELISA were comparable to those from RT assay. In addition, this assay was much more sensitive than the RT assay.

**Enzyme-linked immunosorbant assay (ELISA) for p27**

1. Principle

The measurement of p27 by ELISA was developed by Lutz et al. (48). Three monoclonal antibodies against different
epitopes of p27 are used in this assay. The first one is coated on the bottom of the ELISA wells. The other two antibodies, which are coupled to horseradish peroxidase as an enzymatic indicator, are added to the wells along with p27 samples. As a result of incubating these components together, antibody-p27-antibody complexes are formed. The number of the complexes formed is a function of the amount of p27 present in the reaction mixture. The antibodies that do not participate in the complex formation are washed away at the end of the incubation period. A color develops when the substrate for the peroxidase, together with a chromogen, is added to the wells. The intensity of the color is proportional to the amount of p27 in the sample. Using a standard curve based on p27 protein, the amount of p27 in the sample can be estimated.

2. Material

The ELISA kit was purchased from the Synbiotic Co. (San Diego, CA). The kit was composed of 5 reagents as listed below:
Reagent A: anti-p27 monoclonal antibodies conjugated with horseradish peroxidase.
Reagent B: p27 positive control.
Reagent C: normal cat serum as negative control.
Reagent D: chromogen (tetramethylbenzidine) for color development.
Reagent E: hydrogen peroxide as substrate.

3. Procedure

The ELISA was performed according to the instructions of the manufacturer with some modifications in order to get the optimal results. The major steps of the modified procedure were listed below.

Standards used in the ELISA: A purified p27 standard was obtained from Dr. Eric Bean of the Synbiotic Co.. This was used for the calibration of the secondary standard. A FeLV infected N-FL-74 cell culture (79) (provided by Dr. Mark Lewis, the Department of Veterinary Pathology and Microbiology, the Ohio State University) was used to prepare the secondary standard. Cells were removed by centrifugation at 20,000 x g for 10 minutes. The supernatant was collected and centrifuged again at 100,000 x g for one hour to remove intact FeLV particles. This 100,000 x g supernatant was saved and used as the secondary standard of p27. Based on the primary standard, we determined that the stock secondary standard contained 6655 ng/ml of p27. This was used as the standard for all the experiments performed.

To prepare the standard curve, the stock solution was diluted with complete McCoy's 5A medium to 1/30 of the
original concentration. This was used as the first concentration in the standard curve. This solution was then diluted by mixing one part of this solution with one part of the medium to get the second point. This was continued to the 7th concentration. This standard curve covered the range of 221.8 ng/ml to 3.5 ng/ml of p27, with McCoy's 5A medium alone as zero concentration.

Standardization of the p27 ELISA:

Initial experiments with the p27 ELISA kit were done according to the manufacturer's instructions. Further experiments indicated that with some modification of the conditions, the sensitivity and reproducibility were improved. As described previously, there are several different steps involved in this assay. In the first step, three different monoclonal antibodies are incubated with the antigen, p27. In the second step, the substrate for horseradish peroxidase, together with the chromogen, are added and incubated. The manufacturer suggested reading at this step. However, we found that addition of sulfuric acid caused the formation of a more intense color. We therefore added this step. We determined the optimal incubation times for each step in order to get maximum sensitivity and reproducibility.

We first determined the time needed for the antibody-antigen reaction. There is a time-dependent increase of
binding between the antibodies and p27 (fig.5). The increase appeared to continue up to at least 3 hours. We chose a 3 hour incubation for our subsequent experiments. However, it is possible that a longer incubation time might further increase the sensitivity of the assay.

In this assay, the formation of color is a function of the amount of the indicator enzyme present. However, prolonged incubation consumes all the substrate no matter how much enzyme is present. As a result, the color appears the same in every sample after prolonged incubation. On the other hand, the lower concentrations of p27 require an incubation time which is long enough to produce a measurable color. Our results indicated that for routine experiments, 15 minutes of incubation at this step was appropriate (fig.6). The color of the solution at this stage was greenish-blue, which had absorption peaks at both 405 nm and 690 nm.

The enzymatic reaction is stopped by addition of 1 N sulfuric acid. This converts the color of the solution from greenish-blue to yellow which had an absorption peak at 450 nm. The absorption of the acid treated sample was much greater than that of the untreated sample. As a result, a lower concentration of p27 could be determined. The intensity of this yellow color changed slightly after prolonged standing (fig.7). We routinely did the measurement 30 minutes after the addition of sulfuric acid.
The p27 ELISA was performed as described in the Methods. The p27 positive control which came with the ELISA kit was used in this experiment. 50 µl of reagent A was added to the antibody-coated wells followed by addition of 50 µl of samples. The antigen-antibody reaction was started at staggered time periods and all were stopped at the same time. This resulted in antigen-antibody reaction time ranging from 0.5 to 3 hours. To stop the reaction, the wells were washed with deionized water 5 times and blotted dry between each wash. 50 µl of reagent D (chromogen) was then added to each well followed by 50 µl of reagent E (substrate). This enzymatic reaction was carried out for 15 minutes and stopped by addition of 50 µl of 1 N sulfuric acid to each well. The samples were read at 450 nm 30 minutes after sulfuric acid addition. There was a time-dependent increase in antigen-antibody binding as shown by the increase of reading at 450 nm. Each point represents the mean ± S.D. of four samples.
Figure 6. Incubation of the horseradish peroxidase, substrate and chromogen.

The reaction conditions of this experiment were similar to those of the experiment shown in fig. 5. p27 positive control was incubated with the antibodies for three hours. After the washes, reagent D and reagent E were added to each well. The enzymatic reaction was carried out for the indicated times and stopped by the addition of 1 N sulfuric acid. The samples were read 30 minutes after the sulfuric acid addition. Each point represents the mean ± S.D. of four samples.
Figure 7. Change of readings at 450 nm after sulfuric acid addition.

The reaction conditions of this experiment were similar to those of the experiment shown in fig. 5. The p27 samples were incubated with antibodies for 3 hours. The enzymatic reaction with horseradish peroxidase was carried out for 15 minutes then stopped with 1 N sulfuric acid. The same set of samples was read at several times after the sulfuric acid addition. Each point represents the mean ± S.D. of four samples.
Figure 8. Standard curve for p27 ELISA.

The cells from a 5-day old N-FL-74 culture were removed by 20,000 x g centrifugation for 10 minutes. The supernatant was centrifuged again at 100,000 x g to remove the FeLV particles. This 100,000 x g supernatant was used as the source of our p27 standard. The p27 concentration in this preparation was 6655 ng/ml as calibrated against the affinity-purified p27. This solution was diluted to 1/30 in complete 81 C cell medium and stored in the freezer as our stock for secondary standard. To set up a standard curve, this stock was serially diluted in the complete 81 C medium and used in the ELISA. The standard curve was fitted using a polynomial curve fitter program (Curve Fitter, Interactive Microware, Inc.) with n=2. The coefficient of correlation of this curve was 0.99. Each point represents the mean ± S.D. of two samples.
The ELISA procedure: To minimize the handling time for each step, the reagents were added using an automatic pipeter (the Eppendorf Repeater). To initiate the assay, 50 ul of Reagent A was added to the ELISA wells followed by 50 ul of sample (or standard). The antibody-antigen mixture was incubated at room temperature for three hours. At the end of the incubation, the wells were washed with distilled water five times and blotted dry between each wash. Then 50 ul of Reagent D was added to the wells followed by 50 ul of Reagent E. This was incubated at room temperature for 15 minutes. During this period, a bluish-green color developed in the wells. At the end of the 15 minute incubation period, 50 ul of 1 N sulfuric acid was added to the wells. This stopped the reaction and converted the bluish-green color to an intensive yellow color. The absorption at 450 nm was read 30 minutes after the sulfuric acid addition by an ELISA reader (Flow Laboratories, Multiskan MC). A typical standard curve using our secondary standard is shown in fig.8. With polynomial curve fitting, the coefficient of correlation was 0.99.

Correlation of p27 ELISA with RT assay: In order to use the p27 ELISA assay as a quantitative measure of infectivity, it must be compared with a standard assay, the RT assay. The comparison between these two assays was
carried out using the 3201 cell line, derived from feline T-cells. These cells contained significant RT activity as well as p27 in the culture supernatant after FeLV infection. The cells were infected and the medium was collected and analyzed both for RT and p27.

The infection of 3201 cells and the measurement of the RT activity is detailed in the previous sections of Methods. On day one, 15 ml of infected cells (2 x 10^6 cells per ml) were prepared. Starting from day 2, 7.5 ml of medium containing cells were removed from the culture and replaced with 7.5 ml of fresh medium. The medium collected was centrifuged at 400 x g to remove the cells. This cell-free medium was saved for the RT assay and ELISA. There was a time-dependent increase in RT activity from the supernatant of 3201 cells after they were infected with FeLV, as indicated in fig.9. A parallel ELISA was done by using an aliquot of the supernatant from the same experiment. There was a time-dependant increase in p27 level in the infected culture, as shown in fig.10. Since this ELISA was done before the use of sulfuric acid and the availability of a p27 standard curve, the absorbance readings at 405 nm were used to determine the relative concentration of p27. When the RT activity from each day's sample was plotted against the corresponding p27 level,
Figure 9. Expression of RT activity from 3201 cells after FeLV infection.

20 ml of 3201 cells (2 x 10^6 cells/ml) were infected with FeLV as described in the Methods. Following infection, 7.5 ml of the cell suspension was taken from the culture and replaced with 7.5 ml of fresh medium each day for 8 days. The cell suspension collected was centrifuged at 400 x g for 10 minutes and the supernatant was used for RT analysis. Each point represents the mean of two samples. There was a time-dependent increase of RT activity in the culture medium.
Figure 10. Expression of p27 from 3201 cells after FeLV infection.

An aliquot of supernatant obtained from the experiment in fig. 9 was analyzed by p27 ELISA. The results indicated that there was a time-dependent increase of p27 in the culture medium. Each point represents the mean of two samples.
Figure 11. Correlation between the expression of RT and p27 from FeLV infected 3201 cells.

A comparison of the results from fig.9 and fig.10 indicated that there was a good correlation between RT and p27 expression. The coefficient of correlation was 0.96.
there was a very good correlation between the RT and ELISA assays. This indicated that both methods are suitable for measuring viral infectivity (fig.11).

The amount of sample needed to do the RT assay was much more than what was needed for p27 ELISA. As described in the Methods, it took 7.5 ml of culture supernatant to measure RT activity. On the other hand, it took only 50 ul to measure the p27 by ELISA. While both assays gave comparable results, the RT assay needed 150 times as much volume for a single sample. Based on this calculation, the ELISA appeared to be 150 times more sensitive than the RT assay. Because ELISA was more sensitive and this method did not involve the use of radioactive material, we decided to use this as our assay in measuring viral infectivity.

**Infection of 81 c cells by FeLV and the measurement of infectivity with p27 ELISA:**

The culture supernatant from permanently infected N-FL-74 cells (79), provided by Dr. Mark Lewis of the Department of Veterinary Pathology and Microbiology, the Ohio State University, was used as the source for FeLV. The cells in the culture were removed by centrifugation at 400 x g for 20 minutes. The supernatant was pipetted into 2.5 ml fractions and stored at -70°C. This viral solution was titrated for infectivity before the actual experiments.
There were several factors which altered infectivity including the number of cells used, the amount of virus used, the total exposure time of cells to virus, pretreatment of the cells with DEAE-dextran, and the day on which infectivity is measured. First, the expression of p27 was a function of the initial number of cells infected. Up to a point, there was an increase in p27 as we increased the number of cells seeded as shown in fig.12. The p27 did not increase when we plated down more than 50,000 cells per well. We thus decided to use this number of cells per well in our subsequent experiments. At this density, cells usually became confluent 4 days after plating. Second, there was a dose-dependent relationship between the amount of virus used to infect the cells and the expression of p27. This is demonstrated in fig.13. Routinely, we titrated the virus and diluted it to a concentration which yielded approximately 100 ng/ml of p27 after a 7-day incubation. Third, pretreating the cells with DEAE-dextran led to a great increase in viral infectivity, as shown in fig.14. This method is commonly used in virology experiments when a higher infectivity is desired (80). Fourth, longer exposure of host cells to virus led to a higher level of infectivity, as shown in fig.15. For our routine experiments, we infected the cells for one hour without DEAE-dextran pretreatment. Free viruses were washed away after the incubation and fresh medium was added
Figure 12. Infectivity as a result of the number of cells plated.

A confluent 81 c cell culture was split to 1/3 of the original cell density and incubated overnight. This culture was trypsinized the second day and a series of cell suspensions ranging from 2,500 to 50,000 cells per ml were prepared. One ml from each of these suspensions was plated and resultant culture was incubated for another 24 hours prior to FeLV infection. At the time of infection, the medium in each of the wells was replaced with 0.5 ml of fresh medium then 0.2 ml of FeLV was added to the culture and incubated for an hour. After this one-hour infection period, the free virus was washed away with PBS. Fresh medium was added to the culture, which was incubated for another 7 days. The p27 concentration from the 7th day's medium was analyzed by ELISA. Each point represents the mean ± S.D. of four samples. The results indicate that the expression of p27 was a function of the number of the initial cells infected.
Figure 13. Infectivity as a result of virus titer.

Cells were prepared as described in fig.12, except 50,000 cells were plated into each well. The FeLV stock was serially diluted in complete medium and used for infection. The infection period was one hour. The medium was analyzed for p27 after 7 days. Each point represents the mean ± S.D. of four samples. The results indicate that the amount of p27 produced was a function of the titer of virus used to infect the cells.
Figure 14. Effect of DEAE-dextran preincubation on FeLV infectivity.

