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The Myxoma Virus M-T4 Gene Encodes a Novel RDEL-Containing Protein That Is Retained within the Endoplasmic Reticulum and Is Important for the Productive Infection of Lymphocytes

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To investigate the contribution of the myxoma virus M-T4 gene to viral virulence, both copies of the M-T4 gene were inactivated by disruption and insertion of the *Escherichia coli* guanosine phosphoribosyltransferase gene. Infection of European rabbits with the recombinant M-T4-deleted virus, vMyxlacT4$^-$, resulted in disease attenuation. In contrast, infection of rabbits with vMyxlac elicited the classical features of lethal myxomatosis. A notable decrease in the number of secondary lesions in animals infected with vMyxlacT4$^-$ suggested an inability of the virus to disseminate *in vivo.* Infection of either a rabbit CD4$^+$ T cell line, RL-5, or primary rabbit peripheral blood lymphocytes with vMyxlacT4$^-$ resulted in the rapid induction of apoptosis. Sequence analysis of M-T4 revealed both an N-terminal signal sequence and a C-terminal -RDEL sequence, suggesting that M-T4 resides in the endoplasmic reticulum. The M-T4 protein was found to be sensitive to endo H digestion and confocal fluorescence microscopy demonstrated that M-T4 colocalized with calreticulin, indicating that M-T4 is retained within the endoplasmic reticulum. Our results indicate that M-T4 is the first example of an intracellular virulence factor in myxoma virus that functions from within the endoplasmic reticulum and is necessary for the productive infection of lymphocytes.

INTRODUCTION

In order to replicate in the presence of a vigorous host immune response, many viruses have evolved diverse strategies to evade the workings of the immune system (reviewed in Marrack and Kappler, 1994; Smith, 1994; Spriggs, 1994 and 1996). Poxviruses have proven to be particularly successful at immune evasion and contain a large number of open reading frames that encode proteins essential for subversion of the immune response (reviewed in Pickup, 1994; Alcamí and Smith, 1995; Barry and McFadden, 1997). One of the most obvious strategies of immune evasion by poxviruses is the apparent capture, manipulation, and expression of host genes that normally function to assist the elimination of invading pathogens. To date studies have demonstrated that poxviruses encode an array of proteins that interfere with the regulation of apoptosis, growth factors, cytokine networks, complement-mediated lysis, and cytotoxic T lymphocyte recognition, in order to confer a selective advantage for the virus in an immunocompetent host (McFadden, 1995).

Myxoma virus is a member of the poxvirus family that causes a virulent and lethal disease in the European rabbit, known as myxomatosis (Fenner and Ratcliffe, 1965). Myxomatosis is characterized by extensive virus replication at the initial site of infection, followed by infection of resident leukocytes and virus dissemination in order to initiate secondary sites of infection (Fenner and Ratcliffe, 1965; McFadden, 1988). Clinical features include the presence of a fulminant lesion at the initial site of infection evident as early as 3 days postinfection, followed by the development of multiple internal and external secondary lesions that are particularly obvious around the ears, eyes, and nares of the animal 8–10 days postinfection. Infection is accompanied by extensive cellular immune dysfunction resulting in supervening gram-negative bacterial infection and death 10–13 days postinfection. Like other members of the poxvirus family, myxoma virus encodes a diverse array of proteins that operate to subvert the host's immune response (reviewed in McFadden and Graham, 1994; McFadden *et al.,* 1995). Typically the open reading frames that encode these virulence factors are located near the terminal ends of the double-stranded DNA virus genome, and deletion or disruption of the open reading frame routinely has no effect on virus replication in tissue culture, but an attenuation is observed when the deleted virus is utilized to infect European rabbits.

Secreted myxoma virus proteins have thus been identified with homology to the tumor necrosis factor (TNF) receptor and the interferon-γ (IFN-γ) receptor that function as bona fide soluble receptors by binding and se-

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questering these cellular cytokines thus inhibiting their biological activities (Upton *et al.,* 1991, 1992). Myxoma virus also encodes proteins with homology to epidermal growth factor and a serine proteinase inhibitor, both of which are secreted from infected cells (Opgenorth *et al.,* 1992; Macen *et al.,* 1993). Recently, a secreted myxoma virus protein encoded by the M-T1 open reading frame has been shown to bind various members of the chemokine family, although it demonstrates no homology to known cellular chemokine receptors (Graham *et al.,* 1997). It is likely that more secreted proteins necessary for immune evasion remain to be discovered. In addition to an abundance of secreted virus-encoded proteins, myxoma virus also encodes cell-associated proteins that have been shown to play a role in immune subversion. Serp-2 is an intracellular serine proteinase inhibitor with significant homology to cowpox virus crmA protein, which inhibits human interleukin-1β converting enzyme (Petit *et al.,* 1996). The M11L open reading frame encodes a membrane-associated protein which is important for the evasion of inflammation (Opgenorth *et al.,* 1992; Graham *et al.,* 1992), and the M-T5 open reading frame encodes a protein with homology to the poxvirus family of host range proteins which is necessary for protein synthesis, the productive infection of lymphocytes and inhibition of apoptosis (Mossman *et al.,* 1996a). In addition, the expression of the myxoma virus-encoded tumor necrosis factor receptor homolog and M11L are necessary for the prevention of virus-induced apoptosis in a $CD4^+$ T cell line (Macen *et al.,* 1996). Since an important feature of myxoma virus pathogenesis and virulence is the ability to replicate in leukocytes, it is not surprising that myxoma virus also encodes multiple proteins essential for this function (Macen *et al.,* 1996; Mossman *et al.,* 1996a; Schreiber *et al.,* 1996). In fact, many viruses encode proteins that function to evade programmed cell death or apoptosis in specific cells (reviewed in Cuff and Ruby, 1996; Gilet and Brun, 1996; White, 1996; McFadden and Barry, 1997; Teodoro and Branton, 1997).

In this report we describe the identification of a novel myxoma virus-encoded gene, M-T4, which functions as a virulence factor. M-T4 localizes specifically to the endoplasmic reticulum (ER) of infected cells and prevents apoptosis induction in infected lymphocytes. We show that M-T4 contains a C-terminal ER retention sequence, -RDEL, and that the presence of M-T4 is necessary for both virus virulence *in vivo* and virus replication in lymphocytes *in vitro.*

MATERIALS AND METHODS

Cells and viruses

vMyxlac is a myxoma virus (strain Lausanne) containing the *Escherichia coli* lac Z gene inserted between the myxoma virus growth factor and M-T9 genes, and its construction has been described elsewhere (Opgenorth *et al.,* 1992). Vaccinia virus (strain WR) was obtained from the American Type Culture Collection. Vaccinia virus 601 (VV601) is an engineered derivative of vaccinia virus (strain WR) containing the *E. coli* lac Z gene inserted into the virus thymidine kinase gene (Macen *et al.,* 1993). The construction of both vMyxlac M-T4$^-$ and the vaccinia virus that overexpresses M-T4 (VVT4) are described below. All viruses were routinely propagated in baby green monkey kidney (BGMK) cells (a gift from S. Dales, University of Western Ontario, London, Canada) grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum (Gibco BRL Life Technologies Inc.), 200 units/ml penicillin (Ayerst Laboratories), and 200 μg/ml streptomycin (Sigma Chemical Co.). Viruses were isolated as previously described (Stuart *et al.,* 1991). RL-5 cells were obtained from National Institutes of Health: AIDS Research and Reference Reagent Program and were routinely cultured in RPMI 1640 (Gibco BRL Life Technologies Inc.) supplemented with 200 units/ml penicillin and 200 μg/ml streptomycin and 10% fetal calf serum. Rabbit RK-13 cells were provided by S. Sell and cultured in DMEM supplemented with 10% fetal calf serum, 200 units/ml penicillin, and 200 μg/ml streptomycin. Thymidine kinase deficient (TK$^-$) H143 cells were obtained from D. Panicali and cultured in DMEM supplemented with 10% fetal calf serum, 200 units/ml penicillin, 200 μg/ml streptomycin, and 25 μg/ml 5′-bromo-2′-deoxyuridine (BUdR) (Sigma Chemical Co.).

Primary rabbit peripheral blood leukocytes were isolated from 30 ml of rabbit blood. Rabbit blood was layered over an equal volume of Ficoll-Plaque (Pharmacia) and centrifuged at 400 *g* for 1 h. The buffy coat containing the mononuclear cell fraction was collected and cells were washed twice in phosphate-buffered saline (PBS) prior to culturing the cells in RPMI 1640 containing 10% fetal calf serum, 200 units/ml penicillin, and 200 μg/ml streptomycin. The nonadherent cells were collected after 4 h by washing the adherent monolayer with RPMI 1640.

Virus infection

RL-5 cells and nonadherent peripheral blood leukocytes were incubated with virus in 1.0 ml of cell culture medium for 1 h at 37°C at a multiplicity of infection (m.o.i.) of 10 plaque forming units (PFU)/cell. Following this initial infection period, cells were diluted to 5×10^5 cells/ml with fresh medium and the infection was allowed to proceed at 37°C for the indicated time. Confluent monolayers of RK-13 and BGMK cells were incubated with virus in 0.5 ml cell culture medium for 1 h at 37°C at a m.o.i. of 10 after which medium was added back to the cells and the infection was allowed to proceed at 37°C. All infected cells were harvested with 1X SSC containing

0.15 M NaCl and 15 mM sodium citrate. The efficiency of virus infection was monitored by colorometric analysis using 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) since all viruses contained a lacZ gene. In all cases RL-5, RK-13, and BGMK cells were found to be greater than 95% infected, while only approximately 50% of nonadherent primary peripheral blood leukocytes appeared to be infected under the above conditions.

Sequence analysis of M-T4

The myxoma virus M-T4 gene (Genbank Accession No. AF002684) was sequenced from three independent PCR amplified products to ensure the lack of PCR errors. Briefly, myxoma virus genomic DNA was used as the template for primers BS3.6 5′(ACGTCGAATGAGAGATTC) and BS3.4 5′(TTCGGGGAACTCAATCC) which are complementary to sequences 5′ and 3′ to the M-T4 gene. The DNA was amplified by 25 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C in a buffer containing 30 pmol each of primers BS3.6 and BS3.4, 50 mM KCl, 2.5 mM $MgCl_2$, and 20 mM Tris–HCl, pH 8.4. The amplified product was cloned directly into pT7Blue T-vector (Novagen) (pT7Blue MT4), which was subsequently used for sequencing. Sequencing was performed on an ABI 373 DNA sequencer with *Taq* cycling to greater than fivefold redundancy. Genetics Computer Group (Madison, WI) GelAssemble program was used to compile sequence data. The Genbank (release 90) data base was used to obtain additional sequences. All sequences were aligned with Genetics Computer Group Bestfit and PileUp programs.

