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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

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Myxoma virus is a pathogenic poxvirus that induces a lethal myxomatosis disease profile in European rabbits, which is characterized by fulminating lesions at the primary site of inoculation, rapid dissemination to secondary internal organs and peripheral external sites, and supervening gram-negative bacterial infection. Here we describe the role of a novel myxoma virus protein encoded by the M-T5 open reading frame during pathogenesis. The myxoma virus M-T5 protein possesses no significant sequence homology to nonviral proteins but is a member of a larger poxviral superfamily designated host range proteins. An M-T5$^-$ mutant virus was constructed by disruption of both copies of the M-T5 gene followed by insertion of the selectable marker p7.5Ecogpt. Although the M-T5$^-$ deletion mutant replicated with wild-type kinetics in rabbit fibroblasts, infection of a rabbit CD4$^+$ T-cell line (RL5) with the myxoma virus M-T5$^-$ mutant virus resulted in the rapid and complete cessation of both host and viral protein synthesis, accompanied by the manifestation of all the classical features of programmed cell death. Infection of primary rabbit peripheral mononuclear cells with the myxoma virus M-T5$^-$ mutant virus resulted in the apoptotic death of nonadherent lymphocytes but not adherent monocytes. Within the European rabbit, disruption of the M-T5 open reading frame caused a dramatic attenuation of the rapidly lethal myxomatosis infection, and none of the infected rabbits displayed any of the characteristic features of myxomatosis. The two most significant histological observations in rabbits infected with the M-T5$^-$ mutant virus were (i) the lack of progression of the infection past the primary site of inoculation, coupled with the establishment of a rapid and effective inflammatory reaction, and (ii) the inability of the virus to initiate a cellular reaction within secondary immune organs. We conclude that M-T5 functions as a critical virulence factor by allowing productive infection of immune cells such as peripheral lymphocytes, thus facilitating virus dissemination to secondary tissue sites via the lymphatic channels.

Survival of a virus within a susceptible host requires specific strategies to evade antiviral and inflammatory immune responses elicited against a viral infection (2, 15, 30, 33, 42). Poxviruses are among the largest of the DNA viruses that infect eukaryotes (34) and have proven to be an excellent system in which to study immune system evasion for several reasons. Poxviruses encode more proteins than are necessary for progeny virion formation, and many of these extra, nonessential proteins are used exclusively for subverting host immune defenses within the vertebrate host. Poxvirus immune system evasion strategies include interference with cytokines and growth factors, inhibition of the complement cascade, reduction of inflammation, and repression of cellular immune recognition (2, 4, 15, 30, 33, 42, 44, 48).

The substantial encoding capacity of the poxvirus genome allows for adaptation to multiple cellular environments, which may explain the broad host range characteristic of many poxviruses. Vaccinia virus, an orthopoxvirus whose evolutionary history remains obscure, is capable of replicating in a broad range of mammalian and avian cells but is defective for replication in Chinese hamster ovary (CHO) cells (9). Cowpox virus, a related orthopoxvirus which is capable of replicating in CHO cells, encodes a 77-kDa protein, CHOhr, which can confer to recombinant vaccinia virus constructs the ability to productively infect CHO cells (43). In addition to the cowpox virus CHOhr gene, two additional related but distinct vaccinia virus genes, C7L and K1L, have been implicated in the determination of the vaccinia virus host range in vitro (39). Ectromelia virus and rabbitpox virus also encode related proteins involved in host range restriction (1, 5), indicating the existence of a larger superfamily of viral genes dedicated to the control of the poxvirus host range. While proteins encoded by this family of poxvirus genes have been defined by virtue of their ability to determine host range in cultured cells, their relevance in vivo is speculative, since the natural host(s) of many poxviruses remains unknown.

Myxoma virus, a member of the genus *Leporipoxvirus*, is one of the few poxviruses whose natural history has been well established; therefore, it provides an excellent model system in which to study virus-host interactions. Myxoma virus evolved within the South American rabbit (*Sylvilagus brasiliensis*), and infection of this natural host results in benign lesions which may persist for many months (11, 28, 29). However, infection of European rabbits (*Oryctolagus cuniculus*) induces a rapidly lethal infection known as myxomatosis, which is characterized by extensive fulminating internal and external lesions, severe immunodysfunction, and supervening gram-negative bacterial

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infection. Because of the extreme lethality of myxomatosis, myxoma virus was exploited in Australia in the early 1950s in an attempt to eradicate the feral European rabbit population, which had become endemic (11). However, because of a rapid co-evolution between the European rabbit and myxoma virus, more-resistant rabbits have recently returned as major agricultural and economic pests (12).