Cells were prepared as described in fig.12. To initiate this experiment, the medium was removed from each well, replaced with 0.5 ml of DEAE-dextran medium (0.03 mg/ml) and incubated at 37°C for 30 minutes. After the incubation period, the DEAE-dextran medium was replaced with 0.5 ml of complete medium. The cells were then infected with FeLV for one hour and the medium was analyzed for p27 after 7 days. The open bar indicates the degree of infectivity without pretreating the cells with DEAE-dextran. The diagonal bar represents the increase of infectivity when the cells were pretreated with DEAE-dextran before FeLV infection. Each bar represents the mean ± S.D. of four samples.
The cells in this experiment were prepared as described in fig.14. After DEAE-dextran pretreatment, the cells were infected with FeLV for different lengths of time. Free viruses were washed away at the end of the incubation, and the culture was incubated for another 7 days. The result indicated that there was a higher infectivity when the infection period was increased. Each point represents the mean ± S.D. of four samples.

Figure 15. Infectivity as a function of the length of infection period.
Figure 16. Expression of p27 from 81 c cells after FeLV infection.

50,000 cells were plated and infected with FeLV as described in the Methods. The medium from a set of samples was collected every other day and analyzed for p27 concentration. The results indicated that p27 concentration in the medium increased gradually and reached a plateau on day 7. Further incubation of the culture did not increase the p27 concentration. Each point represents the mean ± S.D. of four samples.
to the wells. Finally, there was a gradual increase in p27 in the medium after the cells were infected with FeLV. The highest level of p27 was usually reached between day 7 and day 9, as shown in fig.16. For our routine experiments we tested the level of p27 on day 7 to determine the degree of infectivity.

In summary, for a routine experiment, we plated down 50,000 cells per well into the 24-well plates one day before the experiment. On the day of the experiment, the cells were infected with FeLV for one hour. Then the free viruses were washed away by PBS, and fresh medium was added to the culture. This culture was incubated in a 37°C incubator for 7 days before it was harvested. The medium was not changed during this 7-day incubation.

**Protein isolation**

This work was done in cooperation with Dr. Hitoshi Ebata in the laboratory of Dr. David Johnson, the Department of Physiological Chemistry, the Ohio State University. The calmodulin-like protein was isolated by a two-step column chromatographic purification with a FPLC system (Fast Protein Liquid Chromatography), as described below.

100 ml of sucrose gradient purified FeLV (55) was tip sonicated for 5 minutes, then centrifuged at 20,000 x g for
20 minutes at 4°C. The supernatant was collected and dialyzed against 2 mM CaCl₂, 20 mM MOPS and 0.2 mM PMSF, pH 7.2 in the cold room for 4 hours.

1. Phenylsepharose column:

The FeLV sample prepared in the previous section was applied to a phenylsepharose column (25) (10 ml size, pre-equilibrated with buffer A: 20 mM MOPS, pH 7.2, 0.2 mM PMSF, 2 mM CaCl₂) then eluted with buffer A. The flow rate was adjusted to 2 ml/min, and the samples were collected every 2 minutes. This was continued until no more protein came out of the column (about 18 fractions), then the column was eluted with buffer B (20 mM MOPS, pH 7.2, 0.2 mM PMSF, 2 mM EGTA). The flow rate was adjusted to 1 ml/min, and samples were collected every 2 minutes.

The fractions collected from the phenylsepharose column were analyzed for the ability to activate heart PDE by the fluorimetric PDE assay. 50 ul of each of the fractions was used in a 30 minute fixed-time PDE assay. The fractions which had the highest activities were pooled together and applied to the Mono-Q ion exchange column.

2. Mono-Q ion exchange column (Pharmacia, HR 5/5):

This column was pre-equilibrated with buffer A (20 mM MOPS, pH 7.0, 0.1 mM PMSF) before use. After the sample (fractions #11-16 from the previous column, a total volume
of 12 ml) was applied, the column was eluted with buffer A. The flow rate was 1 ml/min and 1 ml fractions were collected. When no more protein came out of the column (after about 6 fractions), the column was eluted with buffer B (20 mM MOPS, pH 7.0, 0.1 mM PMSF and 1.5 M KCl). The fractions collected were analyzed for the ability to activate heart PDE as described in the previous section. 10 ul of each fraction was used for each sample.

**Iodination of FeLV**

1. Principle

The iodination of FeLV was done according to the method of Markwell (53), which employed 'Iodo-beads' as the iodination reagent. Iodo-beads are N-Chloro-benzenesulfonylamine derivatized polystyrene beads, which provide the oxidizing power in the iodination of tyrosine residues in proteins.

2. Material

The Iodo-beads were purchased from the Pierce Chemical Co. (Rockford, IL). $^{125}\text{I}$ (1 mCi) was purchased from Amersham (Arlington Height, IL). Dialysis tubing (Spectrapor, M.W. cutoff: 6,000-8,000) was purchased from National Scientific Co. (Cleveland, OH). Dialysis buffer:
50 mM Tris, pH 7.2, 0.9% NaCl. Sucrose gradient purified FeLV was provided by Dr. Mark Lewis of the Department of Veterinary Pathology and Microbiology.

3. Procedure

The iodination was performed according to the protocol given by the manufacturer. In brief, 20 Iodo-beads were washed with phosphate buffer, dried, and put into a 12 x 75 tube containing 1 mCi of $^{125}$I (in 1 ml buffer containing 50 mM Tris buffer, pH 7.2 and 0.9% NaCl). To initiate the iodination, 10 ml of freshly prepared FeLV solution was added to the reaction tube, mixed well, and incubated at room temperature. 15 minutes later, the reaction was terminated by removing the Iodo-beads from the reaction tube. Free $^{125}$I in the reaction mixture was removed by dialysis against a total of 8 liters of buffer with 5 changes over a period of 6 hours. An examination of the resultant solution indicated that 60% of the radioactivity was protein associated, which was precipitated by 5% trichloroacetic acid solution.
RESULTS

Identification of a calmodulin-like protein in purified FeLV

Initial Observations:

Initial experiments carried out in our laboratory by Dr. Mark Lewis indicated that when cat lymphocytes were stimulated with mitogen, their cAMP was increased. This increase was inhibited by UV-inactivated FeLV. On the basis of this observation, we considered the possibility that the virus contains a factor which can alter cyclic nucleotide metabolism. To test this possibility, experiments were designed to determine if the viral lysate could activate cAMP phosphodiesterase, the enzyme which catalyzes the breakdown of cAMP to 5'AMP.

Activation of PDE by purified FeLV:

Initial experiments were designed to test if sucrose gradient-purified feline leukemia virus had the ability to activate phosphodiesterase (PDE). In order to obtain maximal activation, calmodulin-free PDE was used in this experiment. Our results using a bioluminescence PDE assay
(fig.17) indicated that purified virus was able to activate PDE in a dose-dependent manner. Without FeLV, the basal cAMP hydrolysis rate by PDE was 1 umole/mg protein/hour. In the presence of FeLV, the cAMP hydrolysis rate was increased to 3.8 umoles/mg protein/hour. Since calmodulin is a well established PDE activator, we ran a parallel experiment using authentic calmodulin. Our results (fig.18) indicated that calmodulin activated the PDE in a dose-dependent manner, from 1.2 umoles/mg protein/hour to 4.5 umoles/mg protein/hour. The pattern of PDE activation by FeLV was very similar to that of the authentic calmodulin.

The activation seen in the bioluminescence assay system was verified using another PDE assay. In this system, the decrease in fluorescence is an indication of PDE activity. As shown in fig.19, we started the reaction by adding 100 ug of calmodulin-free PDE into a reaction mixture containing 8 uM of Mant-cGMP. The reaction was carried out for 20 minutes and the basal PDE activity was obtained. At the end of 20 minutes, 2.1 ug of FeLV lysate was added to the reaction mixture, and the reaction was carried out for another 30 minutes. The results (dashed line) indicated that the fluorescence level decreased sharply as soon as the FeLV was added. From this we concluded that the FeLV contained a factor which could activate PDE. As a comparison, we repeated the experiment
Figure 17. Activation of bovine heart PDE by dissociated FeLV.

Sucrose-gradient purified FeLV was boiled for 10 minutes and spun at 2,000 x g for 10 minutes. The resulting supernatant fraction was tested in this experiment. The reaction mixture contained 0.6 µg of PDE, 225 µM of cAMP, reagent A and reagent B as described in the Methods. The reaction was carried out at 37°C for one hour and terminated by boiling the samples for 5 minutes. The amount of 5'AMP formed was measured by the luciferin-luciferase assay. Each point represents the mean ± S.D. of three samples. The results indicated that the purified FeLV contained a factor which was able to activate PDE in a dose-dependent fashion.
Figure 18. Activation of bovine heart PDE by calmodulin.

The experiment was done as described in fig. 17 except that calmodulin was used as the PDE activator. Each point represents the mean ± S.D. of three samples.
Figure 19. Activation of heart PDE by calmodulin and by FeLV.

Sucrose-gradient purified FeLV was disrupted by sonication, centrifuged at 20,000 x g, and the supernatant was used as the source of FeLV. 50 ul of the FeLV lysate (2.1 ug) was used in this experiment. The reaction mixture consisted of 100 ug of PDE, 8 uM of cGMP in a buffer containing 10 mM of MOPS, 1 mM of CaCl$_2$, 5 mM of MgCl$_2$ and 90 mM of KCl. The total volume for this reaction mixture was 1 ml. PDE was added at time zero and the reaction was run for 20 minutes to get a basal PDE activity. After this, FeLV (dashed line) or 2 nM calmodulin (solid line) was added to the reaction mixture. There was a rapid decrease in the fluorescence level when PDE was activated either by the FeLV or calmodulin.
using authentic calmodulin as the PDE activator. Our results (solid line) indicated that 2 nM of authentic calmodulin was able to activate the PDE. The pattern of PDE activation by FeLV was essentially the same as that by authentic calmodulin. The results from this experiment confirmed the results we saw in the bioluminescence PDE assay.

Questions raised after this initial observation:

Based on our initial experiments, we concluded that FeLV contained a factor which was able to activate bovine heart PDE. The pattern of PDE activation caused by FeLV was very similar to the pattern caused by calmodulin. We considered several possible explanations for this phenomenon.

The first possibility we considered was that the purified FeLV was associated with calmodulin. Although calmodulin is present in all eukaryotic cells, the presence of calmodulin in lower organisms, such as virus, has never been reported. Alternatively, the PDE activation might be due to a calmodulin-like protein instead of the authentic calmodulin. For example, oncomodulin, a calmodulin-like protein present in some transformed cells, is able to activate heart PDE (57). A third possibility was that the virus contained a unique viral protein which was neither
calmodulin, nor oncomodulin, but had the ability to activate heart PDE in a way similar to calmodulin.

There were several other possible explanations for our initial observations. Since viruses usually contain enzymes that are crucial for viral functions, it was possible that the FeLV contained PDE. Alternatively, the FeLV might contain proteolytic enzymes which could activate the heart PDE in the reaction mixture. The activation of heart PDE by proteolytic enzymes has been reported (38). Finally, several reports indicated that PDE could be activated by some phospholipids (63,90). There existed a possibility that the FeLV contained such phospholipids.

In order to determine which of the above explanations was responsible for the initial observations we made, we designed a series of experiments to test each of the possibilities. Our basic hypothesis was that the FeLV contained calmodulin, and this was responsible for the PDE activation we saw in the initial experiments. To test this hypothesis, we first designed a series of experiments to rule out other possible explanations for the observed PDE activation. Second, we designed experiments to compare the FeLV factor and calmodulin. Finally, we isolated the factor from FeLV and examined its biochemical characteristics directly.
Evidence that FeLV does not contain PDE:

In order to test if the FeLV sample had PDE activity of its own, we repeated the fluorimetric PDE assay by incubating the FeLV sample with Mant-cGMP in the absence of PDE. Our result indicated that there was no measurable PDE activity associated with the sample (data not shown).

Evidence that PDE activation was not due to the presence of proteolytic enzymes in the FeLV:

Further experiments demonstrated that the FeLV could be boiled for 10 minutes and still retain its ability to activate PDE (see below). Since most enzymes can be inactivated by boiling, our results suggested that the PDE activation by FeLV was not due to any proteolytic enzymes present in the virus.

Evidence that PDE activation was not due to phospholipids:

It has been reported that some phospholipids, such as lysophosphotidyl choline, have the ability to activate PDE (63,90). We therefore considered the possibility that the PDE activation we saw was due to phospholipid contamination. The activation of PDE by phospholipids is calcium independent, and can be abolished by detergent and
phospholipase C. This enabled us to design a series of experiments to test the possibility that phospholipids were responsible for the PDE activation we saw.

**Calcium dependence:**

In order to test if the activation by feline leukemia virus was calcium dependent, we included the calcium-chelator, EGTA, in the reaction mixture. The results (fig. 20) demonstrated that the basal cAMP hydrolysis rate by PDE was 1.2 umoles/mg protein/hour. In the absence of EGTA, 1 ug of FeLV was able to activate PDE to 350% of its basal activity (open bar). The addition of EGTA to the reaction mixture caused a dose-dependent decrease in PDE activity. When the EGTA concentration was 100 uM or more, there was no detectable PDE activation by FeLV. As a comparison, we ran a parallel experiment using 0.05 unit of calmodulin as the PDE activator. Our results indicated that in the absence of EGTA, calmodulin (solid bar) was able to activate PDE to the same extent as FeLV. The addition of EGTA caused a dose-dependent decrease of the calmodulin-stimulated PDE activity. If removal of calcium by EGTA inhibited the PDE activation by either calmodulin or FeLV, then addition of calcium back into the reaction mixture should reverse the inhibitory effect. Our subsequent experiments (fig. 21) demonstrated that while 100 uM of EGTA abolished the PDE activation of FeLV, addition
Figure 20. Calcium dependence of the activation of PDE by FeLV.

This experiment was similar to the experiment shown in fig. 17, except that a fixed amount of calmodulin (0.05 unit) or FeLV (1 ug) was used. The calcium concentration in the sample was 30 μM. Various concentrations of EGTA were added to the reaction mixture to chelate calcium. Cross bar: PDE alone. Solid bar: calmodulin + PDE. Open bar: FeLV + PDE. Each bar represents the mean ± S.D. of three samples.
Figure 21. Reversal of EGTA inhibition by calcium.