Virus construction

In order to construct a myxoma virus with disrupted copies of the M-T4 gene (vMyxlacT4$^-$), the M-T4 open reading frame cloned into pT7Blue T-vector was used as a template to generate the right and left fragments of M-T4 by PCR amplification. Primers T4.1 5′(CATATGAAAATGTACACGCGATTATC), T4KO.1 5′(TTACGAGATCTAGTAGAGCGGATACTTTGTGTTCTT) and T4.2 5′(GGATCCTTATAATTCATCTCGTAATA), T4KO.2 5′(CTACTAGATCTCGTAATTAATCGACACGTCGTGCGT) were used to create the left (5′M-T4) and right (3′M-T4) fragments, respectively. Primers T4KO.1 and T4KO.2 were engineered to contain a *Bgl*II restriction site so that a *Bgl*II cassette containing the *E. coli* guanosine phosphoribosyltransferase gene (gpt) under the control of the vaccinia virus 7.5 promoter could be easily inserted. The resulting DNA (pMT4gpt) and vMyxlac were subsequently used in construction of vMyxlacT4$^-$ by previously described methods (Mossman *et al.,* 1996b). PCR analysis using T4.1 and T4.2 primers (described above) was used to confirm truncation of the M-T4 gene and insertion of the *E. coli* gpt gene in the recombinant virus. The vMyxlacT4$^-$ virus was used to generate a T4 revertant virus, vMyxT4R, in which the M-T4 gene was restored into a wild-type myxoma virus background as previously described (Macen *et al.,* 1996). In addition, a recombinant vaccinia virus (strain WR) expressing the M-T4 gene was constructed (Macen *et al.,* 1993). The entire M-T4 open reading frame was subcloned into the *Hind*III and *Sma*I sites of PMJ601 (Davison and Moss, 1990). This allowed insertion of M-T4 into the vaccinia virus thymidine kinase gene (TK) under the control of a vaccinia virus synthetic late promoter. Recombinant vaccinia virus containing M-T4 was selected on TK$^-$ H143 cells in the presence of 25 μg/ml BUdR and plaque purified three times using 300 μg/ml X-gal to visualize recombinant viruses expressing β-galactosidase. PCR analysis with T4.1 and T4.2 primers was performed on DNA isolated from plaque purified virus to ensure the presence of the intact M-T4 gene in the recombinant virus.

Detection of apoptosis

In order to detect virus-induced apoptosis in RL-5 cells, DNA fragmentation was monitored by an agarose gel assay as previously described (Mossman *et al.,* 1996a). Briefly, genomic DNA was isolated from 2×10^6 RL-5 cells infected with vMyxlac or vMyxlacT4$^-$ 16 h postinfection. Cells were lysed in 10 mM Tris, pH 7.5, containing 100 mM NaCl, 1 mM EDTA, and 1% SDS. Following lysis of cells, the supernatants were treated with 0.5 mg/ml of proteinase K for 1 h at 55°C and then with 0.5mg/ml RNase for 1 h at 37°C. Genomic DNA was precipitated with 2.5 vol of 95% ethanol and redissolved in 10 mM Tris, pH 8.0, prior to agarose gel electrophoresis.

DNA fragmentation in RL-5 cells and primary rabbit peripheral blood leukocytes was also monitored by flow cytometric analysis via the TUNEL method (Gavriell *et al.,* 1992; Sgonc *et al.,* 1994). Cells (1×10^6) were infected with vMyxlac or vMyxlacT4$^-$ and 16 h postinfection the cells were harvested and washed in PBS containing 1% FCS. Cells were then fixed in 200 μl of 2% paraformaldehyde for 30 min at room temperature with constant agitation. Following fixation, cells were washed three times in PBS containing 1% FCS prior to permeabilization in 100 μl of 0.1% Triton X-100 and 0.1% sodium citrate for 2 min on ice. The cells were washed again in PBS containing 1% FCS and then incubated for 1 h at 37°C in 30 μl of 30 mM Tris, pH 7.2, 140 mM cacodylate, 0.6 nmol of fluorescein-12-dUTP, 3 nmol dATP, 1 mM $CoCl_2$, and 25 units of terminal deoxynucleotidyltransferase (Boehringer-Mannheim). After incubation cells were washed in PBS containing 1% FCS prior to flow-cytometric analysis performed on a FACScan flow cytometer equipped with an argon-ion laser with 15 mW of excitation at 488 nm. Data was acquired on 10,000 cells per sample with light

scatter signals at linear gain and fluorescence signals at logarithmic gain.

RNA isolation and northern blotting

BGMK cells (5×10^6) were infected at an m.o.i. of 10 PFU/cell with vMyxlac or vMyxlacT4$^-$. Total RNA was isolated at 2, 4, 7, and 10 h postinfection with TRIzol Reagent (Gibco BRL Life Technologies Inc.), according to the manufacturer's instructions. In addition, total RNA was also isolated from infected cells treated with 40 μg/ml cytosine arabinoside (Ara C) (Sigma Chemical Co.) 16 h postinfection. For Northern blotting analysis 3 μg of isolated total RNA was used for each time point and blotting was performed as previously described (Mossman *et al.,* 1995). Full-length M-T4 was amplified by PCR and 25 ng was used as a template for random priming in the presence of [α-^{32}P]dCTP (ICN Biomedical Inc.) and *E. coli* Klenow (GIBCO BRL Life Technologies).

Single-step growth curve analysis

RK-13 and RL-5 cells (5×10^5) were infected in triplicate with vMyxlac and vMyxlacT4$^-$ at an m.o.i. of 10 for 1 h. After the initial 1-h infection, excess virus was removed by washing the cells three times with cell culture medium. Infected cells were harvested at 0, 4, 8, 24, and 48 h postinfection and viral titers were determined by foci formation on BGMK cells as previously described (Opgenorth *et al.,* 1992).

vMyxlacT4$^-$ pathogenesis

Female New Zealand White rabbits (*Oryctolagus cuniculus*) were obtained from a local supplier and housed in level-C containment facilities in accordance with guidelines published by the Canadian Council on Animal Care. Eight rabbits were injected with 10^3 PFU/site intradermally in each thigh with vMyxlacT4$^-$. Similarly, four rabbits were injected with the parental virus vMyxlac. Rabbits were monitored on a daily basis for symptoms of myxomatosis, and rabbits demonstrating extensive disease progression were euthanized by intravenous administration of euthanyl. For histological analysis a second set of nine rabbits were infected; six rabbits infected with vMyxlacT4$^-$ and three rabbits infected with vMyxlac. Two rabbits from the vMyxlacT4$^-$-infected group and one rabbit from the vMyxlac-infected group were sacrificed on days 3, 7, and 11 postinoculation and subjected to a complete postmortem examination. Tissue sections were harvested from each rabbit, embedded in paraffin, and 5-μm sections were stained with hematoxylin and eosin.

Histological analysis

Tissue sections harvested from the nine rabbits subjected to postmortem analysis were analyzed by immunohistology. The Mouse Extravidin Peroxidase Staining kit (Sigma Chemical Co.) was used according to the manufacturer's instructions. Anti-β-galactosidase antibody (Promega) at 1:500 was used to detect the presence of virus expressing *E. coli* β-galactosidase (vMyxlac and vMyxlacT4$^-$). Monoclonal antibody specific for rabbit CD43 (Spring Valley Inc.) was used at 1:300 in order to detect rabbit T cells, monocytes, and macrophages.

Preparation of M-T4 antiserum

In order to generate antiserum specific for M-T4 a peptide was synthesized encompassing the C-terminal 10 amino acids of M-T4 (-YVAKVLRDEL) by the Alberta Peptide Institute (University of Alberta, Canada). Two rabbits were initially immunized by injecting 500 μg of peptide conjugated to KLH dissolved in 0.5 ml of PBS and 0.5 ml of Freund's complete adjuvant (Sigma Chemical Co.) per rabbit. At 2-week intervals the animals were boosted with 500 μg of antigen, in Freund's incomplete adjuvant (Sigma Chemical Co.), and antiserum was collected 2 weeks following the third boost.

Immunoblotting

To detect M-T4 protein, 1×10^6 BGMK cells were infected with vMyxlac, vMyxlacT4$^-$, VV601, or VVT4 at an m.o.i. of 10. After 1 h, excess virus was washed free of the cells with serum-free medium and the cells were overlayed with 1 ml of serum free medium. Cellular lysates were collected by directly harvesting the cells into 100 μl of 1× sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gel loading buffer at 4, 8, 12 h postinfection. At the same time supernatants containing secreted proteins were also collected and concentrated fourfold by precipitation with 5 vol of ice-cold acetone prior to SDS–PAGE. Proteins were transferred to nitrocellulose (Micron Separations Inc.) by using a semidry transfer apparatus (Tyler Corp.) for 1 h at 50 mA. Membranes were blocked in PBS containing 0.1% Tween 20 (ICN Biomedicals Inc.) and 5% skim milk for 16 h. Anti-T4 antiserum was used at 1:10,000 and incubated with the membrane for at least 2 h, after which the blot was washed three times in PBS containing 0.1% Tween 20. The membranes were probed with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories) at a 1:5000 dilution and developed with Amersham's chemiluminescence detection system (Amersham Inc.), according to the manufacturer's directions.

Endoglycosidase H (endo H) analysis

BGMK cells (10^6) were either mock infected or infected with VV601 or VVT4 at an m.o.i. of 10 in the presence of 50 μCi of [^{35}S]cysteine [^{35}S]methionine (ICN) in medium free of cysteine and methionine (ICN). At 6 h postinfec-

tion the cells were harvested and lysed in NP-40 lysis buffer containing 150 mM NaCl, 50 mM Tris–HCl (pH 8.0), and 1% NP-40. Lysates were cleared of nuclei by centrifugation at 12,000*g* for 30 min before the addition of 4 μl of anti-MT4 antiserum. Following a 1-h incubation at 4°C, 30 μl of protein A–Sepharose (Sigma Chem. Co.) was added and the lysates were further incubated for 30 min at 4°C. The protein A–Sepharose beads were collected by centrifugation and washed three times in NP-40 lysis buffer. Prior to treatment with Endo H, the M-T4 containing Sepharose beads were denatured by boiling for 10 min in 0.5% SDS and 0.1% 2-mercaptoethanol. The denaturing buffer was neutralized by the addition of 50 mM sodium citrate (pH 5.5) and 2,000 units of Endo H_f (New England Biolabs) was added. The reaction was allowed to proceed for 1 h at 37°C, after which it was terminated by the addition of an equal volume of SDS gel loading buffer. Samples were analyzed by SDS–PAGE followed by autoradiography.

Immunofluorescence and confocal microscopy

Polyclonal antiserum specific for calreticulin was a generous gift from M. Michalak (University of Alberta, Edmonton, Canada) (Michalak and MacLennan, 1980). Fluorescein (FITC)-conjugated affinity pure donkey-anti-rabbit IgG and Lissamine Rhodamine (LRSC)-conjugated affinity pure donkey anti-goat IgG were purchased from Jackson ImmunoResearch Laboratories, Inc. BGMK cells were grown directly on cover slips and were either mock infected or infected with VV601 or VVT4 at an m.o.i. of 10 for 12 h. Following infection the cover slips were washed three times in PBS containing 1% FCS prior to fixing in ice-cold absolute methanol for 5 min. Coverslips were washed as before and then blocked with PBS containing 4% FCS and 50 mM ammonium chloride for 30 min at 37°C. For double labeling, coverslips were washed three times in PBS containing 1% FCS and the rabbit anti-M-T4 antiserum (1:250 dilution) and goat anti-calreticulin antiserum (1:20) were added directly to the coverslips. The coverslips were incubated with primary antibody for 60 min at room temperature after which they were once again washed and donkey-anti-rabbit FITC-conjugated secondary antibody (1:100 dilution) and donkey-anti-goat LRSC-conjugated secondary antibody (1:100 dilution) were added directly to the coverslips. The secondary antibodies remained on the coverslips for 1 h at room temperature. The coverslips were washed and mounted in 0.0025% *N*-propyl galate (Sigma Chem. Co.) to prevent photobleaching prior to analysis by confocal fluorescence microscopy. Confocal fluorescence microscopy was performed at the University of Alberta Faculty of Medicine confocal laser scanning microscope facility and data was analyzed using CLSM software (CLSM, Leica, Heidelberg).