This experiment was similar to that shown in fig.20. Cross bar: PDE alone. Solid bar: Calmodulin + PDE. Open bar: FeLV + PDE. Each bar represents the mean ± S.D. of three samples.
of 100 uM calcium reversed this inhibitory effect (open bars). With authentic calmodulin as the PDE activator, we saw exactly the same patterns (solid bars). Based on these experiments, we concluded that the activation of PDE by FeLV was calcium dependent, which suggested that the activation was not due to phospholipid.

Effect of detergent on FeLV activity:

Pichard and Cheung reported that the detergent Lubrol-PX at a concentration of 0.1% prevented the activation of PDE by phospholipids (63). Although this detergent partially interfered with the detection of 5'AMP in our assay system, the behavior of FeLV in the presence of detergent was very similar to that of authentic calmodulin (result not shown).

Effect of phospholipase C:

The activation of PDE by phospholipid can be abolished by phospholipase C as demonstrated by Wolff and Brostrom (90). However, incubation of PDE with phospholipase C may sometimes lead to a partial digestion of PDE and cause a non-specific activation of this enzyme, as shown by Pichard and Cheung (63). Apparently, the outcome is dependent on the preparation of the PDE. The PDE we used could be
activated by phospholipase C (data not shown). As a result this is not a useful way to differentiate the property of calmodulin from phospholipids.

Effect of ether extraction:

It was reasoned that if the activity we saw was a result of phospholipid, we should be able to remove the activating factor by ether extraction. Our result indicated that FeLV sample retained 85% of its activity after it was extracted 3 times with diethyl ether (data not shown).

From the fact that the activation of PDE by FeLV was calcium dependent, could not be abolished by ether extraction and behaved in a similar way under detergent treatment, it was concluded that this activation was not due to viral-associated phospholipids.

These results established that the PDE activation by FeLV was not due to an intrinsic PDE activity associated with the FeLV particles, and it was not likely that a viral-associated proteolytic activity was responsible for the activation. In addition, non-specific PDE activation by phospholipids was ruled out.

We next evaluated the similarities between the active factor and authentic calmodulin. Calmodulin has a number of distinctive characteristics. For example, it is a heat
stable protein which is able to activate a number of enzymes, such as PDE and the calcium-magnesium ATPase from red blood cells, and its activity can be inhibited by a series of structurally different calmodulin antagonists. These properties enabled us to design a series of experiments to test the hypothesis that the activator from FeLV was calmodulin.

**Similarities between the viral factor and calmodulin:**

1. **Heat stability:**

   Calmodulin is a heat stable protein which can be boiled and still retain the ability to activate PDE. To test the heat stability of FeLV, we put the sample into boiling water for 10 minutes and tested the ability to activate PDE. Our results indicated that FeLV was still active after this treatment (fig.17).

2. **Trypsin treatment:**

   In order to determine if the activating factor in the FeLV is a protein, we first treated FeLV with the proteolytic enzyme trypsin and then measured its ability to activate PDE. The FeLV was treated with 0.001 % of trypsin at room temperature for one hour and then boiled for 5 minutes to stop the reaction. As a control, we boiled the trypsin for 5 minutes before incubating it with FeLV.
Basal PDE activity was monitored for 18 minutes, then the activators were added (fig. 22). The results indicated that when the FeLV was digested with trypsin, it lost the ability to activate PDE (dashed line). On the other hand, if the trypsin was inactivated first, the FeLV retained the ability to activate PDE (solid line). Based on the observation that trypsin inhibited the activating factor associated with FeLV, we concluded that the viral factor was a protein.

3. Inhibition of FeLV activity by calmodulin antagonists:

In order to examine the similarities between calmodulin and the viral protein, we tested a series of structurally different calmodulin antagonists to see if they could inhibit the activity of the protein. The compounds tested were trifluoperazine, chlorpromazine, W-7, calmidazolium, propranolol, and compound 48/80.

Inhibition by trifluoperazine (TFP):

The first group of calmodulin antagonists we tried was the phenothiazines, with TFP as the representative agent. In the presence of calcium, TFP is able to bind to calmodulin at a 2:1 ratio (85) and inactivate calmodulin. In order to test the effect of TFP on the viral protein, we included different concentrations of TFP in the PDE activation assay. The drug was first incubated with FeLV
Figure 22. Effect of trypsin treatment on FeLV activity.

The reaction conditions of this experiment were similar to those in fig.19. PDE was added at time zero and the reaction was carried out for 18 minutes. 55 ul (2.31 ug) of trypsin-treated FeLV (dashed line) or control FeLV (treated with inactivated trypsin) was then added to the reaction mixture. The results indicated that the active factor in FeLV could be digested by trypsin.
Figure 23. Inhibition of calmodulin and FeLV activity by trifluoperazine (TFP).

Calmodulin (or FeLV) was incubated with various concentrations of TFP before the addition of PDE and cAMP. The experiment was run under the same conditions as those described in fig. 20. The results indicated that the calmodulin antagonist TFP inhibited the activation of PDE either by calmodulin (solid square) or FeLV (open circle) in a dose-dependent manner. Each point represents the mean of three samples.
Figure 24. Inhibition of calmodulin and FeLV activity by W-7.

The conditions of this experiment were similar to those in fig.23. The calmodulin antagonist W-7 inhibited the activation of PDE by calmodulin (solid square) and FeLV (open circle) in a similar way. Each point represents the mean of three samples.
Table 1. Inhibition of calmodulin and FeLV activity by calmodulin antagonists.

The conditions of these experiments were similar to those in fig.23. The concentrations of calmodulin antagonists needed to inhibit 50% of calmodulin (or FeLV) activity were obtained by a linear regression of the dose-response curve.

<table>
<thead>
<tr>
<th>CaM antagonists</th>
<th>IC50 CaM</th>
<th>IC50 FeLV</th>
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<tbody>
<tr>
<td>chlorpromazine</td>
<td>75 uM</td>
<td>80 uM</td>
</tr>
<tr>
<td>propranolol</td>
<td>480 uM</td>
<td>400 uM</td>
</tr>
<tr>
<td>calmidazolium</td>
<td>0.15 uM</td>
<td>0.14 uM</td>
</tr>
<tr>
<td>compound 48/80</td>
<td>1.2 ug/ml</td>
<td>1.1 ug/ml</td>
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</table>
(or calmodulin), then PDE was added, followed by the substrate, cAMP. The reaction was carried out for one hour after the addition of the substrate. Our results (fig.23) indicated that TFP inhibited both authentic calmodulin (solid square) and FeLV (open circle) in a dose-dependent fashion. Without TFP, calmodulin activated the PDE to a cAMP hydrolysis rate of 4.8 umoles/mg protein/hour. 25 uM of TFP was able to decrease the PDE activity to 1.2 umoles/mg protein/hour, which was the basal PDE activity. The pattern of inhibition was essentially the same when we used FeLV as the PDE activator.

Inhibition by W-7:

W-7, a naphthalene sulfonamide compound, belongs to a new generation of calmodulin antagonists. Although it is not as potent as TFP, it is believed to have a higher specificity (77). In order to test the effect of W-7 on the viral protein, we incubated this compound in the reaction mixture and performed an PDE assay as described in the previous section. Our results (fig.24) indicated that this compound inhibited both authentic calmodulin and FeLV in a dose-dependent manner. 100 uM of W-7 was able to completely inhibit the activities of both authentic calmodulin and FeLV.
Inhibition by other calmodulin antagonists:

In order to extend the experiments we did with TFP and W-7, we determined the effect of a series of structurally different calmodulin antagonists on the PDE-activating effect of the viral protein. Our results indicated that these compounds could inhibit both the FeLV and the authentic calmodulin (table 1).

Since a series of chemically different calmodulin antagonists inhibited the activity of both calmodulin and FeLV, we concluded that FeLV contained a protein which is functionally very similar to calmodulin.

4. Activation of human RBC ATPase by calmodulin and FeLV:

In addition to PDE, calmodulin is able to activate a number of other enzymes, including the calcium-magnesium ATPase from human red blood cells, myosin light chain kinase from smooth muscle and phosphorylase kinase in the muscle. Further characterization of the viral protein required a demonstration of the effect of this protein on other calmodulin-dependent enzymes. We therefore tested the effect of the viral protein on the ATPase from human RBC. The activity of this enzyme is dependent on calcium concentration. There is evidence that, under physiological conditions, this is the mechanism by which the RBC maintains a constant calcium level. When this enzyme senses a higher concentration of calcium present inside the
RBC, it will become activated and start to pump the calcium out, with the hydrolysis of ATP as the energy source. In the presence of calmodulin, the enzymatic activity is greatly enhanced (11).

Activation of RBC ATPase by calmodulin:

To prepare the ATPase, human RBC were collected from whole blood and lysed by hypotonic shock. The RBC membranes were treated with EDTA to remove intrinsic calmodulin. After several washings, the resultant membrane preparation was used as the source for ATPase. This ATPase was incubated with different concentrations of calmodulin, then 20 nanomoles of ATP were added to the reaction mixture and incubated at 37°C for 10 minutes. The reaction was stopped by immersing the tubes in boiling water for 5 minutes and the ATP left in each sample was measured by the luciferin-luciferase assay. The difference in ATP before and after the incubation was calculated, expressed as umoles/mg protein/10 minutes, and used as an indicator of the activity of the ATPase. The results (fig.25) indicated that calmodulin was able to activate the ATPase in a dose-dependent manner. The activation was from 0.8 umoles/mg protein/10 minutes to 2.7 umoles/mg protein/10 minutes.
Human RBC ATPase was prepared as described in the Methods. The reaction mixture contained 6.7 ug of RBC membrane, 20 nanomoles of ATP and various concentrations of calmodulin in 150 ul of reagent X (11 mM of Tris buffer, 3 mM of MgCl₂ and 0.1 mM of CaCl₂). The RBC membrane was preincubated with calmodulin for 5 minutes at 37°C, then ATP was added and the mixture was incubated for another 10 minutes. The reaction was stopped by boiling the samples for 5 minutes. The ATP which remained in the sample was measured by luciferin-luciferase as described in the Methods. Each point represents the mean ± S.D. of three samples. The results indicated that the calmodulin was able to activate ATPase in a dose-dependent manner.
Figure 26. Activation of RBC ATPase by FeLV.

The conditions of this experiment were similar to those in fig. 25, except FeLV was added instead of calmodulin. FeLV was boiled for 10 minutes before the experiment in order to remove the endogenous ATPase associated with the virus. Each point represents the mean ± S.D. of three samples. The results indicated that FeLV was able to activate ATPase in a dose-dependent manner.
Activation of RBC ATPase by FeLV:

Sucrose gradient purified FeLV was used in this experiment. The virus appeared to contain intrinsic ATPase activity which could be removed by boiling (see Methods). Therefore, the FeLV was boiled for 10 minutes, and centrifuged. The supernatant was used in this experiment. Our results (fig.26) indicated that the FeLV was able to increase ATPase activity in a dose-dependent manner. The activation was from 1.2 umoles/mg protein/10 minutes to 2.7 umoles/mg protein/10 minutes.

In summary, our results indicated that, in addition to bovine heart PDE, the active factor in the FeLV also activated human RBC ATPase. The extent of activation by FeLV was similar to the extent of activation by authentic calmodulin. On the basis of these results, we concluded that FeLV contains a factor which is functionally similar to calmodulin.

Having established that FeLV contains a protein which is functionally similar to calmodulin, we next examined the structural similarities between these two proteins. One way to determine the structural similarity is to see if antibodies raised against calmodulin can recognize the viral protein. We used a calmodulin radioimmunoassay (RIA) to answer this question.
5. FeLV lysate cross-reacts with a calmodulin antibody:

In order to determine if the FeLV sample contained any protein which was immunochemically similar to the authentic calmodulin, we tested the sample with a calmodulin radioimmunoassay. The calmodulin RIA kit was purchased from New England Nuclear, and the assay was performed according to their protocol. As suggested by the protocol, the sample was heated at 90°C for 30 minutes before the RIA was performed. This procedure can increase the binding between the antibody and calmodulin. Our results indicated that the sucrose gradient purified FeLV does, in fact, bind to antibodies raised against calmodulin. The purified FeLV contained 23 ng of calmodulin per mg of protein (table 2).

Calmodulin level in other retroviruses:

In order to determine if this calmodulin immunoreactivity was present in other retroviruses, we collected a number of retroviruses from different hosts, such as cats, mice, non-human primates and human. The viruses from cat were Kowakami-Theilen FeLV (KT-FeLV), and Rhabdomyosarcoma 114 (RD-114), those from mouse were Rauscher leukemia virus (RLV), Balb C murine leukemia virus (Balb), and Murine leukemia virus-New Zealand Black (MuLV-NZB), those from non-human primates were M7/A204, and Gibbon Ape leukemia virus (GaLV) and the human retrovirus
Table 2. Calmodulin content in viral preparations as determined by radioimmunoassay.

Purified retroviruses from cat (KT-FeLV, RD-114), mouse (RLV, Balb, MuLV-NZB), primate (M7/A204, GaLV) and human (HTLV-1) were heated at 90°C for 30 minutes and then subjected to RIA. Each number represents the mean of two samples.

<table>
<thead>
<tr>
<th>Virus Isolate</th>
<th>ng calmodulin/mg protein</th>
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<tr>
<td>KT-FeLV</td>
<td>23</td>
</tr>
<tr>
<td>RD-114</td>
<td>17</td>
</tr>
<tr>
<td>RLV</td>
<td>12</td>
</tr>
<tr>
<td>Balb</td>
<td>15</td>
</tr>
<tr>
<td>MuLV-NZB</td>
<td>13</td>
</tr>
<tr>
<td>M7/A204</td>
<td>10</td>
</tr>
<tr>
<td>GaLV</td>
<td>13</td>
</tr>
<tr>
<td>HTLV-1</td>
<td>30</td>
</tr>
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</table>
was human T-cell leukemia virus type I (HTLV-I). Our results (table 2) indicated that all viruses tested cross reacted with calmodulin antibody.

**Summary:**

There is a heat-stable protein present in the FeLV sample which is functionally and structurally similar to calmodulin. This protein was able to activate heart PDE and human RBC ATPase. The activation of PDE was calcium-dependent and could be inhibited by a variety of calmodulin antagonists. The protein cross-reacted with antibodies raised against calmodulin.

Further proof for the presence of calmodulin in FeLV requires isolation from the purified virus and examination of the physical and biochemical properties of the purified protein.

**Isolation of the calmodulin-like protein and partial characterization**

This work was done in cooperation with Dr. Hitoshi Ebata in the laboratory of Dr. David Johnson, the Department of Physiological Chemistry, the Ohio State University.

1. **Isolation of the calmodulin-like protein**

   Because of its similarity to calmodulin, the active factor in FeLV was isolated according to published methods
for isolating calmodulin. The FeLV sample was sonicated, centrifuged and dialyzed as described in the Methods. This sample was applied to a phenyl-sepharose column (25) in the presence of calcium. The hydrophobic sites of calmodulin are exposed in the presence of calcium, which facilitates the binding of calmodulin to the phenyl-sepharose column. The sample was eluted from the column with EGTA. Removal of calcium from calmodulin can close the hydrophobic sites, which will lead to the dissociation of calmodulin from the column. The fractions eluted with EGTA were tested for the ability to activate heart PDE by a fluorimetric PDE assay. When the first fraction eluted with EGTA was designated as fraction #1, a major peak of activity appeared in fractions 11-16 (fig.27). The active fractions were then pooled and applied to an ion-exchange column (Mono-Q column). This column was eluted with 1.5 M KCl with a continuous gradient. The fractions collected were again tested for the ability to activate PDE. There was a major PDE-activating peak in fractions #19-21 (fig.28).

2. Properties of the isolated protein:

We determined the effect of this isolated protein on several enzymes which can be activated by calmodulin. The enzymes tested included bovine heart PDE, bovine brain PDE and calcineurin.
Figure 27. Purification of the calmodulin-like protein: A. Phenylsepharose column.

Sucrose-gradient purified FeLV was disrupted by sonication and prepared for column chromatography as described in the Methods. 80 ml (3.36 mg) of the FeLV sample was applied to phenylsepharose column. The column was first eluted with buffer A (2 mM CaCl₂, 20 mM MOPS and 0.2 mM PMSF) until no more protein came out of the column. Then the column was eluted with buffer B (2 mM EGTA, 20 mM MOPS and 0.2 mM PMSF) and 2 ml fractions were collected for the fluorimetric PDE assay. 50 ul of each fraction was used in the PDE assay. The result was expressed as percentage of basal PDE activity. The basal PDE activity was 0.46 nanomoles cGMP hydrolyzed/30 minutes.
Figure 28. Purification of the calmodulin-like protein:
B. Ion exchange (Mono-Q) column.

Fractions 11-16 of the phenylsepharose column (fig.27) were pooled and applied to an ion exchange column (Mono-Q). The column was eluted with a continuous gradient (1.5 M KCl, 20 mM MOPS, 0.1 mM PMSF, pH 7.0) and 1 ml fractions were collected. 10 ul of each fraction collected was used for the fluorimetric PDE assay. The basal PDE activity in this experiment was 0.49 nanomoles cGMP hydrolyzed/30 minutes.
Figure 29. Activation of heart PDE by the Mono-Q purified active factor and its inhibition by melittin.

Cyclic AMP and the active factor with or without melittin were added to the reaction cuvette at time zero. 25 ul of PDE was added in the 18th minute and the reaction was observed for 55 minutes. The bottom line indicates the activity of PDE in the presence of 5 ul of the purified viral factor. The top line indicates the inhibition of PDE activation by the viral factor in the presence of melittin. The dashed line indicates the activity of PDE alone. The activity of PDE alone was not affected by melittin. The results indicated that 1 uM of melittin was able to block the activation of PDE by the active factor.
Activation of heart PDE by the purified factor and its inhibition by a calmodulin antagonist, melittin:

We had previously demonstrated that the active factor in the FeLV lysate could activate heart PDE, and the activation could be inhibited by a number of calmodulin antagonists. We attempted to verify this using the purified factor. Fractions #19-21 from the Mono-Q column (fig.28) were pooled, and an aliquot was used in this experiment. The reactions were run under three conditions. First, PDE alone was used as a control. To start the reaction, cGMP was added to the reaction cuvette and the reaction was carried out for 18 minutes. Since there was no PDE present in the reaction mixture, the fluorescence level did not decrease. At the end of the 18th minute, PDE was added to the reaction mixture and the reaction was carried out for another 40 minutes. The results (fig.29) indicated that upon the addition of PDE, there was a slow decrease in the fluorescence level (dashed line). This is the basal PDE activity.

Second, to measure the effect of the active factor on PDE, we added cGMP and 5 ul of the active factor to the reaction cuvette. This reaction was observed for 18 minutes, then PDE was added to the reaction mixture and the reaction was carried out for another 40 minutes. The results (bottom line) indicated that the active factor greatly increased PDE activity.
Finally, to determine the effect of melittin on the active factor, we incubated cGMP and 5 ul of the active factor with 1 uM of melittin. The incubation was carried out for 18 minutes, then PDE was added to the reaction mixture and the reaction was monitored for another 40 minutes. The results (top line) indicated that 1 uM of melittin completely blocked the effect of the active factor. The combination of melittin, the active factor and PDE together gave essentially the same result as PDE alone (dashed line).

Based on this experiment, we concluded that the factor we isolated could activate heart PDE. Furthermore, the activation was inhibited by a calmodulin antagonist, melittin. This suggested, but did not prove, that the factor we isolated is calmodulin.

Dose-response of the active factor and authentic calmodulin:

In order to determine the relative potency of the viral factor compared to calmodulin, we generated a dose-response curve using heart PDE as the effector enzyme. Fractions #19-21 from the Mono-Q column (fig.28) were pooled and a dose-response curve on PDE activation was done. The results were compared to the activation seen with calmodulin. The continuous fluorimetric PDE assay was employed in this experiment. The basal rate of cGMP
hydrolysis by PDE alone was 0.05 nanomoles/10 minutes. This was designated as 0% activity. The PDE could be maximally activated to have a cGMP hydrolysis rate of 1 nanomoles/10 minutes by 0.5 nM of calmodulin. This was designated as 100% activity.

As shown in fig.30, the purified active factor was able to activate the PDE in a dose-dependent fashion (open circle). When this dose-response curve was compared to the curve generated using the authentic calmodulin (solid square), it revealed that the activity of 1 ul of the active factor was roughly equivalent to 0.1 nM of authentic calmodulin.

Activation of calcineurin and brain PDE by the active factor:

In addition to heart PDE, calmodulin is able to activate a number of other enzymes. To test if the protein we isolated could activate other calmodulin-dependent enzymes, we chose calcineurin (61) and brain PDE. The results we obtained from these experiments were not conclusive. The isolated protein from the first viral preparation did not activate either enzyme. When we repeated the protein purification, the isolated protein activated both calcineurin and brain PDE. The reasons for the difference between these two preparations are not clear at this time.
Figure 30. Activation of heart PDE by calmodulin and the Mono-Q purified active factor.

The increase in the initial rate of hydrolysis of cGMP by heart PDE as a function of the concentration of either the authentic calmodulin or the active factor is shown in this experiment. The basal rate of PDE alone was 0.05 nanomoles cGMP hydrolyzed/10 minutes, and 100% (maximal activation) corresponded to a rate of 1 nanomoles of cGMP hydrolyzed/10 minutes. The ability of different concentrations of calmodulin (solid square) or the viral active factor (open circle) was expressed as % of maximal PDE activation.
Summary:

The active factor purified using a two-step procedure appeared to be similar to calmodulin. It was able to activate heart PDE in a dose-dependent fashion. The maximal degree of heart PDE activation was similar to the activation we obtained from the authentic calmodulin. The activity was inhibited by the calmodulin antagonist, melittin. Since we did not have enough protein to run a gel electrophoresis, the purity and the molecular weight of the protein we isolated was not confirmed.
Do calmodulin antagonists lyse viruses?

There are a number of reports indicating the presence of calcium-binding proteins in viruses (see Introduction). Most reports suggest that calcium-binding protein(s), together with calcium, might play an important role in viral assembly and disassembly. Given the finding that FeLV contains a calmodulin-like protein, we hypothesized that this protein was responsible for maintaining the integrity of the FeLV particle.

To test this hypothesis, we chose to use the release of reverse transcriptase from the virus as a measure of viral lysis. FeLV is surrounded by an envelope, which consists of a lipid-bilayer containing the membrane proteins, p15E and gp70. Inside the envelope is a protein shell composed of the gag gene products, such as p27. Inside the protein shell is reverse transcriptase (RT), viral RNA and a nucleic acid-binding protein. When the virus is intact, very little RT activity can be detected. On the other hand, if the virus is broken, the RT will be released to the medium and can be measured.
Lysis of FeLV by calmodulin antagonists:

The sucrose gradient purified FeLV used in these experiments were obtained from Dr. Mark Lewis, the Department of Veterinary Pathology and Microbiology, the Ohio State University. To start the experiment, the FeLV was incubated with the drug to be tested at 37°C for one hour. At the end of the incubation, an aliquot of the reaction mixture was removed and tested for RT activity. As a control, the RT activity from untreated virus was considered as 0% lysis. RT activity from a sample lysed with the detergent Triton X-100 was considered as 100% (total) lysis (fig.1). The ability of an agent to lyse FeLV was expressed as % of total lysis.

The procedure for the RT assay is detailed in the Methods. For a typical RT assay, the reaction mixture contains RT, a polymer of adenosine as the template, a short stretch of thymidine polymer as the primer for RT and 3H-thymidine as the substrate. RT can build a 3H-thymidine polymer from this reaction mixture. This polymer can be precipitated by trichloroacetic acid and collected by filtration. The radioactivity retained on the filter paper is a function of the RT activity.

Trifluoperazine:

When we incubated the sucrose gradient purified FeLV with TFP, we found that TFP at a concentration of 200 uM
could lyse purified FeLV (fig.31). The degree of lysis was about 33% of what we found with Triton X-100. At this concentration, TFP inhibited RT activity slightly, which suggests the actual lysis might be slightly higher than 33%. Since TFP is able to inhibit calmodulin activity completely at 25 uM or less (fig.23), the lysis seen here may not be a result of calmodulin inhibition. A more likely explanation is that TFP at high concentration can disrupt membrane structure (69), which leads to viral disintegration.

Melittin:

Melittin, a peptide isolated from bee venom, is a very potent calmodulin antagonist. We found that it was able to lyse FeLV completely at a concentration of 25 uM, and 2.5 uM led to a 50% lysis (fig.32). However, this agent can inhibit calmodulin activity in the nanomolar range (3). As a result, the lysis may not be a direct result of calmodulin inhibition. A more likely explanation is that melittin, like TFP, is not acting as a calmodulin antagonist, but as a detergent. The lysis of retroviruses by high concentrations of melittin has been reported previously (19).
Figure 31. Lysis of purified FeLV by trifluoperazine.

Unfrozen sucrose gradient purified FeLV was treated with different concentrations of TFP at 37°C for 1 hour. The activity of RT released from the virus treated with Triton X-100 was used as 100% lysis, and the degree of lysis from each concentration of TFP was compared to this. TFP at 200 μM caused the lysis of approximately 33% of total virus. There was a 10% inhibition of RT activity by this concentration of TFP. Each point represents the mean ± S.D. of three samples.
Experimental conditions in this experiment were similar to those in fig.31; except melittin was used to lyse the virus. Melittin at 2.5 uM caused 50% of the virus to lyse. There appeared to be no inhibition of RT activity by melittin at concentrations up to 25 uM. Each point represents the mean of two samples.
W-7:

We did not get a good dose-response using W-7. Since the FeLV sample we used for this experiment was previously frozen and thawed, the virus was partially disrupted and there was a very high RT background. Under these conditions, it appeared that 200 μM of this agent had no effect on viral lysis, while 600 μM caused total viral lysis (data not shown).

Does EGTA lyse FeLV?

The activity of calmodulin is calcium-dependent. If the calmodulin-like factor is responsible for maintaining viral integrity, then removal of calcium should lead to viral lysis. To test this possibility, we incubated purified FeLV with EGTA and measured the RT released. The result indicated that EGTA at a concentration of 250 μM did not lyse the virus (data not shown). We did not try concentrations higher than this, because EGTA slightly inhibited RT activity at this concentration.

Summary:

In this series of experiments we tested three structurally different calmodulin antagonists (TFP, W-7 and melittin) for their ability to abolish viral integrity. Our results indicated that these agents could not disintegrate the virus at concentrations which inhibited
calmodulin activity. As a result, we were not able to demonstrate the role of the calmodulin-like protein in FeLV integrity.
Inhibition of viral infection by calmodulin antagonists

Although calmodulin antagonists were able to disrupt the viral particles only at concentrations which were well above the range of calmodulin antagonism, we considered the possibility that viral function could be impaired by this group of drugs. In fact, there are a number of reports describing the ability of calmodulin antagonists to decrease viral infection in vitro. The proposed mechanisms for the inhibition of viral infectivity all concerned cellular events during viral replication. For example, Nemerow, et al. (59) observed that a number of calmodulin antagonists, such as TFP and calmidazolium, were able to inhibit the transformation of B lymphocytes by Epstein-Barr virus. They proposed that these drugs were able to inhibit the entry of the virus into the host cells. On the other hand, Bohn et al. (4) demonstrated that TFP could inhibit the replication of measles virus. They proposed that TFP could inhibit the final budding process of the virus. Based on these reports and our finding of the calmodulin-like protein in FeLV, we tested the possibility that
calmodulin antagonists can decrease FeLV infectivity, and, if so, whether the virus or the host cell was the primary site of action.

**Effect of calmodulin antagonists on FeLV infectivity:**

81 C cells were used in this series of experiments. The cells were grown in 24-well plates at a density of 50,000 cells per well. In a typical experiment, the drug, at a volume of 0.5 ml, was first incubated with 81 C cells for one hour, then virus was added to the culture, which was incubated for another hour. At the end of this incubation, the drug and free viruses were washed away by PBS. Fresh medium without drug was added to the culture and the cells were further incubated for 7 days. At the end of this incubation, the amount of p27 was measured by ELISA.