RESULTS

Myxoma virus M-T4 contains an endoplasmic reticulum retrieval sequence

The T4 gene from Shope fibroma virus (SFV) was previously sequenced (Upton *et al.,* 1987) and found to contain a consensus carboxyl terminal endoplasmic reticulum (ER) retrieval sequence (reviewed in Pelham, 1989, 1990; Nilsson and Warren, 1994). Many soluble proteins that reside in the lumen of the ER contain an -RDEL or -KDEL tetrapeptide C-terminal sequence that is sufficient for retrieval back into the ER from the early Golgi compartment. Since the T4 gene from SFV contained at its C-terminus a putative ER retrieval sequence (-RDEL) (Upton *et al.,* 1987), and since SFV and myxoma virus are closely related viruses, we were interested to locate and sequence the equivalent gene in myxoma virus. Sequence information from SFV and myxoma virus was used to construct primers complementary to regions 5′ and 3′ of the area suspected to contain the equivalent open reading frame in myxoma virus, and the polymerase chain reaction was used to amplify a product directly from myxoma virus genomic DNA. This product was subcloned into the pT7blue plasmid for sequencing. Sequence analysis indicated that the myxoma virus T4 open reading frame, designated M-T4, encodes a protein of 237 amino acids with a predicted molecular mass of 26 kDa (Fig. 1). The M-T4 protein possesses a putative signal sequence of 28 amino acids, and a C-terminal -RDEL motif with no obvious hydrophobic membrane-spanning clusters similar to the T4 open reading frame in SFV. The M-T4 gene of myxoma virus is the fourth open reading frame from the end of the genome in the terminal inverted repeat and is thus present as two identical copies. The majority of the gene is present in the *Bam*HI-CC fragment, extends 120 nucleotides into the *Bam*HI-S fragment and is oriented to be transcribed toward the termini, similar to the T4 open reading frame in SFV (Macaulay *et al.,* 1987).

Data base searches failed to reveal homology between M-T4 and any nonviral proteins, however, significant homology was observed between M-T4 and the T4 genes from SFV and capripox virus, a poxvirus that infects sheep (Fig. 2). The percent identity between the T4 amino acid sequence of SFV and myxoma virus was found to be 77%, while the percentage identity between M-T4 and capripox T4 was found to be 36%. In addition, the B9R amino acid sequence from vaccinia virus strains WR and Copenhagen showed 28% identity to the amino terminal end of M-T4, indicating that B9R may be a truncated version of the myxoma virus M-T4 open reading frame that has lost the C-terminal RDEL motif (Howard *et al.,* 1991) (Fig. 2). The amino acid alignment in Fig. 2 indicates that 11 of 12 cysteine residues are present at the same position in M-T4, SFV-T4, and capripox T4, and the first 3 cysteines are conserved among

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1   ATGAAAATGTACACGCGATTATCGAGGGTAGGAACCATGGAGTCCTTCGTGCTACTTCTC   60
    M  K  M  Y  T  R  L  S  R  V  G  T  M  E  S  F  V  L  L  L

61  GCGTTCCTATCCCTCGTTGGTGGCTACGTCATCGATCCGTGTACGTCGCAAGAACGATCT  120
    A  F  L  S  L  V  G  G  Y  V  I  D  P  C  T  S  Q  E  R  S

121 ACGTGGCACGTATCGATCAAACTATGTATATACGTCCAAGAACACAAAGTATCCGCTAAC  180
    T  W  H  V  S  I  K  L  C  I  Y  V  Q  E  H  K  V  S  A  N

181 GGTTGTCGTCTCGAACAGGGTCCGGGTGGGTTTATCGCCACGGGGAACGGGTTTAAGATT  240
    G  C  R  L  E  Q  G  P  G  G  F  I  A  T  G  N  G  F  K  I

241 TTCGCACACGACGAATGCTCTCACAACGAACACAGTTTTCTACTAACGGACGTCCGAGAA  300
    F  A  H  D  E  C  S  H  N  E  H  S  F  L  L  T  D  V  R  E

301 GCCGTGTACGCGTCGGGGCACGGCATGTACGTGGAAATATCGGGCAATGTTCCTTATCTA  360
    A  V  Y  A  S  G  H  G  M  Y  V  E  I  S  G  N  V  P  Y  L

361 GATTCCGTACATCAGTGTGCCAGAAATATGACCGTCGCGGTATCGTGCGACAACCCCGTC  420
    D  S  V  H  Q  C  A  R  N  M  T  V  A  V  S  C  D  N  P  V

421 CCTAACGCCTACGGCAAGAACACGGACAAACTACGTCCGGGCGTCGCGATCGCCACGTTA  480
    P  N  A  Y  G  K  N  T  D  K  L  R  P  G  V  A  I  A  T  L

481 ATCGACACGTCGTGCGTACGAAGTCACAGTTTCGCATACTCCGTAAACACGTTGTGTACG  540
    I  D  T  S  C  V  R  S  H  S  F  A  Y  S  V  N  T  L  C  T

541 GAACGACTCTCCGGAGAGTCGTGCGAACAATTAGACTGTCACATGGTCAAAGGATCCCAA  600
    E  R  L  S  G  E  S  C  E  Q  L  D  C  H  M  V  K  G  S  Q

601 CACGAACACTACTTACAAACGTGCGACCGAAACGTTCCCGATCGGCGTACGTTTAAGGCG  660
    H  E  H  Y  L  Q  T  C  D  R  N  V  P  D  R  R  T  F  K  A

661 TATAAACCTCATCAACGGCCCTACGTCGCAAAAGTATTACGAGATGAATTA           711
    Y  K  P  H  Q  R  P  Y  V  A  K  V  L  R  D  E  L
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FIG. 1. The nucleotide sequence and deduced amino acid sequence of the M-T4 gene (Genbank Accession No. AF002684). The location of the predicted signal sequence and the C-terminus ER retention sequence (-RDEL) are indicated by the presence of a solid line.

the T4 proteins from myxoma virus, SFV, capripox virus, and B9R of vaccinia virus, suggesting the possibility of conservation at the structural level among these proteins.

Construction and verification of an M-T4 deletion virus

Myxoma virus expresses a number of virulence factors which have been shown to interact with various elements of the immune system (reviewed in McFadden and Graham, 1994; McFadden *et al.,* 1995). Virulence genes are routinely identified by their deletion and subsequent virus attenuation in infected rabbits. In order to characterize the M-T4 gene and assess its role in myxoma virus virulence both copies of the M-T4 gene were inactivated by insertion of the *E. coli* guanosine phosphoribosyl transferase (Eco gpt) gene. The 5′ (180 base pair) and 3′ (230 base pair) fragments were amplified with complementary primers prior to insertion of Eco gpt into an intervening *Bgl*II site. The resulting disrupted M-T4, containing Eco gpt (pMT4gpt), was then used to generate a recombinant virus (vMyxlacT4$^-$) by homologous recombination and selection in the presence of mycophenolic acid. A similar strategy has been used successfully to disrupt other open reading frames in myxoma virus (Upton *et al.,* 1991; Mossman *et al.,* 1996a, 1996b; Macen *et al.,* 1993). To ascertain that both copies of the gene were in fact disrupted, PCR analysis was performed on DNA isolated from plaque-purified virus (data not shown).

Northern blotting analysis of total RNA isolated from vMyxlac-infected cells demonstrated that a single M-T4 transcript of approximately 900 bases is present at 2 h postinfection and can still be detected at late times (Fig. 3A, lanes 2–5). No M-T4 transcript was detected in RNA isolated from either mock-infected (Fig. 3A, lane 1) or vMyxlacT4$^-$-infected cells 4 h postinfection (Fig. 3A, lane 6), suggesting that any truncated message transcribed from the remaining portion of the M-T4 gene is probably unstable and rapidly degraded. In the presence of araC, an inhibitor of DNA replication, and thus late virus gene expression, the M-T4 transcript could still be detected in RNA isolated 16 h postinfection (Fig. 3B, lanes 1 and 2). These experiments indicate that the M-T4 gene is transcribed at early times as a relatively stable message and that no M-T4 mRNA can be detected from vMyxlacT4$^-$-infected cells.

M-T4 functions as a myxoma virus virulence factor

Infection of European rabbits with myxoma virus results in a lethal and progressive disease termed myxomatosis (Fenner and Ratcliffe, 1965), and the deletion of

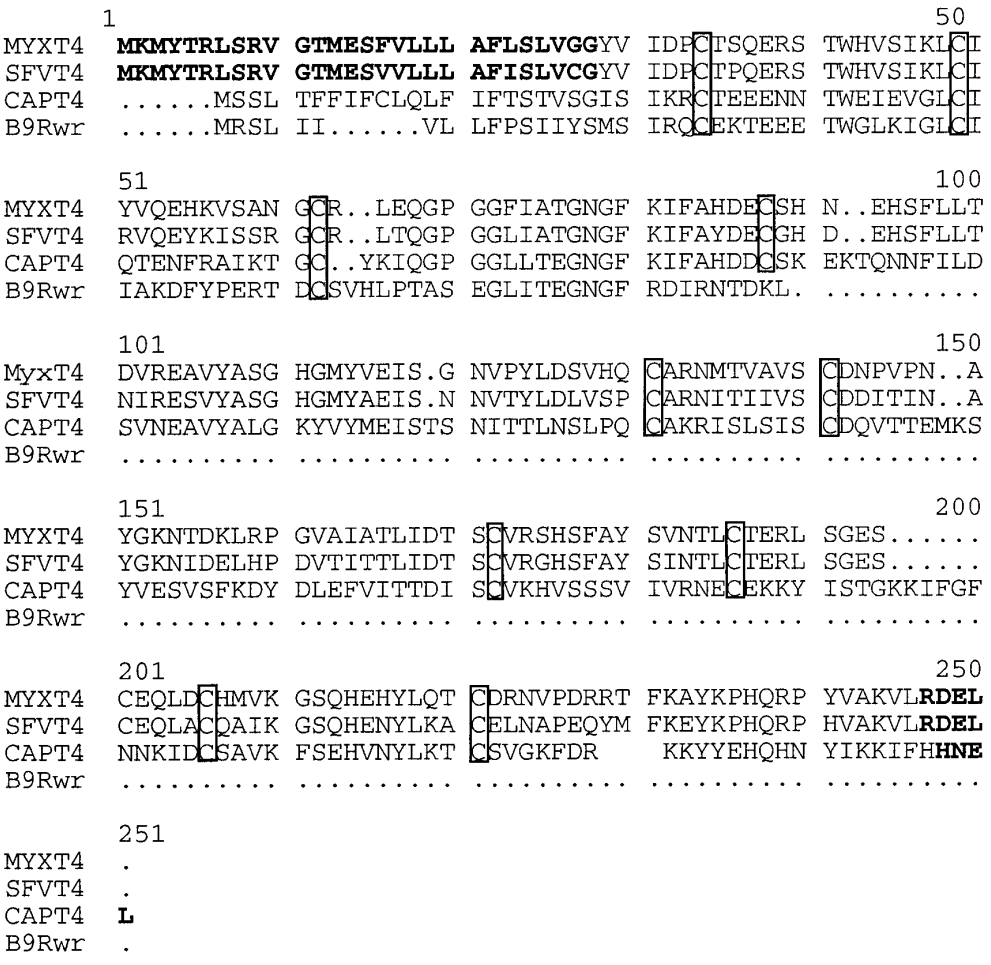

FIG. 2. The M-T4 open reading frame is homologous to open reading frames in other poxvirus family members. Amino acid sequence alignment between the M-T4 open reading frame and the T4 open reading frames of Shope fibroma virus (SFVT4), capripoxvirus (CAPT4), and the B9R open reading frame in vaccinia virus strain WR (B9Rwr) is shown. Predicted signal sequences and ER retention sequences are shown bolded. Conserved cysteine residues among the four open reading frames are highlighted by boxes and gaps introduced to maintain alignment are indicated by dots.

virus genes necessary for virus virulence routinely results in a decrease in disease pathogenesis. To determine the effect of an M-T4 disruption *in vivo,* eight rabbits were inoculated with vMyxlacT4$^-$. As a control, four rabbits were inoculated with the parental vMyxlac virus. A reduction in virulence was observed in European rabbits infected with vMyxlacT4$^-$ as compared to rabbits infected with the parental virus (Table 1). Day 3 postinfection both sets of rabbits displayed small lesions at the primary site of inoculation. By day 6 postinfection, the vMyxlac-infected rabbits demonstrated many of the features of myxomatosis, including the presence of secondary lesions around the eyes, ears, and nares. As the disease progressed, the vMyxlac-infected rabbits exhibited extensive conjunctivitis and rhinitis and difficulty breathing. Three of the vMyxlac-infected rabbits were sacrificed 10 days postinfection with the final rabbit sacrificed on day 13 due to the severity of the disease. In contrast, all animals infected with the vMyxlacT4$^-$ virus recovered fully from infection and displayed few of the classical disease features of myxomatosis. For example, no bacterial infection of the nasal mucosa and conjunctiva were detected throughout the course of the experiment, and only one of eight animals infected with the mutant virus showed any obvious signs of secondary sites of infection, whereas animals infected with the parental vMyxlac virus had many lesions on the ears, nose, and above the eyes. By 21 days postinfection, all animals infected with the M-T4 mutant virus had fully recovered and the primary lesions were resolved.