The procedures for p27 ELISA are detailed in the Methods. In brief, 50 ul of sample was incubated with the horseradish peroxidase-coupled monoclonal antibodies against p27 for 3 hours. The excessive antibodies were washed away at the end of the incubation. The substrate for the horseradish peroxidase, together with the chromogen were added to the ELISA wells. This enzymatic reaction was carried out for 15 minutes then terminated by the addition
of 1 N sulfuric acid. The color developed during this period, which was an indication of p27 concentration, was measured by an ELISA reader.

1. Effect of phenothiazines on FeLV infectivity:
1.1 Presence of phenothiazines during infection:

We first tried the classical calmodulin antagonist, trifluoperazine (TFP). One mM of TFP was prepared in deionized water, sterilized by filtering through a 0.2 μm filter, and diluted with complete medium to get the desired concentration. As described in Methods, the drug was first incubated with the cells for one hour, then the challenging virus was added into the culture. Free virus and the drug were removed by washing at the end of the infection period. Fresh medium without drug was added to the culture and the production of virus was analyzed 7 days later by p27 ELISA. The degree of infectivity in the absence of drug was considered to be 100% of control, and the effect of the drugs were expressed accordingly.

Our results (fig.33) indicated that TFP was able to inhibit FeLV infection in a dose-dependent fashion (open circle). This effect was very prominent at concentrations between 30-50 μM. Concentrations higher than 50 μM appeared to have a significant adverse effect on host cell growth. Cells treated with 50 μM or higher concentrations of TFP usually detached from the bottom of the flask after the
Figure 33. Effect of TFP and TFP-sulfoxide on FeLV infectivity.

81 c cells were prepared as described in the Methods. Before the infection period, the cells were pretreated with 0.5 ml of TFP (open circle) or TFP-sulfoxide (solid square) for 1 hour. 0.2 ml of FeLV was added to the cells, which were then incubated for another hour. Free viruses and drug were washed away at the end of this infection period. Fresh medium without drug was added to the culture, which was incubated for another 7 days. The medium of the 7th day's culture was analyzed for p27 concentration by ELISA. Each point represents the mean ± S.D. of four samples. The result indicated that TFP at a concentration of 30-50 μM inhibited the infection of cells by FeLV. The inactive analog TFP-sulfoxide had no effect.
Figure 34. Effect of chlorpromazine and promethazine on FeLV infectivity.

The conditions for this experiment were similar to those in fig. 33. Each point represents the mean ± S.D. of four samples. CPZ (open circle) at concentrations between 40-60 μM was able to inhibit FeLV infection. Promethazine (solid square), a weak calmodulin antagonist, was not effective even at 60 μM.
infection period, and were washed away during PBS washing. The cells which remained appeared to have a slower growth rate, as observed under microscope.

As a control, we determined the effect of TFP-sulfoxide on viral infectivity. TFP-sulfoxide is a TFP analog which does not bind calmodulin, and thus has little inhibitory effect on calmodulin (86). Our results indicated that this agent, under the same experimental conditions, did not inhibit FeLV infection.

In order to verify the results we got from TFP and TFP-sulfoxide, we repeated the experiments using another set of calmodulin antagonists, chlorpromazine (CPZ) and promethazine. CPZ is slightly weaker than TFP in terms of inhibiting calmodulin activity. The calmodulin IC$_{50}$ for TFP is 10 uM and for CPZ is 42 uM. On the other hand, promethazine is a very weak calmodulin antagonist. The IC$_{50}$ for promethazine is 340 uM (82).

Our results (fig.34) indicated that CPZ was able to decrease FeLV infectivity in a dose-dependent fashion. The effect is especially evident at concentrations between 40-60 uM. On the other hand, promethazine has no inhibitory effects in this range.

Based on these two experiments, we concluded that the phenothiazine calmodulin antagonists are able to decrease FeLV infectivity. The inhibitory effect correlates well with the ability of these agents to antagonize calmodulin.
Since the drugs were present only before and during the infection period, it is likely these agents inhibit the early events of viral infection, such as viral binding or entry.

1.2 Presence of TFP throughout the 7-day incubation:

In the previous experiments, drugs were present only just before and during the infection period. There was a possibility that we could see a better inhibition of viral infectivity if we had the drugs in the culture throughout the 7-day incubation. In this case, we might be able to inhibit not only the infectivity of the challenging virus, but also the infectivity of the progeny virus.

To test this possibility, we incubated TFP with the cells for 7 days and determined the p27 level. Our results (not shown) indicated that 50 uM of TFP killed the host cells after 12 hours, and 25 uM TFP killed the cells in 3 days. The highest concentration the cells could survive for 7 days was 10 uM. Further experiments indicated that the presence of 10 uM TFP throughout the 7-day incubation did not decrease FeLV infectivity to any significant extent (fig.35). There was a slight decrease of p27 level in the TFP treated samples (open circle) as compared to the controls (solid square), but the difference was not significant.
Figure 35. Effect of 7-day TFP incubation on FeLV infectivity.

81 C cells were pretreated with 10 μM of TFP for one hour then infected with FeLV as described in fig.33. After the removal of free virus, fresh medium containing 10 μM of TFP was added to the wells. A set of samples was collected every other day and analyzed for p27 concentration. Each point represents the mean ± S.D. of four samples. The results indicated that the presence of 10 μM TFP in the medium (open circle) led to a slight decrease in p27 level as compared to the untreated controls (solid square).
An 81c cell culture was permanently infected with FeLV and grown for 2 1/2 months (15 passages) prior to this experiment. 50,000 cells were plated into separate wells and incubated overnight. The culture was washed with PBS the next day then fresh medium containing 10 μM TFP was added. This medium was collected 24 hours later and replaced with fresh medium containing the same concentration of TFP. This was repeated to day 8. The medium collected was analyzed for p27 content by ELISA as described in the Methods. Each point represents the mean of four samples. The results indicated that TFP treated samples (open circle) produce as much p27 as the untreated control (solid square).
1.3 Effect of TFP on permanently infected cells:

We tested the possibility that TFP could alter viral growth on a permanent infected cell culture. Unlike the cells used in previous experiments, these cells were infected with FeLV permanently and kept releasing viruses into the medium. There existed a possibility that the constant presence of TFP could interrupt the replication cycle by inhibit the budding of mature virus, entry of the virus to the neighboring cells or subsequent replication of the virus in the cells.

The experiment was initiated by plating down the cells into 24-well plates and growing them in medium containing 10 μM of TFP. The medium was collected daily and replaced with fresh medium containing TFP. The medium from each day was analyzed for the concentration of p27. Our results (fig.36) indicated that the samples treated with 10 μM TFP (open circle) had a concentration of p27 which was comparable to the controls (solid square). Based on these results, we concluded that the presence of TFP does not decrease the expression of p27 from permanently infected cells.

1.4 Preparation of TFP in serum-free medium:

The TFP in the previous experiments was prepared in complete medium containing 15% fetal bovine serum. We considered the possibility that a fraction of TFP was bound
to the serum protein. If so, the free drug which was presented to the system was lower than the total drug added. In order to examine this, we prepared the drug in serum-free medium and used this preparation to repeat the TFP titration experiment in 1.1.

Our results (fig.37) indicated that under these conditions TFP was able to decrease viral infectivity in a dose-dependent fashion. The drug caused a clear inhibition of viral infection at 15-20 uM. TFP at a concentration higher than 20 uM appeared toxic to the host cells. Since TFP prepared in serum-free medium was a more accurate reflection of the concentration of drug presented to the system, we decided to continue the experiments under these conditions.

Summary:

Our results indicated that the presence of 50 uM of TFP or 60 uM of CPZ during the infection period protected cells from FeLV infection. On the other hand, TFP-sulfoxide or promethazine at equivalent concentrations had no effect. When the TFP was prepared in serum-free medium, there was a clear decrease of FeLV infectivity at 15-20 uM of TFP. Long term incubation of TFP killed the host cells at concentrations above 10 uM. Incubation of cells with 10
Figure 37. Effect of TFP (prepared in the absence of fetal bovine serum) on FeLV infectivity.

81 c cells were prepared as described in the Methods. Before FeLV infection, the cells were pretreated with TFP for one hour. The TFP was prepared in medium without serum. The cells were then infected with the FeLV for one hour. After the infection period, free viruses and TFP were removed and complete medium without drug was added. The 7th day's medium was collected for p27 measurement. Each point represents the mean ± S.D. of four samples. Under these conditions TFP at 5-20 μM was effective in decreasing viral infectivity. *p<.05, **p<.01, ***p<.005 as compared to control using Student's t-test.
Figure 38. Effect of TFP treatment on the protein content after 7 day incubation.

The protein content of the cells in the experiment described in fig.37 was measured. After removal of the medium on the 7th day post-infection, the culture was washed with PBS (2 ml x 2). The cells in each well were then solubilized by 2 ml of 0.1 N NaOH and analyzed for protein content. Each point represents the mean ± S.D. of four samples. The results indicate that TFP treatment did not affect the final protein content.
Figure 39. Effect of TFP treatment on the $^{3}\text{H}$-thymidine uptake during the 24 hours post-infection.

Cells were prepared and infected with FeLV as described in fig.37. After the infection period, the cells were given complete medium containing 1 uCi/ml of $^{3}\text{H}$-thymidine. The medium was removed 24 hours later and the culture was washed extensively with PBS. The cells were then removed from the wells by trypsin and the $^{3}\text{H}$-thymidine uptake was measured. Each point represents the mean ± S.D. of four samples. The result indicated that TFP at concentrations between 5-15 μM had no effect on $^{3}\text{H}$-thymidine uptake. 20 μM of TFP caused a 40% inhibition of this process.
um TFP for 7 days did not decrease the infectivity. Similarly, 10 um TFP did not decrease the expression of p27 from permanently infected cells.

2. Why does TFP decrease FeLV infectivity?

Our results indicated that TFP had an inhibitory effect on some early process in viral infection, but the site of action was not clear. The effect of the drug might be on the virus or on the host cells. It is possible that TFP could damage the viral particles. The defective viruses might either have difficulty in binding to host cells, or might be able to bind to host cells but be unable to infect the cells. On the other hand, altered host cell functions might interfere with viral replication.

2.1 Effect of TFP on host cell functions:

TFP appeared to have no effect on the host cells at concentrations of 5-15 um. The cells grew well, and the total protein in the 7th-day's culture was the same as that in the control cells (fig.38). On the other hand, 20 um of TFP appeared to detach a small fraction of cells during the 2-hour incubation. The cells which remained appeared to grow more slowly during the 7-day culture period. While most wells reached confluence after 3-4 days of culture, the 20 um TFP-treated cells required 5-6 days to reach confluence. However, when the total amount of protein on
the 7th day's culture was measured, there was no difference between the control cells and the 20 uM TFP-treated cells.

The uptake of $^3$H-thymidine by cells is often used as an indicator of cell growth rate and replication. We used this parameter to determine the effect of TFP on host cell growth rate in the 24-hour period after drug treatment. In this experiment, cells were treated with TFP and infected with FeLV as in previous experiments. At the end of the infection period, the cells were exposed to medium containing $^3$H-thymidine. The cells were harvested 24 hours later, and the amount of $^3$H-thymidine incorporated was counted by a scintillation counter. Our result indicated that TFP at 5-15 uM had no effect on $^3$H-thymidine uptake by the host cells (fig.39). However, 20 uM of TFP significantly decreased the $^3$H-thymidine uptake. This indicated that host cell growth was decreased in the 24 hours after brief exposure to 20 uM of TFP.

In summary, our results indicate that TFP at 5-15 uM appears to have no effect on normal cell growth, since the treated cells incorporate $^3$H-thymidine as well as the control cells, and they appeared to grow normally as observed under microscope. On the other hand, 20 uM had a significant effect on host cell function. These cells did not take up $^3$H-thymidine well and appeared to have a
slower growth rate in general. This might be partly responsible for the decrease of infectivity seen at this concentration in previous experiments.

2.2. Does TFP damage the virus?

Our previous experiments with reverse transcriptase indicated that TFP at 20 uM or less caused the release of less than 2% of RT stored in the virus (fig.31). It is unlikely that this degree of viral lysis was fully responsible for the decreased infectivity we observed. However, it is possible that TFP treatment causes a subtle change in viral structure which leads to a decrease of viral binding or internalization during the infection period. However, this is unlikely, since pretreating the virus with TFP did not decrease FeLV infectivity (data not shown).

There exists an alternative explanation for the decreased infectivity seen with TFP. It is possible that TFP treatment can cause changes in host cell physiology which lead to a general suppression of host cell protein synthesis. This in turn could lead to a decrease in viral protein synthesis, hence an apparently lower viral replication. In other words, the decrease of viral replication may be the result of some non-specific
alteration of host cell functions. If this is the case, then TFP will have an effect if it is given either before or after the infection period.

2.3 Evidence that TFP needs to be present during the viral infection period:

We performed the following experiment in order to determine if TFP causes a non-specific alteration of host cell physiology, which leads to a decrease of viral replication. Three sets of samples were prepared in this experiment. In the first set (fig.40, pre-treated), the cells were exposed to 15 uM TFP for two hours, then the drug was washed away by PBS. Fresh medium without TFP was added to the culture and incubated for two hours. Then the cells were infected with FeLV as usual. This determined the effect of pre-exposing the host cells to TFP on FeLV infectivity. In this condition, only the host cells were exposed to the drug. The virus was never exposed to the drug.

The second set of samples was treated with our standard procedure (fig.40, treated during). The cells were pretreated with TFP for one hour. The drug was left on the cells and virus was added for an additional hour. This determined the effect of TFP when it was in contact with both the host cells and the virus. The third set of
samples was a reverse of the first set (fig.40, post-treated). The cells were infected with the virus first. The virus was then washed away. Two hours after the end of the infection period, the cells were exposed to the drug for two hours. This determined the effect on infectivity of post-exposing the cells to drug. As in the first set of samples, the viruses in this sample were never exposed to the drug.

In summary, we treated the host cells with 15 µM of TFP at different time periods relative to the time of infection. The drug was given either 2 hours before the infection period, 2 hours after the infection period or during the infection period, as indicated in fig.40.

Our results (fig.41) indicated that TFP was effective only when the drug was present together with the cells and the virus. The drug had no effect when it was given before or after the infection period. From these results we concluded that: (1) The inhibition of FeLV infectivity by TFP was not a result of non-specific host cell inhibition, since every sample in this experiment was treated with TFP and handled in exactly the same way; (2) TFP appeared to inhibit the viral-cell interaction, probably due to an effect on the binding or internalization process.
81 C cells were prepared as described in the Methods. Three sets of samples were prepared and treated with TFP at different time periods relative to FeLV infection, as described in the text.

*Note* In order to treat every sample in the same way, the following procedure was necessary:

1. Whenever one sample was given 0.5 ml of 15 uM TFP (prepared in the serum-free medium), the others would be given the same medium. When the sample was washed with PBS to remove the drug, others were washed in the same way.

2. The sample treated with TFP during the infection received 0.5 ml of drug in the first hour, and another 0.2 ml of FeLV was added to the wells during the second hour. As a result, the real TFP concentration in the second hour was diluted to 10.7 uM. In order to create the same condition, the other samples were treated the same way. They were given 0.5 ml of TFP in the first hour and 0.2 ml of medium in the second hour.

Figure 40. Scheme for TFP treatment before, during and after viral infection.
Figure 41. Effect of TFP treatment before, during and after viral infection.

81c cells were treated with 15 μM of TFP either 2 hours before, during or 2 hours after FeLV infection, as detailed in the text and illustrated in fig. 40. Each bar represents the mean ± S.D. of four samples. The results indicated that in order for TFP to be effective the drug must be present during the infection period. The sample with TFP during the infection period had significantly lower p27 level (p<.005 as compared to control as determined by Student's t-test).
Figure 42. Effect of W-7 on FeLV infectivity.

The experimental conditions were the same as that of fig.37, except that W-7 prepared in serum-free medium was used in this experiment. Each point represents the mean ± S.D. of four samples. The results indicated that W-7 at 40-50 μM was able to decrease FeLV infectivity.
Figure 43. Effect of W-7 treatment on the $^3$H-thymidine uptake during the 24 hours post-infection.

A set of cells were treated with W-7 prepared in the serum-free medium and infected with FeLV as described in fig.42. The effect of W-7 on the $^3$H-thymidine uptake was determined as described in fig.39. Each point represents the mean ± S.D. of four samples. The results indicated that W-7 at 40-50 uM could inhibit $^3$H-thymidine uptake.
2.4 Binding of FeLV to the host cells:

In order to determine if TFP could inhibit the binding of FeLV to the host cells, we labeled the virus with $^{125}\text{I}$ and monitored the binding of this labeled FeLV to host cells in the presence of TFP. This turned out to be an unsuccessful approach. The purified FeLV was externally labeled with $^{125}\text{I}$ by Iodo-beads, as described in the Methods. They appeared to bind to 81 C cells in a dose-dependent manner. There was a plateau of the binding curve, as would be expected from the binding to limited binding sites. However, when the labeled viruses were incubated with human fibroblast cells in culture, they had a similar degree of binding. Since human fibroblasts are not a host for FeLV, this indicated that the binding seen with 81 C cells might not be specific (data not shown).

3. Effect of W-7 on viral infectivity:

W-7, a non-phenothiazine calmodulin antagonist, was tested for its ability to decrease FeLV infectivity under conditions identical to those used with TFP. Our results indicated that this agent was able to decrease viral infectivity at 40-50 μM (fig.42). However, this concentration range also inhibited cell growth, as indicated by a $^3\text{H}$-thymidine uptake experiment (fig.43). As a result, W-7 might inhibit FeLV infection at the expense of normal cell growth.
4. Effect of other calmodulin antagonists on viral infectivity:

In addition to TFP and W-7, we also tested other agents which could inhibit calmodulin activity on this infectivity assay. The examples we tested include calmidazolium and quinacrine. These experiments were done with the drugs prepared in medium containing fetal bovine serum. The results were not conclusive. Under the experimental conditions, calmidazolium was not effective up to 1 uM. Quinacrine at 10 uM had a significant inhibitory effect. However, this agent also inhibits normal cell growth at this concentration (data not shown).

Summary:

Among the calmodulin antagonists tested, only the phenothiazines gave a reproducible inhibition of viral infectivity within a concentration range which did not inhibit normal cell growth. As a result, we currently do not have enough evidence to support the hypothesis that this inhibition is a calmodulin-associated phenomenon. The phenothiazines appeared to decrease FeLV infectivity by inhibiting the viral binding and/or internalization process. Since the drugs did not appear to lyse the viruses in the concentration range tested, and pretreating the virus with this agent did not decrease FeLV infectivity, we believe the effect of TFP is due to
inhibition of some cellular events. These drugs might change the characteristics of a normal cell membrane, which, in turn, might impair the viral binding or internalization.
SUMMARY OF THE RESULTS

1. Calmodulin as the calcium-binding protein in FeLV.

The lysate of sucrose gradient purified FeLV contains a heat-stable protein which is functionally similar to calmodulin. This factor is able to activate bovine heart cAMP phosphodiesterase in a manner similar to authentic calmodulin. The activation is calcium dependent and can be inhibited by a series of calmodulin antagonists. The calmodulin antagonists tested include trifluoperazine, chlorpromazine, W-7, calmidazolium, compound 48/80 and propranolol. The IC₅₀'s for inhibition of PDE activation by FeLV lysate and authentic calmodulin are very similar in all cases. The FeLV lysate is also able to activate calcium-magnesium ATPase from human red blood cell membrane. The extent of activation is similar to that seen with the authentic calmodulin.

With a calmodulin radioimmunoassay we were able to determine that FeLV lysate contained 23 ng of calmodulin per mg of protein. A screening of other retroviruses including those isolated from primates, mice and cats, indicated that all viruses tested contained calmodulin-like immunoreactivity.
The calmodulin-like protein was purified by a two-step column chromatography. The sample was first purified by a phenylsepharose column and followed by an ion exchange column. The purified protein was able to activate heart cAMP PDE in a dose-dependent manner. The activation was calcium dependent and could be inhibited by a calmodulin antagonist, melittin. The protein concentration was too low to be detected in the SDS-PAGE. As a result, the molecular weight of this protein is currently unknown.

2. The role of calmodulin in maintaining viral integrity.

The degree of retroviral lysis was monitored by the release of reverse transcriptase from the viral particle. Using the RT activity from Triton X-100 lysed virus as 100% lysis, the relative ability of drugs to lyse the virus were compared. We demonstrated that TFP at a concentration of 200 uM caused a 33% lysis of the purified FeLV. TFP at this concentration had a slight (10%) inhibition of RT activity. As a result, the real extent of lysis might be slightly higher than it appeared.

Another calmodulin antagonist tried was melittin, a peptide isolated from bee venom. This peptide was able to cause a 5% viral lysis at 0.25 uM, 50% lysis at 2.5 uM and 100% lysis at 25 uM. There appeared to be no inhibition of RT activity by this agent at the concentrations tested.
A third calmodulin antagonist, W-7, caused a total viral lysis at 600 uM. There was no apparent lysis at 200 uM or less. Finally, there was no viral lysis by 250 uM of EGTA. Higher concentrations of EGTA interfered with the RT assay.

3. Calmodulin antagonists alter FeLV infectivity.

A commercially available p27 ELISA kit was characterized and modified for quantitative use. We determined that 3 hours was a reasonable incubation time between the antibodies and the p27 antigen. The incubation time for horseradish peroxidase and the substrate was determined to be 15 minutes. The assay sensitivity was increased by using sulfuric acid to terminate the reaction. The reading at 450 nm was taken 30 minutes after sulfuric acid addition. The resultant ELISA was able to measure p27 concentration ranging from 0 to 200 ng/ml.

The infection of 81 c cells by FeLV was carefully characterized. The factors which affect the degree of infectivity include: a. the titer of virus used to infect the cells; b. the number of cells seeded for the infection; c. the length of infection period; d. the presence of DEAE-dextran; e. the time of incubation post-infection. For a routine infectivity experiment, we seeded 50,000 cells per well into the 24-well plate. The cells were infected with FeLV for one hour. The free virus was removed after this
infection period, and the p27 level was determined 7 days later by ELISA. The degree of infection determined by p27 ELISA correlated very well with the RT assay. The coefficient of correlation was 0.96. However, the ELISA turned out to be a preferred assay because of its sensitivity and simplicity.

The effect of calmodulin antagonists on FeLV infectivity was determined. Pretreatment of cells with trifluoperazine caused a dose-dependent decrease in viral infectivity. Similar results were obtained using another phenothiazine, chlorpromazine. TFP-sulfoxide (a TFP analog which does not bind calmodulin) and promethazine (a weak calmodulin antagonist) had no effect under the same conditions.

When TFP was prepared in medium containing serum, it took 50 uM of TFP to demonstrate a clear inhibition of viral infection. However, when TFP was prepared in the medium without serum, the inhibition of FeLV infection could be seen at 20 uM or less. When the drug was prepared in the medium without serum, TFP at 20 uM appeared to damage the host cells to some extent. This was determined by the $^3$H-thymidine uptake by the cells during the first 24 hours post infection. There was no apparent alteration of host cell function by 15 uM of TFP. The effect of TFP could only be seen when the drug was present together with the virus and the host cells. The presence of the drug
before or after the infection period did not decrease FeLV infectivity. This suggested that this drug could inhibit viral binding or internalization.

Another calmodulin antagonist tested was W-7. We determined that this drug did not inhibit $^3$H-thymidine uptake at a concentration of 30 uM or less. This agent had little inhibitory effect on viral infection at this concentration. Other calmodulin antagonists tried include calmidazolium and quinacrine. The effectiveness of these agents could not be established with the existing data.
DISCUSSION

The origin of the calmodulin-like protein in FeLV:

Our results indicate that FeLV contains a calcium-binding protein which is similar to calmodulin. The origin of the virus-associated calmodulin is not clear. There are several possibilities. First, it is possible that the virus encodes for calmodulin. This is unlikely, since the FeLV genome does not contain calmodulin-like calcium-binding sites (personal communication with Dr. David Johnson). Second, it is possible that the calmodulin is simply a contaminant artifact which occurs during the purification process. The amount of calmodulin present in the virus is considerably less that in the cells. We estimated that purified FeLV contained 23 ng of calmodulin per mg of protein. This is about 0.002% of the total protein. On the other hand, the calmodulin content of cells is approximately 0.1-1% of total protein. The low amount of calmodulin present in the purified FeLV raised the possibility that this was a contamination of cellular calmodulin during virus purification.

To explore this possibility, we incubated sucrose gradient purified virus with $^{125}$I-labelled calmodulin, then went through sucrose gradient isolation again. In this
experiment, the virus was purposely contaminated with calmodulin. If calmodulin is a contaminant of the virus, it should bind to the virus, and co-purify with the virus. Our results (data not shown, see ref. (42)) indicated that the virus and the $^{125}$I-calmodulin did not co-purify under these conditions. This suggested that the calmodulin found in the virus was not simply an artifact of the purification process. In addition, samples which contained intact FeLV did not activate PDE. The activity was only seen when the virus was disrupted with sonication in the presence of EGTA (personal communication with Dr. David Johnson). This indicated that the viral calmodulin is tightly associated with the virus instead of being a loosely bound contaminant. One possible explanation for the presence of calmodulin in FeLV is that the virus may incorporate cellular calmodulin during the budding process. This will require further confirmation.

It is not clear if viruses in general contain calmodulin. The results we obtained with the calmodulin RIA indicated that calmodulin immunoreactivity was present in a variety of retroviruses (table 2). However, viruses from other classes were not tested. While we found that purified FeLV contains a protein which can activate cAMP PDE, Krizanova, et al. (40) reported that purified influenza virus contained a factor which could inhibit the activity of calmodulin activated heart PDE. Briefly, they
first demonstrated the activation of PDE by calmodulin. Then, in the presence of purified influenza virus and ATP, the activity of the calmodulin-activated PDE was shown to decrease. For reasons not clear to the authors, the presence of ATP was necessary to see the inhibition. If the virus was heated at 56°C for 30 minutes, the inhibitory effect disappeared. The inhibitory effect also disappeared if the calmodulin was used at a concentration higher than that needed to saturate the PDE. They did not determine the effect of virus on calmodulin-deficient PDE. From their results, it appeared that influenza virus contained a calmodulin-binding protein instead of calmodulin or calmodulin-like protein.

**Potential role of calmodulin (or calmodulin-like protein) in FeLV:**

The exact role of calmodulin (or calmodulin-like protein) in the virus is not clear. A number of possibilities were considered. The calmodulin might serve to maintain viral integrity, act to facilitate viral binding, or enhance viral endocytosis.

1. **Maintenance of viral integrity:**

   There are a number of papers describing the role of calcium and/or calcium-binding protein(s) in maintaining viral integrity. For example, Brady, et al. (6) demonstrated
that incubation of polyoma virus with the calcium chelator, EGTA (10 mM), together with a reducing agent, dithiothreitol (3 mM), was able to break down 75% of the virus within 5 minutes. Both EGTA and the reducing agent had to be present to see the effect. Addition of calcium into the buffer prevented the viral dissociation. Once dissociated, the virus could be reassembled in a medium containing calcium, dimethyl sulfoxide and Triton X-100 (5,93). The reassembled virus was morphologically indistinguishable from normal virus, had the same buoyant density in the CsCl gradient, and partially regained hemagglutinating and plaque-forming activity. Recently, the same group of researchers demonstrated that the major viral capsid protein, VP1, was the calcium binding protein (47). Since the amino acid sequence of this protein is known, the possible calcium-binding sites were identified. Based on these observations, they proposed that calcium is present in the mature virion and is very important in maintaining viral integrity.