Since infection of European rabbits with the M-T4 mutant virus resulted in a significant difference in pathogenic profile, nine additional rabbits were infected with the mutant virus and the parental vMyxlac virus. Rabbits in this study were then subjected to postmortem examination and histological analysis at 3, 7, and 11 days postinfection. Since infection with the vMyxlacT4$^-$ virus clearly resulted in a decrease in virus spread we were particularly interested in events at the primary site of infection. We examined tissue sections of primary site lesions from animals infected with both the parental vMyxlac virus and the vMyxlacT4$^-$ virus. Tissue samples

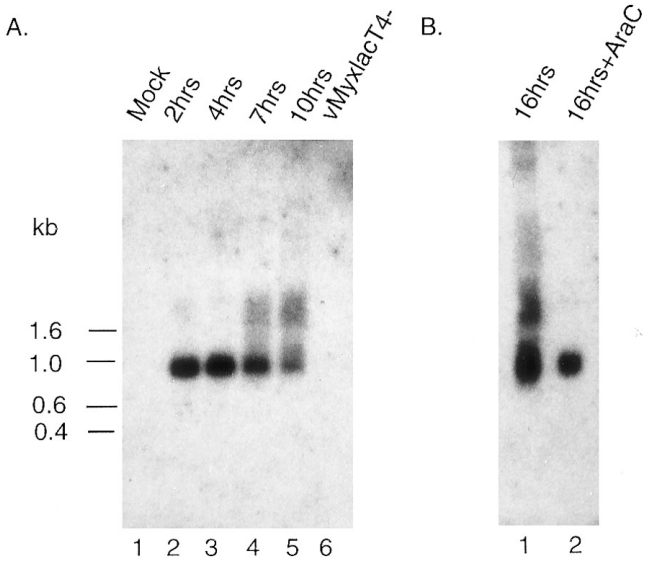

FIG. 3. Northern blotting analysis of RNA isolated from vMyxlac- and vMyxlacT4$^-$-infected BGMK cells. (A) RNA was isolated from mock-infected cells (lane 1), cells infected with vMyxlac for 2 h (lane 2), 4 h (lane 3), 7 h (lane 4), 10 h (lane 5), vMyxlacT4$^-$ 4 h (lane 6) and probed with a radiolabeled M-T4 probe. A single transcript could be detected as early as 2 h postinfection, but could not be detected in vMyxlacT4$^-$-infected cells. (B) RNA was isolated from vMyxlac-infected cells 16 h postinfection in the presence (lane 1) and absence (lane 2) of 40 μg/ml of AraC.

stained with hematoxylin and eosin revealed a striking difference between the two sets of rabbits at the primary site of inoculation. As early as 3 days postinfection primary site lesions from rabbits infected with the parental vMyxlac virus showed extensive edema due to virus infection and only a minor infiltration of heterophils (Fig. 4A). In contrast, primary lesions from rabbits infected with vMyxlacT4$^-$ showed a significant increase in the number of infiltrating heterophils (Fig. 4B), suggesting the initiation of a more vigorous cellular inflammatory response in animals infected with the M-T4 mutant virus.

These same tissue sections were subsequently analyzed by immunoperoxidase staining using a primary antibody specific for rabbit CD43. This antibody recognizes rabbit CD43 which is found on the surface of T cells, as well as monocytes and macrophages. Day 3 primary lesions from rabbits infected with the M-T4 mutant virus showed an increased number of CD43-positive cells (Fig. 4D) compared to comparable tissue sections infected with vMyxlac (Fig. 4C), demonstrating that a significant proportion of the infiltrating heterophils in the vMyxlacT4$^-$ primary lesions are CD43-positive. In addition, since both the parental virus, vMyxlac, and the mutant virus, vMyxlacT4$^-$, contain the *E. coli* lacZ gene it was possible to observe the presence of virus in tissues by immunoperoxidase staining with an antibody specific for β-galactosidase. Day 3 primary site tissues demonstrated the presence of virus in tissue sections from both rabbits infected with the mutant virus and the parental virus (Figs. 4E and 4F); however, the amount of virus in tissue sections infected with the mutant M-T4 virus was decreased compared to tissue sections infected with the parental virus. These results reflect the observations made at the gross level and further substantiate our conclusion that the loss of M-T4 from the virus genome results in virus attenuation due to the inability to curtail the local early cellular inflammatory response.

vMyxlacT4$^-$ virus is defective for replication in a CD4$^+$ rabbit T cell line

Historically the deletion of virulence factors from the genomes of poxviruses has no effect on the ability of the virus to replicate in cultured cell lines *in vitro.* Thus we assessed the ability of vMyxlacT4$^-$ to grow in tissue culture cell lines. In a single step growth curve, no defect was noted in the replication of the M-T4 mutant virus in cultured rabbit RK-13 fibroblast cells compared with the parental vMyxlac virus (Fig. 5A). A similar observation was made when BGMK cells were infected with vMyxlac and vMyxlacT4- (unpublished data). Replication of the M-T4 mutant virus in cultured RL-5 cells, a CD4$^+$ rabbit T cell line (Kaschka-Dierich *et al.,* 1982), however, was

TABLE 1

Pathogenicity of vMyxlac and vMyxlacT4$^-$ in Infected European Rabbits

Day	vMyxlac	vMyxlacT4$^-$
0	Four adult female NZW rabbits inoculated intradermally at two sites with 10^3 PFU/site.	Eight adult female NZW rabbits inoculated intradermally at two sites with 10^3 PFU/site.
3	Small primary lesions at site of inoculation, no other symptoms.	Small primary lesions at site of inoculation, no other symptoms.
6	Primary lesions large (3 cm) well defined and necrotic in center. Initial signs of secondary lesions around eyes, ears, nose.	Smaller primary lesions (1.5 cm), some necrosis in center. One rabbit with secondary lesions on ears.
10	Primary lesions very large (5 cm), necrotic area increased, multiple secondary lesions, moderate to severe bacterial infections of nasal mucosa, conjuctiva, difficulty breathing. Three animals sacrificed due to severity of disease.	Primary lesions resolving. No bacterial infection detected. One rabbit with secondary lesions on ear. Overall animals very healthy.
13–21	Final animal sacrificed.	All rabbits recovered. Primary lesions resolved and secondary lesions on one rabbit resolved.

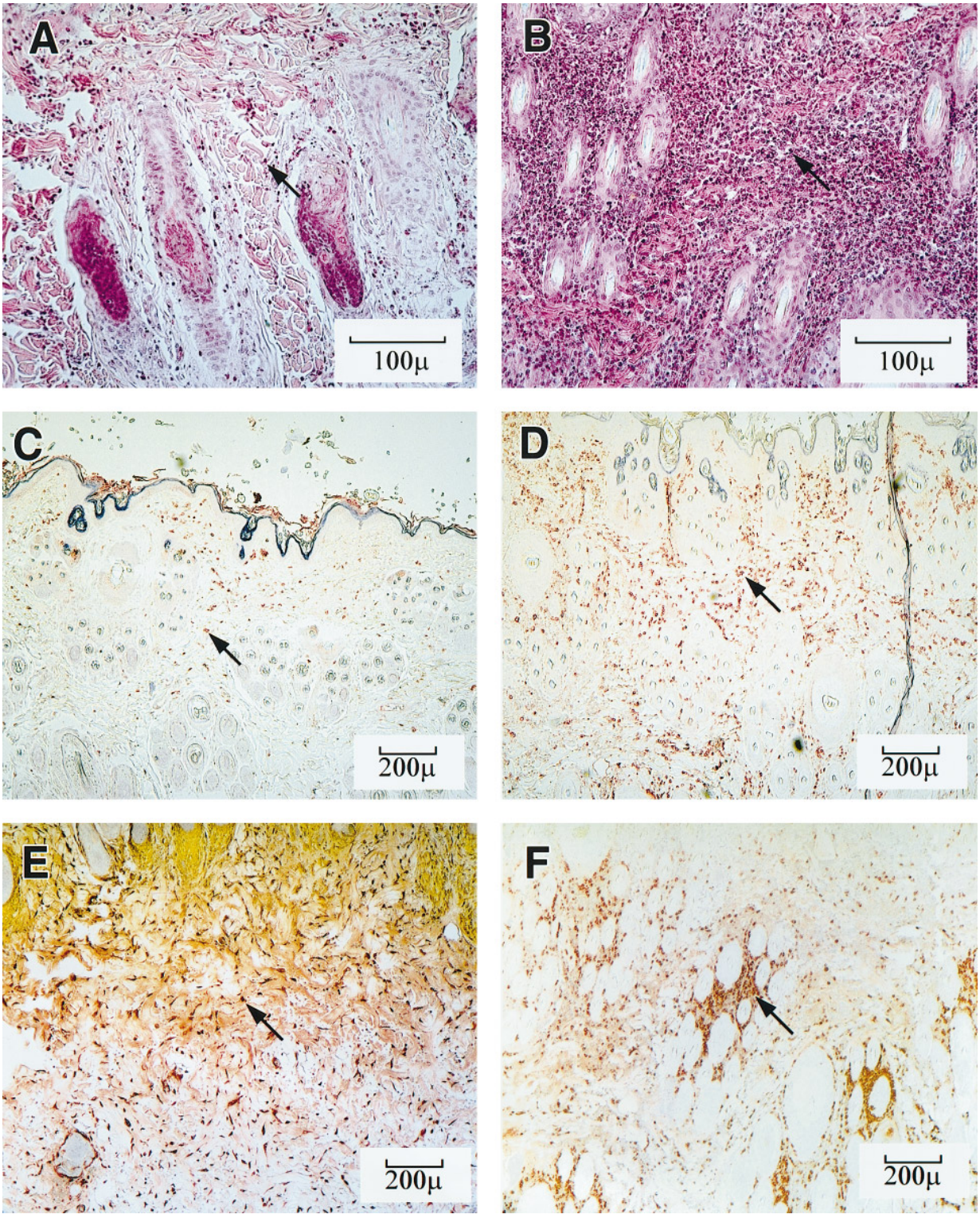

FIG. 4. Immunohistological analysis of primary virus-infected lesions. Tissue sections were harvested from the primary lesions of animals infected with vMyxlac (A) or vMyxlacT4$^-$ (B) 3 days postinfection and stained with hematoxylin and eosin. Tissue sections harvested from the vMyxlacT4$^-$ (B)-infected rabbit shows a dramatic accumulation of heterophils (arrows) in contrast to infection with vMyxlac (A). These same tissue sections were subjected to immunoperoxidase staining with an antibody specific for CD43. Tissue sections harvested from the vMyxlac-infected rabbit (C) demonstrated few CD43 positive infiltrating cells (arrows), whereas tissue sections harvested from the vMyxlacT4$^-$-infected rabbits (D) indicated an increased number of CD43-positive infiltrating cells. These same tissue sections were also subjected to immunoperoxidase staining with an anti-β-galactosidase antibody. Areas with high levels of β-galactosidase correspond to areas of virus infection since both viruses express β-galactosidase. Tissue sections from vMylac (E)-infected tissues exhibit more virus antigen (arrows) than tissue sections from vMyxlacT4$^-$ (F).

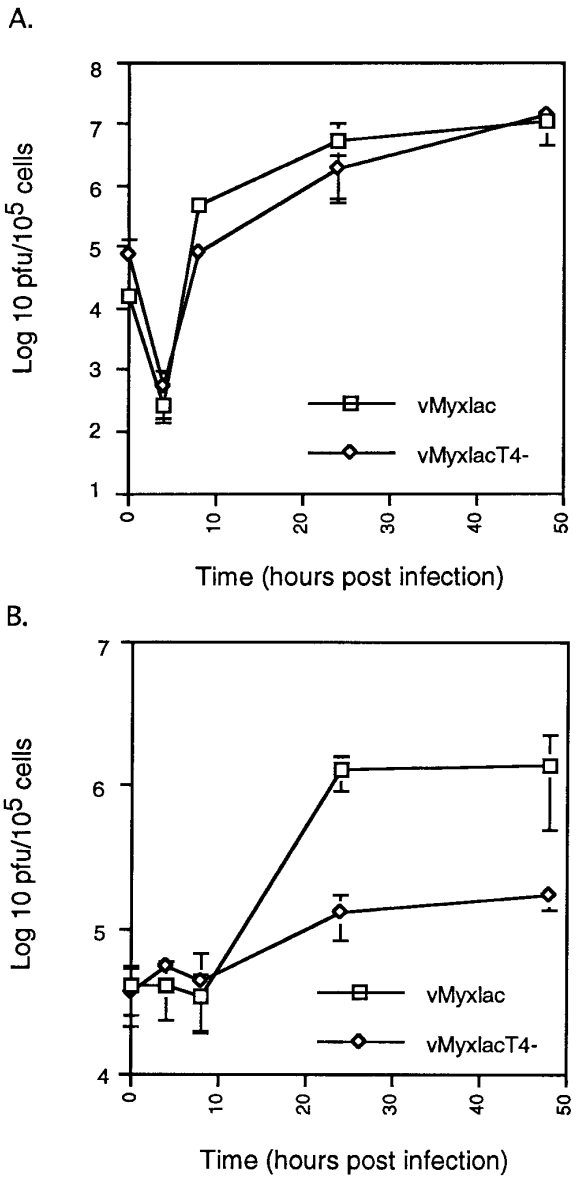

FIG. 5. The replication of vMyxlacT4$^-$ is normal in permissive rabbit fibroblasts but defective in a rabbit T cell line. RK-13 cells (A) and RL-5 cells (B) were infected with either vMylac or vMyxlacT4- at a m.o.i. of 10. The cells were harvested at 0, 4, 8, 24, and 48 h postinfection and virus titers were determined on BGMK cells. All titrations were performed in triplicate.

found to be significantly reduced (Fig. 5B), suggesting that the presence of M-T4 was in fact necessary for replication of the virus in this lymphocytic cell line. We have routinely observed that the levels of myxoma virus replication in lymphocytes is much lower than in fibroblasts (Mossman *et al.,* 1996a), an observation also seen in this study. However, the ability to spread infectious virus to secondary sites is simply a function of the infected lymphocytes surviving long enough to travel to other sites where subsequent rounds of replication in nonlymphoid cells can then occur.

Infection of lymphocytes with vMyxlacT4$^-$ results in apoptosis

Since the M-T4 mutant virus grew normally in a cultured fibroblast cell line, but not in RL-5 cells, and since infection of RL-5 cells with several other mutant myxoma viruses has been shown previously to result in the induction of apoptosis in these cells (Macen *et al.,* 1996; Mossman *et al.,* 1996a), we investigated whether RL-5 cells infected with the M-T4 mutant virus were also undergoing apoptosis. Such a response could account for the distinct disease profile seen in rabbits infected with the M-T4 mutant virus. Induction of programmed cell death, or apoptosis, can function as an antiviral mechanism via the premature death of infected cells and subsequently limit the production and dissemination of virus. In fact, a number of viruses encode proteins that protect against cellular induced apoptosis (reviewed in Cuff and Ruby, 1996; Gilet and Brun, 1996; White, 1996; McFadden and Barry, 1997; Teodoro and Branton, 1997).

One obvious feature of apoptosis is the degradation of DNA into oligonucleosomal fragments, which is observed as DNA laddering when total genomic DNA is subjected to agarose gel electrophoresis. Figure 6 indicates that DNA laddering was observed in genomic DNA isolated from RL-5 cells infected with vMyxlacT4$^-$ 16 h postinfection (Fig. 6, lane 2), but not in DNA isolated from RL-5 cells infected with the parental vMyxlac virus (Fig. 6, lane 1), indicating that RL-5 cells infected with the mutant virus were indeed undergoing the terminal stages of apoptosis.

We also monitored DNA fragmentation in RL-5 cells by

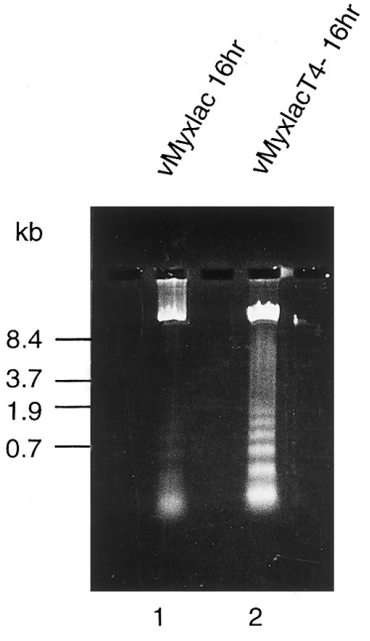

FIG. 6. Infection of RL-5 cells with vMyxlacT4$^-$ results in DNA fragmentation. Genomic DNA was isolated from RL-5 cells infected with vMyxlac (lane 1) or vMyxlacT4$^-$ (lane 2) 16 h postinfection and subjected to agarose gel electrophoresis.

quantitating the terminal deoxynucleotidyl transferase-mediated incorporation of fluorescein dUTP on to the ends of fragmented DNA by flow cytometric analysis (Fig. 7). It would be predicted using this technique that cells undergoing DNA fragmentation would show an increase in fluorescence intensity due to the incorporation of fluorescein-labeled dUTP. Using this approach both mock-infected and parental vMyxlac infected RL-5 cells showed an equivalent low level of background fluorescence (Fig. 7), indicating the lack of DNA fragmentation in cells infected with vMyxlac. Infection of RL-5 cells with the M-T4 mutant virus (vMyxlacT4$^-$) 16 h postinfection, however, resulted in a dramatic increase in relative fluorescence, indicative of cells undergoing DNA fragmentation. In direct contrast, infection of RL-5 cells with the vMyxT4R revertant virus showed minimal apoptosis, indicating that replacement of M-T4 into the viral genome was sufficient to inhibit DNA fragmentation. Changes in cell morpholgy were also monitored by flow cytometric analysis and infection of RL-5 cells with vMyxlacT4$^-$ resulted in the emergence of a population of cells that were smaller in size, another common feature displayed by cells undergoing apoptosis (unpublished data). These studies demonstrate that RL-5 cells infected with the M-T4 mutant virus, but not RL-5 cells infected with the parental virus or revertant virus, undergo DNA fragmentation as monitored by both agarose gel electrophoresis, and terminal deoxynucleotidyl transferase-mediated incorporation of fluorescein-labeled dUTP.

While the observation that RL-5 cells infected with the M-T4 mutant virus induces apoptosis is significant, RL-5 cells are a cultured T cell line derived from a herpesvirus-induced rabbit lymphoma (Kaschka-Dierich *et al.,* 1982). To ascertain whether or not apoptosis is strictly a feature of vMyxlacT4$^-$-infected RL-5 cells or whether infection of primary rabbit T lymphocytes with the M-T4 mutant virus also results in apoptosis, primary rabbit peripheral blood lymphocytes were harvested. The mononuclear cells were fractionated into adherent and nonadherent populations in order to separate the monocytes from the lymphocytes. The nonadherent population, mainly containing lymphocytes, was infected with the parental vMyxlac virus and the mutant vMyxlacT4$^-$ virus. Routinely, we were only able to detect productive infection of approximately 50% of the primary peripheral rabbit lymphocytes as assessed by late expression of β-galactosidase, indicating that not all rabbit lymphocytes are productively infected by myxoma virus, but the identification of specific subsets remains to be clarified. In order to assess whether or not infected primary rabbit lymphocytes were undergoing apoptosis, cells were labeled with fluorescein dUTP and subjected to analysis by flow cytometry (Fig. 8). Nonadherent primary rabbit lymphocytes demonstrated an increase in relative fluorescence when infected with the M-T4 mutant virus as compared to mock-infected cells or infection with the

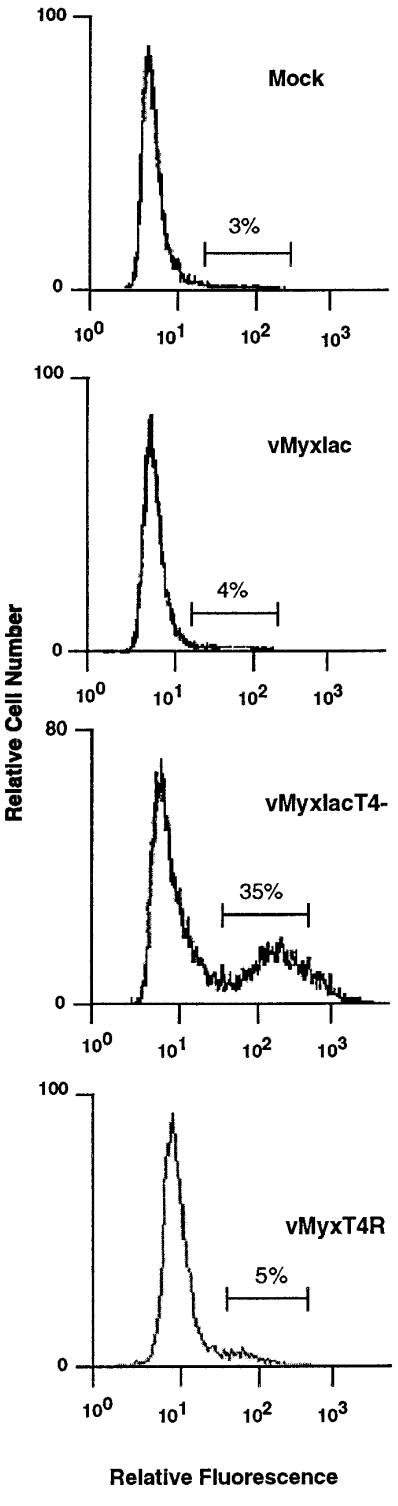

FIG. 7. Flow cytometric analysis of RL-5 cells infected with vMyxlacT4$^-$ indicates virus infection results in the induction of DNA fragmentation. Cells were either mock infected, infected with vMyxlac, vMyxlacT4-, or vMyxT4R at an m.o.i. of 10. At 16 h postinfection cells were harvested and permeabilized as described under Materials and Methods prior to treatment with terminal dexoynucleotidyl transferase and fluorescein-12-dUTP in order to label fragmented DNA. Only RL-5 cells infected with vMyxlacT4$^-$ showed evidence of a shift in relative fluorescence indicative of DNA fragmentation.