Another example of the role of calcium in maintaining viral integrity is from a body of work with rotavirus. Cohen, et al.(10) reported that incubation of purified rotavirus with 0.1 mM of EGTA led to dissociation of more than 75% of the virus within the first 5 minutes of incubation, as indicated by the appearance of viral RNA polymerase activity. The dissociation was inhibited by
addition of calcium into the buffer. This observation was supported by the work of Shirley, et al. (74), who found that incubation of rotavirus with 1 mM of EGTA could remove the outer shell from the virus, causing the virus to lose infectivity. They estimated that the minimum calcium concentration required to keep rotavirus intact was 0.15 mM. Recently, Shahrabadi et al. (70) demonstrated that calcium was incorporated into the mature rotavirus. If the virus was grown in calcium-deprived host cells, the outer capsid protein, VP7, was degraded at an accelerated rate. As a result, the assembly of the mature virus was impaired, the budding of the virus did not take place normally, and the resultant virus was not infectious. These results suggested that calcium is important in maintaining rotavirus integrity.

The role of calcium in viral integrity has been investigated in other viruses, and similar findings were reported. Durham et al. (16-18, 62) demonstrated that a number of plant viruses had the ability to bind calcium, and calcium appeared to stabilize the viral structure. They proposed that calcium binding by the virus controlled the process of viral disassembly in the host cells. Further work by Gallagher and Lauffer suggested that the coat protein of tobacco mosaic virus was the calcium binding site (23). They also came to the conclusion that
calcium binding to this site might play a role in determining the structure and stability of the viral particle.

Calcium appears to be important in maintaining the structural integrity of retroviruses also. Wunderlich and Sydow (92) demonstrated that 1-5 mM of EGTA or EDTA had the ability to lyse a number of retroviruses including bovine leukemia virus and Rauscher murine leukemia virus, as indicated by the release of reverse transcriptase from the virus. They further demonstrated that incubation of Rauscher murine leukemia virus with 1 mM of EGTA or EDTA in vitro decreased the in vivo viral infectivity significantly. Mice infected with the EDTA or EGTA treated viruses had lower RT level in the plasma and the mean survival time doubled.

Based on these observations, it is clear that calcium plays an important role in maintaining viral integrity. Removal of calcium from the virus can lead to viral dissociation, and this may be in fact the mechanism of viral uncoating in the low calcium environment of host cell cytoplasm. This uncoating is essential for viral replication and infection of the host cells. This is in consistent with the finding of Ludert, et al.(46), who demonstrated that an increase in cytoplasmic calcium level caused by the calcium ionophore A23187 inhibited rotavirus uncoating during the early stage of viral infection.
Is calmodulin an important calcium-binding protein in retrovirus?

While there is extensive evidence indicating that viruses have the ability to bind calcium, and the calcium-binding may be important in maintaining the structural integrity of viral particle, the nature of the calcium-binding protein is not clear in most cases. Work with retroviruses indicates that calmodulin might be the calcium-binding protein in these viruses. For example, Wunderlich and Sydow (91,92) demonstrated that the calmodulin antagonist, TFP, at concentrations of 40-200 μM had the ability to lyse retroviruses. Based on these observations, they proposed that retrovirus contains calmodulin, or a calmodulin-like protein which is responsible for maintaining viral integrity. This is in consistent with our finding that feline leukemia virus contains calmodulin or a calmodulin-like protein.

The structure and activity of calmodulin is calcium-dependent. The calcium concentration outside the cell is significantly different from that of inside the cell. The extracellular calcium concentration is approximately 100-1000 μM, while the calcium concentration inside the cell is approximately 0.1-0.01 μM. This is the concentration difference that a virus will encounter during the initial phase of viral entry. It is possible that viral calmodulin can act as a calcium-sensor and change its structure
according to the environment. In the extracellular high-calcium environment, these proteins may bind calcium tightly. When the virus enters the low-calcium intracellular environment, the calmodulin-associated calcium may dissociate from the binding sites. This may lead to a structural change of the viral calmodulin, which, in turn, may lead to a structural change of the viral particle. This may initiate the process of viral disassembly and viral replication.

A series of experiments were carried out in our system to test the possibility that calmodulin is responsible for maintaining the structural integrity of the feline leukemia virus. The basic strategy was to incubate the virus with calmodulin antagonists and look for evidence of loss of viral integrity. Based on our results, we concluded that the agents we tried were able to cause FeLV lysis. However, the concentrations needed for viral lysis were out of the range of calmodulin antagonism. Trifluoperazine, for example, was able to inhibit calmodulin activity totally at a concentration of 25 uM or less. There was only a slight FeLV lysis at this concentration. Melittin was able to inhibit calmodulin activity in the nM range, but 250 nM of melittin caused only a 5% FeLV lysis. The total viral lysis caused by 25 uM melittin was probably due to the detergent effect of this agent. Lysis of retrovirus by high concentration of melittin has been reported before
Although Wunderlich, et al. reported that a number of calmodulin antagonists had the ability to lyse retrovirus, the concentrations they used for most agents were also higher than the range for calmodulin antagonism (91,92). Although, based on RT release, we could not demonstrate the ability of calmodulin antagonists to lyse virus in a reasonable concentration range, this does not necessarily mean that low concentrations of calmodulin antagonists do not damage the viral particle. There exists a possibility that the viral structure is compromised but not to the extent of total viral lysis and total release of RT. This subtle change on viral particle might not be easy to detect by the RT assay system we used. This idea is partly supported by the work of Brady et al. on polyoma virus (6) and Durham et al. on plant viruses (18). Both groups indicated that removal of calcium is a necessary but not a sufficient step for total viral lysis. The work by Brady, et al. indicated that in addition to removal of calcium, a reducing agent was also necessary to see a total viral lysis. It appears that calcium/calcium-binding protein does play a key role in keeping the virus intact, but some other factors also contribute to the viral integrity.
2. Viral acceptor molecule for virus-host cell interaction:

Viral calmodulin might play a role in virus-host cell interactions. For example, there might be a calmodulin-binding protein on the surface of the host cell membrane. This calmodulin-binding protein might bind the calmodulin on FeLV, facilitating the viral entry. If this is true, then the calmodulin associated with FeLV should be accessible to the external environment. In order to test if the viral calmodulin is present on the surface of the virus, we mixed the virus with a gel (W-7 agarose, Sigma, #A-8281) which had the calmodulin antagonists W-7 coupled to it. The gel was removed from solution by centrifugation and the remaining supernatant was tested for the presence of virus by an infectivity experiment. If the calmodulin is present on the surface of the virus, it should bind to the W-7 gel, and be removed by centrifugation. Our results (data not shown) indicated that the supernatant fraction contained as much virus as the control solution. This indicated that the virus was not removed by the gel, which suggested that the viral calmodulin is not bound to the gel. It is therefore unlikely that the viral calmodulin is accessible to the outside of the virus.

Another way to determine if the calmodulin plays any role in the virus-host cell interaction is to run an infectivity experiment and include exogenous calmodulin during the viral infection period. If there is a decrease
in viral infectivity, we can conclude that the interaction between viral calmodulin and the hypothetical host cell calmodulin-binding protein is interfered, which leads to a lower infectivity. On the other hand, if we see a higher infectivity, we may conclude that the virus brings its own calmodulin in order to change the microenvironment of the host cells, which increases the viral infectivity. The exogenous calmodulin we add can further facilitate the infection process. In order to determine the effect of calmodulin on infectivity, we preincubated the host cells with calmodulin (from 0-20 uM) (Calbiochem, #208690) for one hour, then infected the cells in the second hour with FeLV. The calmodulin and free viruses were removed at the end of infection period. Fresh medium without calmodulin was added to the culture and infectivity was examined 7 days later. Our results from these experiments were not conclusive. One experiment indicated that the exogenous calmodulin had no effect, but the other showed slight inhibition. We were not able to demonstrate a role of for viral calmodulin in virus-host cell interactions with the existing data.

3. Facilitation of receptor-mediated endocytosis during viral entry:

It has been demonstrated that calmodulin plays an important role in receptor-mediated endocytosis (43,67),
which is a major route of entry for a number of viruses into their host cells (31,54). Following endocytosis, viruses are delivered to endosomes, then to lysosomes. It is believed that the low pH in the endosome/lysosome initiates an instant fusion between the viral membrane and the membrane of the endocytic vesicles. The viral nucleic acids are then released into the cytoplasm, and viral replication starts. It has been demonstrated that agents which can increase the pH of the endosome/lysosome are able to inhibit the fusion between the viral membrane and the membrane of the endocytic vesicles. Under these conditions, the virus will be digested by lysosomal enzymes, and the infection process is terminated. For example, Helenius, et al. (30) reported that the infection of BHK-21 cells by Semliki Forest virus can be inhibited by either 10 mM of NH₄Cl or 0.1 mM of chloroquine, both of which could increase the pH of endosomes and lysosomes.

Although the route of entry by FeLV into the host cells is not established yet, our limited data (not shown) indicated that the presence of 10 mM of NH₄Cl or 0.1 mM of chloroquine just before and during the virus infection period could lead to a 30% inhibition of infection. The cell growth appeared normal as judged by the total protein from the 7th day's culture. These results suggested that FeLV might enter the host cells by the process of receptor-mediated endocytosis. Under these conditions, the viral
calmodulin associated with FeLV may potentially play a role in the process of receptor-mediated endocytosis. By providing its own calmodulin, the virus can enhance the process, thus ensuring successful viral entry.

In conclusion, we considered several possible roles which might be played by the viral calmodulin. It might be important in keeping viral integrity, in increasing virus-host cell interactions or in facilitating the process of receptor-mediated endocytosis. We currently do not have enough evidence to prove or disprove any of these possibilities.

Inhibition of FeLV infection by TFP:
Possible mechanisms of the antiviral effect of phenothiazines:

A series of experiments were conducted to determine whether calmodulin antagonists can decrease FeLV infectivity. Based on our results, we concluded that TFP, a representative compound of the phenothiazine calmodulin antagonists, had the ability to inhibit FeLV infection. The effect was probably due to an inhibition of viral-cell interaction during the infection period, such as viral binding or internalization. The exact mechanism for this inhibition is unknown, but there are several possibilities.
It is possible that TFP inhibits calmodulin-related receptor-mediated endocytosis. This is consistent with the idea proposed by Nemerow, et al. (59), who demonstrated that the infection of B lymphocytes by Epstein-Barr virus was inhibited by a number of structurally different calmodulin antagonists. They suggested that the calmodulin antagonists decreased the entry of virus into host cells through the inhibition of endocytosis (see below). Our own studies do not provide enough evidence to support this possibility for FeLV, since W-7, another typical calmodulin antagonist, had very little inhibitory effect in a concentration range which was not cytotoxic to the host cells. Experiments using other calmodulin antagonists did not yield conclusive results.

An alternative explanation for the effect of TFP is that this agent can change membrane fluidity. Landry et al.(41) demonstrated that calmodulin antagonists, in general, have a membrane stabilizing effect, as judged by the ability of these agents to protect red blood cells from hypotonic lysis. It is possible that a change in host cell membrane fluidity can impair the process of viral entry, and thus decrease viral infectivity. In fact, Shirazi and Dean (73) demonstrated that 10 uM of TFP was able to inhibit receptor-mediated endocytosis as well as other kinds of pinocytosis by fibroblasts and macrophages. A very similar inhibition could be demonstrated with low
concentration of the detergent, digitonin. They proposed that TFP might operate directly on the cell membrane without the participation of calmodulin.

A third possible explanation for the effect of TFP is provided by the work of McDonald (49), who observed that TFP, at a concentration between 25-100 uM, had the ability to inhibit the function of the fusion protein of Sendai virus. This protein is responsible for inducing fusion between the viral membrane and the host cell membrane. An inhibition of the function of this protein could lead to an impaired viral entry during infection. However, the existence of such a protein for FeLV has not been demonstrated.

Historical evidence for an antiviral effect of phenothiazines:

There are a number of publications describing the effectiveness of phenothiazines in decreasing viral infectivity. Nemerow, et al. (59) demonstrated that the infection of human B lymphocytes by Epstein-Barr virus can be inhibited by a number of structurally different calmodulin antagonists, including trifluoperazine, promethazine, haloperidol and calmidazolium. In their experiments, they preincubated the host cells with the drug for 1 hour, then the purified EBV was added to the cells. The cell culture was incubated for another 14 days, at
which time the culture was examined for colony formation and analyzed for $^{3}H$-thymidine uptake. Under normal conditions the lymphocytes can not propagate and most of the cells will be dead by this time. However, if the cells are infected (transformed) by EBV, there will be an outgrowth of transformed cell colonies and the cell growth can be monitored by $^{3}H$-thymidine uptake.

Their results indicated that the calmodulin antagonists they tried were able to inhibit the EBV infection in a dose-dependent manner. The doses needed for inhibition of viral infection correlated well with the doses needed for inhibition of calmodulin activity. Using $^{32}$S-labeled virus, they demonstrated that these calmodulin antagonists did not interfere with the binding of virus to the host cells. Based on observations with electron microscopy, the internalization of the virus appeared to be inhibited. Addition of TFP 5 minutes after the initiation of the infection process had no inhibitory effect. These observations suggested that calmodulin antagonists could inhibited the early events of viral entry.

Krizanova et al.\(^{(39)}\) reported that chlorpromazine (CPZ) at concentrations of 20-50 uM inhibited the infection by influenza virus. In this system, the host cells were infected with virus in the presence of the drug. The free virus and drug were removed after a 1-hour infection period. Three days later the plaques which formed on the
monolayer culture were counted as an indication of infectivity. They reported that the drug did not cause structural damage to the virus, because pre-treating the virus with CPZ did not decrease infectivity. Based on observations obtained by electron microscopy, they found that the adsorption of virus onto the host cell membrane was normal. However, the fusion between viral and host cell membranes was impaired. The drug was not effective if given two hours after the infection period. They proposed that CPZ decreased viral entry by inhibiting the process of virus-host cell fusion. Since CPZ has the ability to inhibit the function of calmodulin, they suggested that this is a calmodulin-related phenomenon.

Nugent and Shanley (60) also reported the inhibition of influenza virus infection by CPZ. They infected host cells with influenza virus for one hour. After removal of the virus, CPZ, at concentrations from 10-100 uM, was added to the culture, which was incubated for another 8 hours. Then the medium was removed and human red blood cells were added. Since the virus encodes a hemagglutinin protein, the infected cells were identified by the clusters of tightly packed RBC's. Under these experimental conditions, 10 uM CPZ caused a 50% decrease in infectivity. Since the drug was given after the cells were infected, they claimed CPZ could inhibit the late events of viral replication cycle, presumably by inhibiting some host cell functions.
Again, due to the inhibitory effect of CPZ on calmodulin, they suggested that this was a calmodulin-related phenomenon. They did not discuss the effect of the 8-hour drug incubation on the host cell functions. There exists a possibility that the long term incubation of drugs could shut down the host cell functions partially, which, in turn, reflected a lower virus production.

Another report concerning the inhibition of viral replication by phenothiazines was by Bohn et al. (4) who demonstrated that TFP at 10-15 uM and CPZ at 30-40 uM were able to inhibit the replication of measles virus. They infected the host (HeLa) cells with the virus for three hours, the free virus was removed by washing after the infection period, and the cells were grown for another 18 hours. Thereafter, the culture fluid was replaced by complete medium containing the drugs. The virus released into the medium was measured every 4 hours by a plaque assay using another cell line as an indicator. Their results indicated that there was a decreased amount of virus released into the supernatant from the host cells. However, they also observed a significant inhibition of host cell growth under these conditions. Using electron microscopy, they noticed the viral budding process was significantly inhibited. In addition, they noticed that there was a change in the morphology of the virus.
Based on the results we obtained as well as those published, it can be concluded that phenothiazines have the ability to decrease viral infectivity. It is clear that these agents decrease viral entry. In addition, they might be able to decrease viral budding. The site(s) of action which leads to the decreased infectivity has not been firmly established. Most authors ruled out the possibility that the drugs had a direct effect on the viral particles, or initial binding of the virus to the host cells. It is probable that these agents inhibit the cellular calmodulin which is involved in the viral entry processes. This in turn leads to an inhibition of viral infection. However, as discussed above, the membrane-stabilizing effects and the potential of these agents to interfere with viral fusion protein(s) must also be considered.

Cytotoxicity of the calmodulin antagonists and potential applications:

In view of the role of calmodulin in normal cell growth, the agents which inhibit the functions of calmodulin should be cytotoxic to cells. Chafouleas et al. (7) reported that calmodulin antagonists can prevent the cells from entering the S phase (DNA synthesis) of cell cycle. In fact, this is one of the major problems we encountered when we were trying to examine the antiviral effects of this group of drugs. In our system, incubation
of host cells with 20 uM TFP or 40 uM W-7 for two hours appeared to interfere with cell growth. Similar findings have been reported by others. For example, Wei, et al. (84) reported that incubation of a breast cancer cell line (MDK-MB-231) with either TFP or W-7 for one hour led to a decrease in colony formation by the cells. The concentrations needed to inhibit 50% of colony formation were 50 uM for TFP and 53 uM for W-7. MacNeil et al. (50) reported that W-7 decreased the growth rate of B-16 melanoma cells, as measured by 3H-thymidine uptake. Incubation of the cells with 50 uM of W-7 for 2 hours led to a 30% decrease of 3H-thymidine uptake.

Based on these observations, calmodulin antagonists have been proposed as potential cancer chemotherapeutic agents (27). Leukemia cells are among the cells that are highly susceptible to the calmodulin antagonists. Hait (26) reported that L1210 and L5178Y murine leukemic cells and HL-60 human promyelocytic leukemic granulocytes were killed by relatively low doses of calmodulin antagonists. For example, incubation of the L1210 leukemic cells with calmodulin antagonists for 72 hours led to significant cell death. The concentrations needed to kill 50% of the cells was 5 uM for TFP and 7 uM for CPZ. Since TFP has the ability to inhibit feline leukemia virus infection, and leukemia cells are very susceptible to TFP, this agent might have some utility in the leukemia of viral origin.
Further studies of the calmodulin-like protein:

Considering the importance of calmodulin in normal cellular biochemistry and physiology, the presence of calmodulin (or a calmodulin-like protein) in retrovirus should have physiological significance. The following experiments are designed to study the calmodulin-like protein we isolated by the two-step protein purification. These experiments should provide us with some information about the importance of this calmodulin-like protein in viral functions.

1. Determination of the physical and biochemical properties of this protein:

The physical properties of this protein can be determined once the protein is purified. The molecular weight, the calcium-binding ability and the change induced by calcium binding should be determined. The biochemical characteristics of this protein can also be established from the purified protein. We should be able to apply this protein to the calmodulin-regulated systems and evaluate
the properties of this protein. Based on the results from these experiments, we should be able to determine if the protein we isolated is calmodulin or not.

2. Determination of the function of this protein in the viral life cycle:

The possible involvement of this protein in viral life cycle can be examined in the following ways: a. Production of an antibody against this protein and examination of the effect of the antibody on viral life cycle. b. Identification of inhibitors of this protein and examination the effect of such inhibitors on the viral life cycle.

3. Screening of the presence of the protein in other viruses:

If this protein is functionally important in FeLV, there exists a possibility that there is a similar protein present in other viruses. With the antibody against this protein, we can determine if the protein is present in other retroviruses other than FeLV. Is it in any of the other envelope or non-envelope viruses?

4. Determination of the origin of this protein:

Most of the proteins in a viral particle are encoded for by the viral genome. However, there exists a
possibility that the virus can carry some cellular proteins with it during the budding process. It is of interest to determine the origin of this calmodulin-like protein. There are at least two ways we can determine this:

a. Screening of the presence of this protein in the uninfected host cells by antibody against this protein. This can be done with a standard immunochemical staining;
b. Sequencing of the purified protein. If the importance of this protein in the viral life cycle is established, it is necessary to sequence this protein and search for its origin. From the protein sequence we should be able to determine whether this protein is encoded for by the host cell or by the virus. This piece of information should add more insights to the understanding of viral life cycle and viral-host cell interactions.

Further evaluation of the systems used in the infectivity experiments

There are some problems we encountered and some experience we obtained from developing the system for the infectivity assay. The following sections contains a discussion of these experience.
p27 ELISA:

It is very important to run a standard curve for p27 if the purpose is to do a quantitative measurement of this protein in the sample. Just as with the antigen-antibody reactions in radioimmunoassay, the presence of antigen in the sample does not correlate with the print-out readings from the instrument in a linear fashion. It is thus essential to run a standard curve with a known amount of p27 together with the unknown samples.

The results from RT assay are comparable to that from ELISA. However, for a standard RT assay we have to concentrate the viruses from 7.5 ml of culture medium with high speed centrifugation before we can start the RT assay. This becomes very undesirable when we have a large number of samples to run. On the other hand, there is no pretreatment of the samples needed for the ELISA. In addition, it takes only 50 ul of culture medium for each sample. If we compare the amount of sample needed for RT assay (7.5 ml) with that for p27 ELISA (50 ul), ELISA appears to be 150 times more sensitive than RT assay.

81 C cells as the host cells for FeLV infection:
1. Plaque assay vs. p27 ELISA as a measurement of infectivity:

The 81 C cells can form plaques upon infection with FeLV as described by Fishinger (22). As a result, this
cell line is commonly used for FeLV infection experiments. Unfortunately, the identification of the plaques is highly subjective. In addition, the formation of plaques needs more than functional viruses. It also needs normal growing cells as a contrast for plaque formation. If an agent tested alters host cell growth, it may lead to a false positive or false negative result in plaque counting. As a result, we chose to use p27 ELISA as an alternative assay to determine viral infectivity.

There are some potential problems with the p27 ELISA also. This assay measures the concentration of the viral specific protein, p27. A higher concentration of p27 implies a higher number of virus in the sample. However, this does not indicate whether the virus is properly formed and fully infectious. As a result, this assay will not be appropriate for an agent which interferes with the later stages of the viral life cycle, such as the formation and budding of the virus. This is best demonstrated by the antiviral effect of interferon. Maheshwari and Friedman (52) found that 30 units/ml of interferon decreased the infection of cultured Ly cells by vesicular stomatitis virus. There was a 200-fold decrease of the viral infectivity, as determined by a plaque assay. However, there was only a 10-fold decrease of viral RNA, protein or transcriptase. In this case, the measurement of a specific viral protein should have been misleading. Since in our
experiments the drugs are only present during the infection period, we believe this kind of paradox does not occur.

There was a technical problem with the ELISA assay. The p27 ELISA measures the concentration of p27 in the 7th day's medium. Although the culture is incubated in the incubator with proper temperature and humidity control, the evaporation of medium still occurs. Fluid evaporates from the outer wells of the 24-well plate more than from the inner wells. As a result, the outer 16 wells give a higher reading of p27 from the ELISA than the inner 8 wells. In order to correct for this, we used only the inner wells for our experiments. The outer wells were filled with PBS during the 7-day incubation.

2. Increase of viral infectivity by DEAE-dextran:

Pretreatment of the host cells with DEAE-dextran can greatly increase viral infectivity (80). The real mechanism for this increase is not clear. It may change the charge of host cell membrane and facilitate viral binding. It is a common practice to include this agent in virology studies in order to see a higher viral infection. Because it may mask the effect of the anti-viral drugs we are interested in, we did not include this agent in our experiments.
3. Antiviral effect vs. cytotoxicity of an agent:

The life cycle of a virus has an intimate relationship with the host cell functions. We therefore always face a problem of interfering with normal cell functions while we try to inhibit viral replication. As a result, extreme care must be taken when we try to determine the effect of an antiviral agent. The cytotoxicity of an agent on the host cells can be determined in several ways, such as: a. Observation of the host cells during and after the drug treatment. If the drug is extremely toxic to the cells, usually the cells will detach from the bottom of the culture after the incubation. A moderate toxicity will make the cell round-up and some of them will become loose after washing; b. Determination of the viability by trypan-blue dye exclusion. The dead cells will take up the dye and can be distinguished from the live cells. With this method the viability of the cells can be determined; c. Measurement of $^3$H-thymidine uptake by the cells. This can tell if the drug inhibits the normal growth of the cells in a specific period of time; d. Determination of the total protein synthesis. This can determine the overall cell growth during a longer period of time.

4. Effectiveness of an antiviral agent:

The *in vitro* antiviral effect of a drug depends heavily on two factors. The first one is the viral titer.
If the host cells are infected with a tremendous number of viruses, no drug will appear to be effective. On the other hand, if the cells are infected with a very small amount of viruses, even the weakest drug will appear to be effective. A second factor in determining the antiviral effectiveness is the choice of host cells. Some host cells are extremely vulnerable to viral infection. It is very difficult to demonstrate the antiviral effect of an agent on these cells. It is therefore very important to clearly specify the experimental conditions when the effectiveness of an antiviral agent is reported.

Further studies using the infectivity assay we developed:

In search for a treatment for retrovirus infection is currently a major clinical focus. The approach we took, and the results we got, from this project should add some insight to the retroviral study. As a follow up, there are at least three directions that we can further pursue this study:

A. A re-evaluation of the antiviral potential of the calmodulin antagonists under different experimental conditions:

There is still much we can do although our results do not support our original hypothesis that calmodulin
antagonists, in general, can decrease viral infectivity. There is a possibility that the experimental conditions we used are too stringent. We always remove the drugs at the end of the infection period. There is no drug present for the following 7-day incubation. The results from our experiments may be different if we change these conditions. For example, the antiviral effect may be enhanced if we leave the drugs in the system for a longer period of time. Since the basic experimental conditions are established already, it is relatively simple to make small modifications. Following such modifications, we can do an overall screening of the calmodulin antagonists and re-evaluate our conclusion.

B. Establishment of the antiviral effects by the phenothiazines:

The ability of the phenothiazines to decrease retrovirus infection is an important finding. It is necessary to determine the mechanism(s) of this antiviral effect. Since this group of drugs has been used for years, their clinical effects have been well characterized. With a careful study of the relative effect of the agents in this group, we may identify a compound with a good antiviral potential and a relatively low toxicity.
C. Testing of agents with any antiviral potentials and evaluation of the effectiveness of drug combination:

With an experimental system such as ours, it is easy to test other potential antiviral agents with different mechanisms. For example, the effect of a potential reverse transcriptase inhibitor can be tried in this system without difficulty. A new AZT-analog can be tested in this system for its effect. In addition, we can combine drugs with different mechanisms and evaluate the effect in this system. For example, the phenothiazines may not be efficacious enough when used alone, but may make a very useful adjunct antiviral agent.

Recent studies of AZT on the in vitro & in vivo FeLV infection:

AZT is currently the most important drug used in the treatment of the HIV infection (AIDS). The effect of this drug has been evaluated in FeLV infection both in vitro and in vivo, as reported by Tavares, et al. (78). For the in vitro study, they used the same cell line (81 C cell) as we used in our experiments. As a result, we believe it is relevant to document their findings here. The cells were infected with FeLV for 45 minutes, then the drug was added to the culture. The medium was changed every three days while constant AZT concentration was maintained. The
degree of infectivity was determined by scoring the plaques formed 12 days after the infection. Under these experimental conditions, they demonstrated that 1 uM of AZT was able to decrease the number of plaques by 60%, and there was a 96.5% decrease in plaque formation after exposure to 10 uM of AZT. They stated that this drug was not cytotoxic to the cells even at 100 uM. However, they did not specify by which standard they assessed the cytotoxic effect of the drugs.

For the in vivo study, they first infected cats with FeLV, then the drug was given 1 hour, 3 days, 7 days or 28 days after infection. The drug was given by subcutaneous injection and the doses given were 10 or 20 mg/kg body weight. Their results indicated that AZT was protective to the cats if given 1 hour after FeLV infection. It was not protective if given 28 days after infection. When the drug was given between these two time points, the effects were dose-related. Since FeLV infection usually starts from local lymphatic tissue, proceeds to circulating lymphocytes, systemic lymphatic tissues then finally to the bone marrow, they proposed that it is crucial to give the drugs before the viral replication in the bone marrow starts.
LIST OF REFERENCES