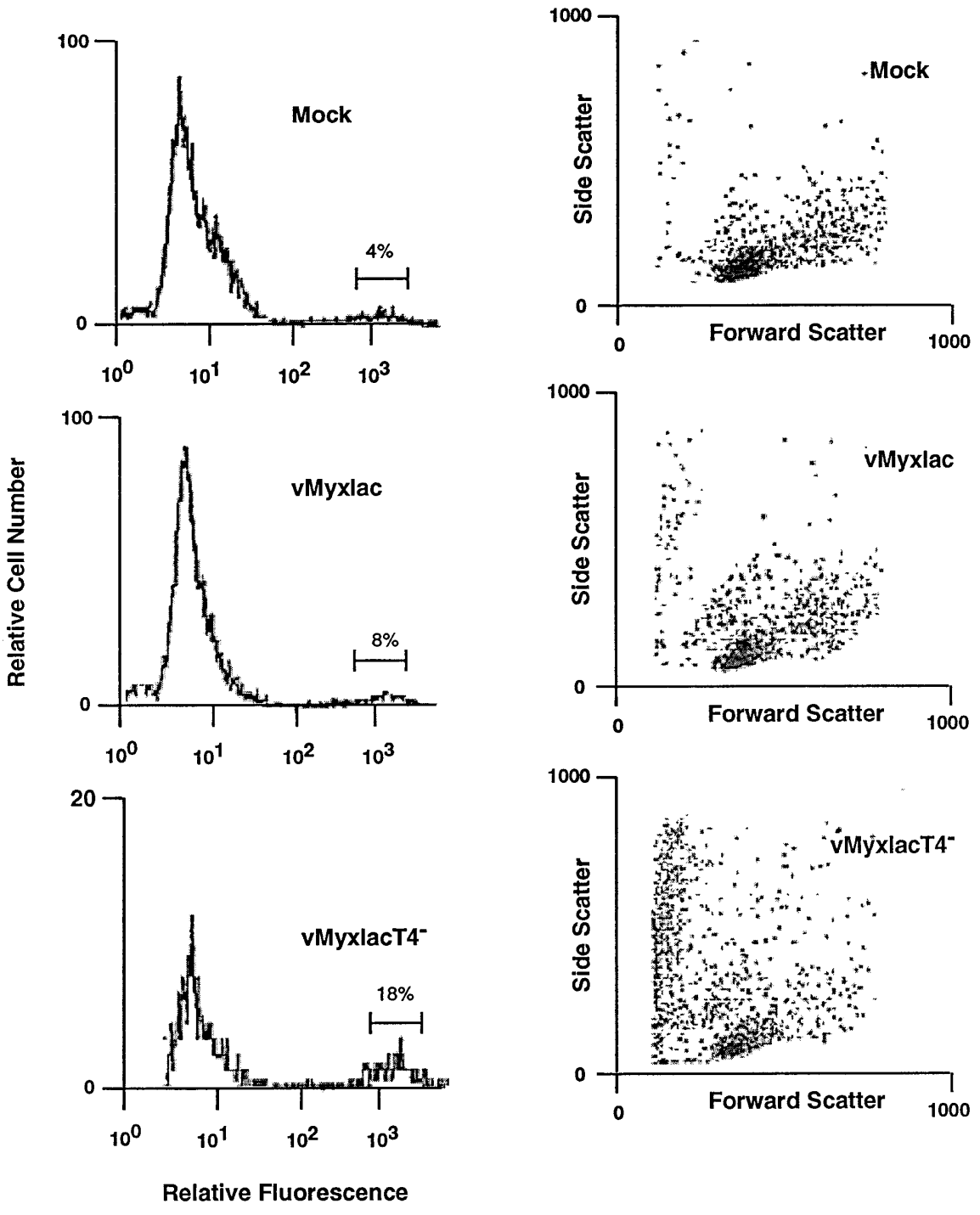

FIG. 8. Flow cytometric analysis of primary rabbit peripheral blood lymphocytes infected with vMyxlacT4$^-$ results in the induction of DNA fragmentation. Nonadherent primary blood lymphocytes were harvested as described under Materials and Methods and either mock infected, infected with vMyxlac, or infected with vMyxlacT4$^-$ at an m.o.i. of 10. At 16 h postinfection cells were harvested and permeabilized as described under Materials and Methods prior to treatment with terminal deoxynucleotidyl transferase and fluorescein-12-dUTP in order to label fragmented DNA. Only primary peripheral lymphocytes infected with vMyxlacT4$^-$ showed evidence of a shift in relative fluorescence indicative of DNA fragmentation. The light scatter profiles of PBLs mock infected, infected with vMyxlac, or infected with vMyxlacT4$^-$ are shown beside the corresponding fluorescence profiles. PBLs infected with vMyxlacT4$^-$ show an increase in a population of shrunken cells, indicative of cells undergoing apoptosis.

parental vMyxlac virus. The number of intact cells following infection with vMyxlacT4$^-$ was found to drop and when changes in cell morphology were monitored by flow cytometry the light scatter profile indicated the emergence of a population of shrunken apoptotic cells after infection with vMyxlacT4$^-$. Although late gene expression was only detected in 50% of the peripheral blood lymphocytes infected with vMyxlac, the extensive

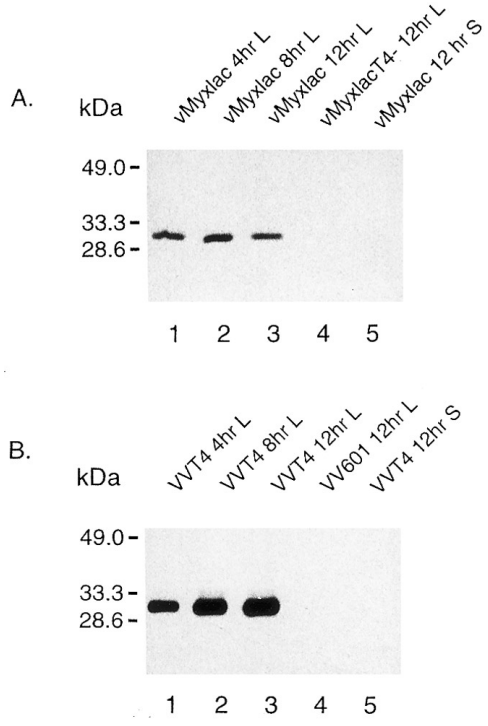

FIG. 9. M-T4 protein is retained within infected cells. (A) BGMK cells were infected with vMyxlac at an m.o.i. of 10 and total cell lysates were harvested at 4 h (lane 1), 8 h (lane 2), and 12 h (lane 3) postinfection. Total cell lysates were also harvested from cells infected with vMyxlacT4$^-$ 12 h postinfection (lane 4). Secreted culture supernatants were harvested from vMyxlac infected cells 12 h postinfection (lane 5). Lysates and supernatants were analyzed by Western blotting with an antibody specific for M-T4. (B) BGMK cells were infected with VVT4 at an m.o.i. of 10 and total cell lysates were harvested at 4 h (lane 1), 8 h (lane 2), and 12 h (lane 3) postinfection. Total cell lysates were also harvested from cells infected with VV601 12 h postinfection (lane 4). Secreted culture supernatants were harvested from VVT4-infected cells 12 h postinfection (lane 5). Lysates and supernatants were analyzed by Western blotting with an antibody specific for M-T4.

amount of apoptosis detected after infection with vMyxlacT4$^-$ suggests that in fact the majority of cells were infected but that late gene expression was present in only a subset of the cells. We conclude that infection of primary rabbit peripheral blood lymphocytes with the M-T4 mutant virus results in apoptosis.

M-T4 protein is not secreted from virally infected cells

Since sequencing information indicated that M-T4 contained both a putative signal sequence and an ER retention sequence, it seemed likely that M-T4 may function as an intracellular virulence factor and more precisely an ER-associated virulence factor. To determine whether or not M-T4 does indeed localize to the ER, polyclonal antiserum was produced in rabbits using a synthetic peptide encompassing the last 10 amino acid residues of M-T4 (-YVAKVLRDEL). In addition, we also constructed a vaccinia virus, VVT4, that was engineered to overexpress M-T4 by placing the M-T4 open reading frame behind a strong synthetic late viral promoter. Total cell lysates and supernatants were harvested from vMyxlac-, vMyxlacT4$^-$-, VV601-, and VVT4-infected BGMK cells 4, 8, and 12 h postinfection. The lysates and supernatants were subjected to Western blot analysis using the generated polyclonal antiserum. Figure 9 indicates the presence of a prominent protein of approximately 30 kDa in lysates from both vMyxlac (Fig. 9A, lanes 1–3) and VVT4-infected cells (Fig. 9B, lanes 1–3). However, this protein was not present in cell lysates infected with vMyxlacT4$^-$ (Fig. 9A, lane 4) or VV601 (Fig. 9B, lane 4), both of which lack complete M-T4 open reading frames. Additionally, this band was not present when the experiment was repeated with preimmune anti-sera (unpublished data). Together these data suggest that the 30-kDa protein is in fact M-T4. The M-T4 protein was detected in vMyxlac-infected cell lysates as early as 4 h postinfection (Fig. 9A, lane 1), but was not detected in culture supernatants harvested 12 h postinfection (Fig. 9A, lane 5), suggesting that although M-T4 contains a signal sequence it is not secreted from virus-infected cells and is therefore cell associated during virus infection. Furthermore, M-T4 is also not secreted from VVT4-infected cells (Fig. 9b, lane 5), indicating that cell association is not simply mediated by another myxoma virus-encoded protein.

M-T4 localizes to the ER

The presence of a tetrapeptide -RDEL C-terminal sequence suggests that M-T4 may be predominantly an endoplasmic reticulum-associated protein since this motif is both necessary and sufficient for endoplasmic reticulum retention (Munro and Pelham, 1987; Andres *et al.,* 1990). Thus we investigated the intracellular localization

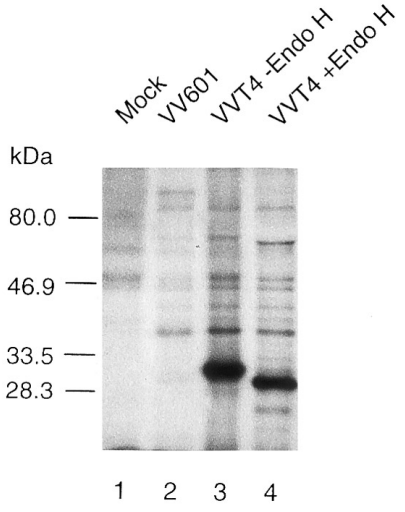

FIG. 10. M-T4 protein is endo H-sensitive. BGMK cells were either mock infected (lane 1), infected with VV601 (lane 2), or infected with VVT4 (lanes 3 and 4) in the presence of ^{35}S Met/Cys. M-T4 protein was immunoprecipitated with antiserum specific for M-T4 and either untreated (lane 3) or treated with endo H (lane 4) prior to SDS–PAGE and analysis by autoradiography.

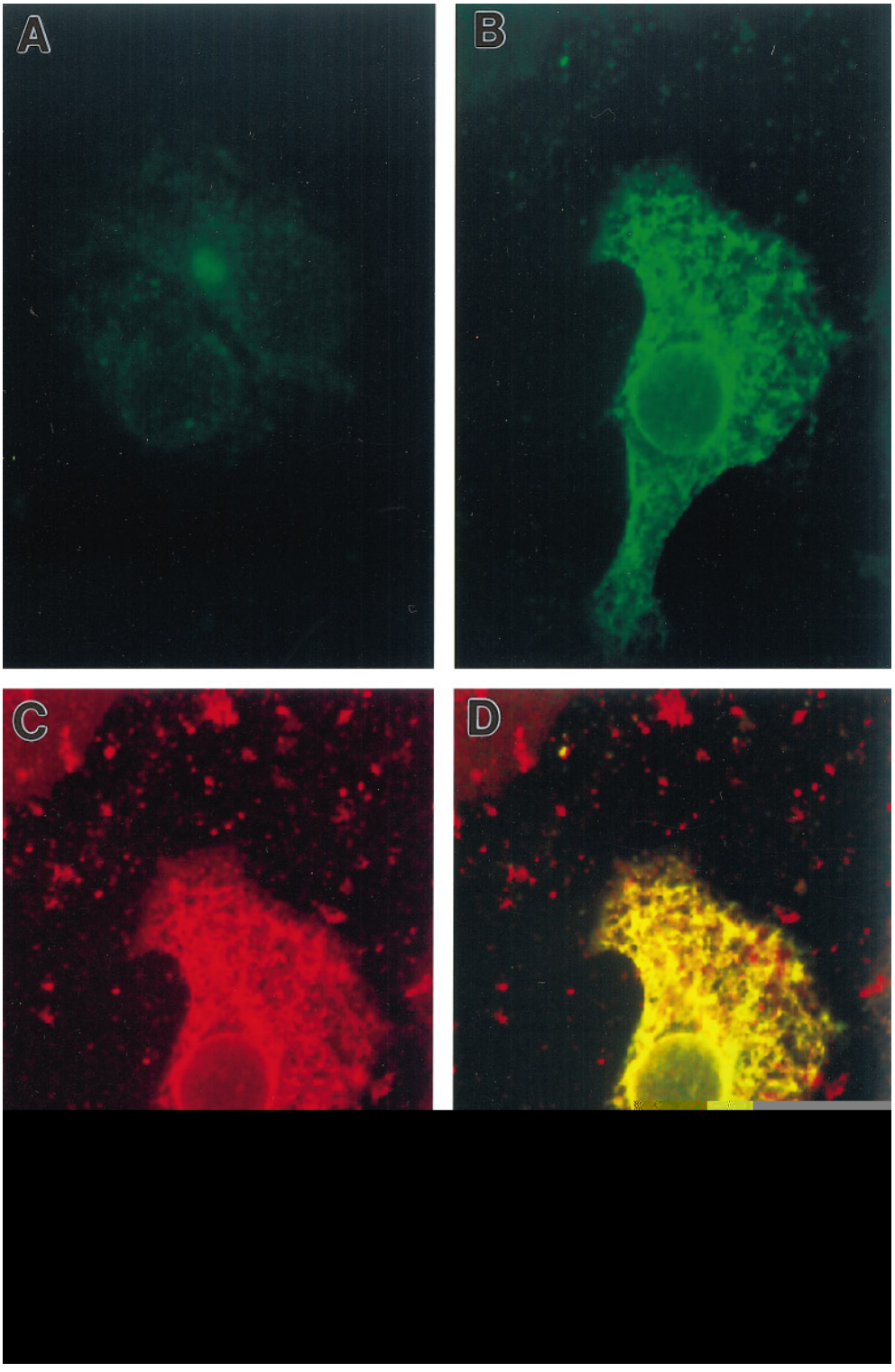

FIG. 11. M-T4 colocalizes with calreticulin. BGMK cells were either infected with VV601 or infected with VVT4 at an m.o.i. of 10. At 12 h postinfection the cells were fixed, permeabilized, treated with rabbit anti-M-T4 and goat anti-calreticulin. (A) BGMK cells infected with VV601 and labeled with rabbit anti-M-T4 and anti-rabbit FITC-conjugated secondary antibody. (B) BGMK cells infected with VVT4 and labeled with rabbit anti-M-T4 and anti-rabbit FITC-conjugated secondary antibody. (C) BGMK cells infected with VVT4 and labeled with goat anti-calreticulin and anti-goat LRSC-conjugated secondary antibody. Cells infected with VVT4 show positive fluorescence with both the anti-M-T4 antibody (B) and the anti-calreticulin antibody (C). When (B) and (C) are combined regions of colocalization appear yellow (D).

of M-T4 by both endo-H sensitivity and confocal fluorescence microscopy. Glycoproteins that enter into the ER are modified by acquisition of N-linked oligosaccharides which can be cleaved enzymatically by endo H. Thus, endo H sensitivity can be used as a diagnostic tool to assess whether or not a protein enters into the ER. To ascertain if M-T4 did indeed enter into the ER, the Endo H sensitivity of ^{35}S-labeled M-T4 protein was assessed. BGMK cells mock infected or infected with VV601 or VVT4 in the presence of ^{35}S Met/Cys were subjected to immunoprecipitation prior to digestion with endo H. Figure 10 demonstrates that no M-T4 protein could be immunoprecipitated from mock-infected cells (Fig. 10, lane 1) or from cells infected with VV601 (Fig. 10, lane 2) as anticipated. However, lysates harvested from BGMK cells infected with VVT4 indicated the presence of a 30-kDa species of M-T4 protein (Fig. 10, lane 3) that was not present in mock-infected or VV601-infected cells. Treatment with endo H resulted in a quantitative shift in the observed migration of M-T4 (Fig. 10, lane 4), indicating that M-T4 was indeed endo H sensitive and was in fact modified by the addition of N-linked oligosaccharides in the ER.

To further assess the intracellular localization of M-T4, infected BGMK cells were subjected to confocal fluorescence microscopy and double labeling was performed with the antibody specific for M-T4 and an antibody specific for calreticulin. Calreticulin is a Ca^{2+} binding protein with a C-terminal -KDEL amino acid sequence which has been previously localized to the lumen of the ER (Fleigel *et al.,* 1989; Milner *et al.,* 1991; reviewed in Nash *et al.,* 1994). In order to examine the intracellular distribution of M-T4 within infected cells, BGMK cells were grown directly onto coverslips and infected with VV601 or VVT4. Due to the demonstrably lower levels of M-T4 expression in myxoma virus-infected BGMK cells compared to that of the VVT4 overexpressor, as quantified by Western blotting, the signal for ER-retained M-T4 protein could not be assessed by immunofluorescence using this antibody. Twelve hours postinfection with VVT4 and VV601, the cells were fixed and permeabilized prior to double labeling with anti-M-T4 and anti-calreticulin. Infection of BGMK cells with VV601 followed by staining with the anti-M-T4 antibody and FITC-conjugated secondary antibody (Fig. 11A) indicated low background fluorescence. BGMK cells infected with the VVT4 virus and stained with the antibody specific for M-T4 demonstrated intense immunofluorescence throughout the extranuclear region (Fig. 11B), which appeared to be similar when these same cells were stained with the anti-calreticulin antibody (Fig. 11C). In fact, when labeling from these cells was combined, colocalization of M-T4 and calreticulin was indeed evident as indicated by the intense yellow color (Fig. 11D). These results clearly suggest that M-T4 colocalizes with calreticulin in infected cells and further indicates that M-T4 is retained as resident ER protein.

DISCUSSION

In order to survive and replicate efficiently within its host, all viruses must possess specific strategies to circumvent the multifaceted immune response that the host elicits. Myxoma virus is a large double-stranded DNA virus that encodes an abundance of proteins, more than are necessary for the simple replication of virus. In fact, many open reading frames encode proteins that are essential for evading antiviral immune responses within the host. In this report we demonstrate that the M-T4 open reading frame of myxoma virus is also necessary for immune evasion since infection of European rabbits with a recombinant virus unable to express a functional M-T4 protein is attenuated *in vivo.* M-T4 can now be added to the growing list of myxoma virus proteins that have been shown to play a role in myxoma virus pathogenesis but is the first example of an ER-retained viral protein to exhibit this property.

Our studies have demonstrated that the M-T4 open reading frame of myxoma virus encodes a 30-kDa glycoprotein that contains both an N-terminal signal sequence and a C-terminal ER retention sequence (-RDEL). The M-T4 protein is likely expressed early during infection since Northern blotting analysis demonstrated the presence of a single transcript as early as 2 h postinfection, and the presence of Ara C, which inhibits late poxvirus gene expression, has no effect on the presence of the transcript (Fig. 3). We were able to detect M-T4 by Western blotting in total cell lysates infected with vMyxlac as early as 4 h postinfection (Fig. 9A), and this expression was unaffected by the presence of AraC (unpublished observation, S. Hnatiuk, M. Barry, and G. McFadden).

Unlike European rabbits infected with the parental virus, rabbits infected with vMyxlacT4$^-$ recovered fully and displayed few of the clinical features normally observed during myxomatosis. In fact, we observed that tissue sections from day 3 primary lesions of rabbits infected with vMyxlacT4$^-$ showed a dramatic increase in the number of infiltrating heterophils (Fig. 4), a result that is consistent with the induction of a robust cellular inflammatory response and subsequent host recovery. One of the most striking observations made during the course of this study was the notable decrease in secondary lesions in animals infected with the M-T4 mutant virus, suggesting that the M-T4 mutant virus was unable to spread to secondary sites of infection. One possible explanation is an inability of the mutant virus to replicate in leukocytes which normally act as vehicles for virus dissemination. In fact, we have observed that the abortive infection of both RL-5 cells and rabbit primary peripheral blood lymphocytes with the vMyxlacT4$^-$ virus resulted in the rapid induction of DNA fragmentation as

monitored by both agarose gel electrophoresis and flow cytometry (Figs. 6, 7, and 8). In contrast, RL-5 cells and rabbit primary peripheral blood lymphocytes infected with vMyxlac did not undergo DNA fragmentation, indicating that the presence of M-T4 protein seems to be necessary for the productive infection of lymphocytes by the virus. Thus one possible explanation for the absence of viral dissemination from the primary lesion may be due to the inability of the mutant virus to efficiently infect lymphocytes which normally serve as the principle vehicles for virus spread during infection.

M-T4 is not the first myxoma virus-encoded protein whose presence is essential for the virus replication in lymphocytes. Previous studies have demonstrated that infection of RL-5 cells with recombinant myxoma viruses that lack either the M-T2 or the M11L open reading frames are also unable to replicate efficiently in these cells resulting in the induction of DNA fragmentation (Macen *et al.,* 1996). The M-T2 open reading frame encodes a secreted version of the tumor necrosis factor (TNF) receptor that probably inhibits apoptosis from an intracellular location since the exogenous addition of purified M-T2 is unable to inhibit apoptosis (Macen *et al.,* 1996). Subsequent studies have shown that the ability of M-T2 to inhibit apoptosis is a function separate from the ability to bind TNF (Schreiber *et al.,* 1996). The M11L open reading frame encodes an early protein that is associated with cell membranes and is involved in the inhibition of inflammation and apoptosis, although its role in either process remains to be characterized. In addition, deletion of the M-T5 open reading frame of myxoma virus, a member of the poxvirus host range superfamily, also results in the induction of apoptosis in infected RL-5 cells and primary rabbit lymphocytes (Mossman *et al.,* 1996a), similar to the results observed with the M-T4 mutant virus. Infection of RL-5 cells with the mutant M-T5 virus was found to result in the inhibition of both host and viral protein synthesis, suggesting that apoptosis, leading to DNA fragmentation, is a secondary response due to the arrest of protein synthesis. The arrest of host and viral protein synthesis has not been detected in RL-5 cells infected with vMyxlacT4$^-$ (unpublished observation K. Mossman, M. Barry, and G. McFadden), indicating that M-T4 functions in a very different manner to M-T5. In addition, M-T5 does not contain a predicted signal sequence or ER retention signal, suggesting a different intracellular localization to M-T4. Construction of an M-T4 revertant virus that does not induce DNA fragmentation in RL-5 cells demonstrates that these open reading frames are all functional in the M-T4 mutant virus and that the specific deletion of M-T4 from the viral genome is responsible for the induction of apoptosis.

Several lines of evidence indicate that M-T4 is an intracellular protein that is predominantly retained within the ER. First, we were unable to detect M-T4 protein in secreted culture supernatants harvested from both vMyxlac- and VVT4-infected cells by Western blotting (Figs. 9A and 9B). Second, M-T4 was found to be fully endo H-sensitive (Fig. 10), a result that is consistent with the ER-associated addition of N-linked oligosaccharides, and finally confocal fluorescence microscopy indicated that M-T4 colocalizes with calreticulin (Fig. 11), a -KDEL containing protein that has previously been localized to the ER. Proteins with N-terminal secretory signals normally enter the ER, undergo modification, and travel to the Golgi apparatus where they undergo further modification to endo H-resistant forms. It has now become clear, however, that a number of proteins enter the ER but are not secreted due to the presence of retention signals, such as the consensus C-terminal sequence motif, -KDEL. Proteins containing this motif are recognized by a receptor in the Golgi compartment which recycles the protein back to the ER (Pelham, 1989, 1990). This sequence has been found to function in the retention of a number of proteins in the ER, including calreticulin. Deletion of the terminal four amino acids results in secretion and, conversely, addition of this sequence motif is sufficient to mediate the accumulation of normally secreted proteins in the ER (Munro and Pelham, 1987; Pelham, 1989; Andres *et al.,* 1990). In addition, variants of the -KDEL sequence such as -DKEL, -KNEL, and most notably -RDEL, also retain normally secreted proteins within the ER (Andres *et al.,* 1990). Sequencing studies have indicated that the M-T4 protein contains such a -RDEL motif, and since confocal fluorescence microscopy studies have demonstrated that M-T4 is indeed associated with the ER it seems likely that ER association is due to the presence of the C-terminal -RDEL sequence. This hypothesis, however, remains to be determined directly via deletion studies. Subsequent studies are also necessary to determine if ER-retention is critical for the antiapoptotic function of M-T4.

In conclusion, our studies have determined that the M-T4 gene of myxoma virus is an important intracellular virulence factor whose expression from the virus genome is necessary for the inhibition of apoptosis in lymphocytes. To our knowledge M-T4 is the first viral protein necessary for the productive infection of lymphocytes that contains a -RDEL motif and localizes to the ER. The ability of viruses to interact with lymphocytes *in vivo* can have dramatic effects on pathogenesis. The exact role of M-T4 in myxoma virus pathogenesis remains to be defined, but the presence of such a novel protein in the ER of virus-infected cells suggests a role in some aspect of cellular protein trafficking possibly pertaining to novel elements of the apoptosis machinery. Thus, the elucidation of the mechanism of M-T4 function should reveal crucial and novel information in regards to myxoma virus pathogenesis, ER function, and viral circumvention of apoptosis.

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REFERENCES

Alcamí, A., and Smith, G. L. (1995). Cytokine receptors encoded by poxviruses: A lesson in cytokine biology. *Immunol. Today* 16, 474–478.

Andres, D. A, Dickerson, I. M., and Dixon, J. E. (1990). Variants of the carboxyl-terminal KDEL sequence direct intracellular retention. *J. Biol. Chem.* 265, 5952–5955.

Barry, M., and McFadden, G. (1997). Virus encoded cytokines and cytokine receptors. *Parasitology,* in press.

Cuff, S., and Ruby, J. (1996). Evasion of apoptosis by DNA viruses. *Immunol. Cell Biol.* 74, 527–537.

Davison, A. J., and Moss, B. (1990). New vaccinia virus recombination plasmids incorporating a synthetic late promoter for high level expression of foreign proteins. *Nucleic Acids Res.* 18, 4285–4286.

Fenner, F. and Ratcliffe, F. N. (1965). 'Myxomatosis.' Cambridge Univ. Press, London, UK.

Fliegel, L., Burns, K., Opas, M., and Michalak, M. (1989). The high affinity calcium binding protein of the sarcoplasmic reticulum. Tissue distribution and homology with calregulin. *Biochim. Biophys. Acta.* 982, 1–8.

Gillet, G, and Brun, G. (1996). Viral inhibition of apoptosis. *Trends Microbiol.* 4, 312–317.

Gavriell, Y., Sherman, Y., and Ben-Sasson, S. A. (1992). Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J. Cell Biol.* 119, 493–501.

Graham, K, A., Opgenorth, A., Upton, C., and McFadden, G. (1992). Myxoma virus M11L ORF encodes a protein for which cell surface localization is critical in manifestation of viral virulence. *Virology* 191, 112–124.

Graham, K. A., Lalani, A. S., Macen, J. L., Ness, T. L., Barry, M., Liu, L. Y., Lucas, A., Clarke-Lewis, I., Moyer, R. W., and McFadden, G. (1997). The T1/35kDa family of poxvirus secreted proteins bind chemokines and modulate leukocyte influx into virus infected tissues. *Virology* 229, 12–24.

Howard, S. T., Chan, Y. S., and Smith, G. L. (1991). Vaccinia virus homologues of the Shope fibroma virus inverted terminal repeat proteins and a discontinuous ORF related to the tumor necrosis factor receptor family. *Virology* 180, 633–647.

Kaschka-Dierich, C., Werner, F. J., Bauer, I., and Fleckenstein, B. (1982). Structure of nonintegrated, circular herpesvirus saimiri and herpesvirus ateles genomes in tumor cell lines and in vitro-transformed cells. *J. Virol.* 44, 295–310.

Macaulay, C., Upton, C., and McFadden, G. (1987). Tumorigenic poxviruses: Transcriptional mapping of the terminal inverted repeats of Shope fibroma virus. *Virology* 158, 381–393.

Macen, J. L., Upton, C., Nation, N., and McFadden, G. (1993). SERP1, a serine proteinase inhibitor encoded by myxoma virus, is a secreted glycoprotein that interferes with inflammation. *Virology* 195, 348–363.

Macen, J. L., Graham, K. A, Lee, S. F., Schreiber, M., Boshkov, L., and McFadden, G. (1996). Expression of the myxoma virus tumor necrosis factor receptor homologue and M11L genes is required to prevent virus-induced apoptosis in infected rabbit T lymphocytes. *Virology* 218, 232–237.

Marrack, P., and Kappler, J. (1994). Subversion of the immune system by pathogens. *Cell* 76, 323–332.

McFadden, G. (1988). Poxviruses in rabbits. *In* "Virus Disease in Laboratory and Captive Animals" (G. Darai, Ed.), pp. 37–62. Nijhoff, Boston.

McFadden, G., and Graham, K. (1994). Modulation of cytokine networks by poxvirus: the myxoma virus model. *Semin. Virol.* 5, 421–429.

McFadden, G. (1995). "Viroceptors, Virokines and Related Immune Modulators Encoded by DNA Viruses." Molecular Biology Intelligence Unit, R. G. Landes Co., Austin, TX.

McFadden, G., Graham, K., Ellison, K., Barry, M., Macen, J., Schreiber, M., Mossman, K., Nash, P., Lalani, A., and Everett, H. (1995). Interruption of cytokine networks by poxviruses: Lessons from myxoma virus. *J. Leuko. Biol.* 57, 731–738.

McFadden, G., and Barry, M. (1997). How poxviruses oppose apoptosis. *Semin. Virol.,* (in press).

Michalak, M., and MacLennan, D. H. (1980). Assembly of sarcoplasmic reticulum. Biosynthesis of the high affinity calcium binding protein in rat skeletal muscle cell cultures. *J. Biol. Chem.* 255, 1327–1334.

Milner, R. E., Baksh, S., Shemanko, C., Carpenter, M. R., Smilie, L., Vance, J. E., Opas, M., and Michalak, M. (1991). Calreticulin, and not calsequesterin, is the major calcium binding protein of smooth muscle sarcoplasmic reticulum and liver endoplasmic reticulum. *J. Biol. Chem.* 266, 7155–7165.

Mossman, K., Upton, C., and McFadden, G. (1995). The myxoma virus-soluble interferon-γ receptor homolog, M-T7, inhibits interferon-γ in a species-specific manner. *J. Biol. Chem.* 270, 3031–3038.

Mossman, K., Lee, S. F., Barry, M., Boshkov, L., and McFadden, G. (1996a). Disruption of M-T5, a novel myxoma virus gene member of the poxvirus host range superfamily, results in dramatic attenuation of myxomatosis in infected European rabbits. *J. Virol.* 70, 4394–4410.

Mossman, K., Nation, P., Macen, J., Garbutt, M., Lucas, A., and McFadden, G. (1996b). Myxoma virus M-T7, a secreted homolog of the interferon-γ receptor, is a critical virulence factor for the development of myxomatosis in European rabbits. *Virology* 215, 17–30.

Munro, S., and Pelham, H. R. B. (1987). A C-terminal signal prevents secretion of luminal ER proteins. *Cell* 48, 899–907.

Nash, P., Opas, M., and Michalak, M. (1994). Calreticulin: Not just another calcium-binding protein. *Mol. Cell. Biochem.* 135, 71–78.

Nilsson, T., and Warren, G. (1994). Retention and retrieval in the endoplasmic reticulum and the Golgi apparatus. *Curr. Opin. Cell Biol.* 6, 517–521.

Opgenorth, A., Graham, K. A., Nation, N., Strayer, D., and McFadden, G. (1992). Deletion analysis of two tandemly arranged virulence genes in myxoma virus, M11L and myxoma growth factor. *J. Virol.* 66, 4720–4731.

Pelham, H. R. B. (1990). The retention signal for soluble proteins of the endoplasmic reticulum. *Trends Biochem. Sci.* 15, 483–486.

Pelham, H. R. B. (1989). Control of protein exit from the endoplasmic reticulum. *Annu. Rev. Cell Biol.* 5, 1–23.

Petit, F., Bertagnoli, S., Gelfi, J., Fassy, F., Boucraut-Baralon, C., and Milon, A. (1996). Characterization of a myxoma virus-encoded serpin-like protein with activity against interleukin-1β-converting enzyme. *J. Virol.* 70, 5860–5866.

Pickup, D. J. (1994). Poxviral modifiers of cytokine responses to infection. *Infect. Agents Dis.* 3, 116–127.

Schreiber, M., Sedger, L., and McFadden, G. (1996). Distinct domains of M-T2, the myxoma virus TNF receptor homolog, mediate extracellular TNF binding and intracellular apoptosis inhibition. *J. Immunol.* 157, 4486–4495.

Sgonc, R., Boeck, G., Dietrich, H., Gruber, J., Recheis, H., and Wick, G. (1994). Simultaneous determination of cell surface antigens and apoptosis. *Trends Genet.* 10, 41–42.

Smith, G. L. (1994). Virus strategies for evasion of the host response to infection. *Trends Microbiol.* 2, 81–88.

Spriggs, M. K. (1994). Cytokine and cytokine receptor genes "captured" by viruses. *Curr. Opin. Immunol.* 6, 526–529.

Spriggs, M. K. (1996). One step ahead of the game: Viral immunomodulatory molecules. *Annu. Rev. Immunol.* 14, 101–131.

Stuart, D., Graham, K., Schreiber, M., Macaulay, C., and McFadden, G. (1991). The target DNA sequence of resolution of provirus replicative intermediates is an active late promoter. *J. Virol.* 65, 61–70.

Teodoro J. G., and Branton P. E. (1997). Regulation of apoptosis by viral gene products. *J. Virol.* 71, 1739–1746.

Upton, C., DeLange, A. M., and McFadden, G. (1987). Tumorigenic poxviruses: Genomic organization and DNA sequence of the telomeric region of the Shope fibroma virus genome. *Virology* 160, 20–30.

Upton, C., Macen, J. L., Schreiber, M., and McFadden, G. (1991). Myxoma virus expresses a secreted protein with homology to the tumor necrosis factor receptor gene family that contributes to viral virulence. *Virology* 184, 370–382.

Upton, C., Mossman, K., and McFadden, G. (1992). Encoding of a homolog of the IFN-γ receptor by myxoma virus. *Science* 258, 1369–1372.

White, E. (1996). Life, death, and the pursuit of apoptosis. *Genes Dev.* 10, 1–15.