Myxoma virus expresses a diverse range of virulence factors, which interact with elements of the host immune system in the establishment of a myxomatosis disease state (32). Some of these virulence factors subvert immune system regulation by modulating host cytokine networks. For example, MGF, the myxoma virus growth factor, functions as a homolog of the epidermal growth factor/transforming growth factor α family (38), and M-T2 and M-T7 function as soluble homologs of the tumor necrosis factor receptor and the gamma interferon receptor, respectively (35, 50, 52). Myxoma virus also expresses cell-associated proteins such as SERP1, a serine proteinase inhibitor, and M11L, a transmembrane protein with unknown biochemical function, which have been shown by deletion analysis to influence pathogenesis by disrupting the host inflammatory responses (16, 26, 37).

Here we describe the identification and characterization of a novel myxoma virus gene, M-T5, which expresses a protein with significant homology with members of the poxvirus family of host range proteins. Deletion analysis of the M-T5 open reading frame indicates that one of the functions of the M-T5 protein is to allow the productive infection of rabbit peripheral lymphocytes; in the absence of M-T5 expression, such cells undergo a rapid apoptotic response. Thus, the myxoma virus M-T5 protein functions as a critical virulence factor in the establishment of myxomatosis in the European rabbit by permitting virus spread via infected lymphocytes and thus dissemination to multiple internal and external peripheral locations.

MATERIALS AND METHODS

Cells and viruses. Myxoma virus (vMyx, strain Lausanne), vMyxlac (a myxoma virus derivative containing the *Escherichia coli lacZ* gene at an intergenic location) (37), and the M-T5 recombinant myxoma viruses (described below) were routinely passaged in primate kidney BGMK cells grown in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum (Gibco BRL). Rabbit kidney RK13 and rabbit $CD4^+$ T-lymphocyte RL5 (21) cells were maintained in Dulbecco's modified Eagle's medium and RPMI 1640 (Gibco BRL), respectively, each supplemented with 10% fetal calf serum (FCS). RK13 cells were obtained from the American Type Culture Collection, whereas RL5 cells were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program. BGMK cells were a gift from S. Dales, University of Western Ontario, London, Ontario, Canada.

Sequence analysis of the myxoma virus M-T5 gene. The myxoma virus M-T5 gene was sequenced on an ABI 373 DNA sequencer with *Taq* cycling to greater than fivefold redundancy. pBamK (described below) was used as a template along with a total of 10 primers directed against both strands of the gene. Initial primers were based on sequences derived from the homologous T5 open reading frame of Shope fibroma virus (49, 51). Sequenced fragments were compiled with GelAssemble (Genetics Computer Group, Madison, Wis.). The PIR (release 45) and GenBank (release 90) databases were used to obtain additional poxvirus sequences. Protein sequences were compared and aligned with Bestfit and PileUp, respectively (Genetics Computer Group).

Construction of M-T5$^-$ recombinant myxoma viruses. The myxoma virus *Bam*HI K fragment (40), containing the complete M-T5 open reading frame plus flanking sequences, was cloned into pBluescript, creating plasmid pBamK. PCR amplification was used to create the fragments M-T5L and M-T5R, using the primer pairs (i) M-T5 5′ (GCCGGATCCGGACGAGGATGGATC) and Del5′ (CTACGCTGCAGGACTGGCGCCATCGCTTGAC) and (ii) S-T5 3′ (CCGG ATCCTACGCATGTAACTTC) and Del3′ (AGTCCTGCAGCGTAGGAATG TATAAGGGAAC), respectively. Del5′ and Del3′ were engineered to contain a complementary linker region with an internal *Pst*I site. Amplified M-T5L and M-T5R fragments were mixed and subjected to one round of PCR in the absence of primers followed by 25 rounds of PCR in the presence of M-T5 5′ and S-T5 3′ primers. The resulting fragment was cloned directly into the pT7Blue T-vector (Novagen), and an *Xba*I-*Eco*RI fragment was subcloned into pBluescript, creating the plasmid pMT5del. A *Pst*I cassette containing the *E. coli* guanosine phosphoribosyltransferase (Ecogpt) gene driven by the vaccinia virus 7.5K promoter (10) was inserted into pMT5del, creating pMT5gpt, which was used for transfection into myxoma virus-infected cells.

M-T5$^-$ mutant myxoma viruses were constructed by mycophenolic acid selection (10) as previously described (35). Both wild-type myxoma virus (Lausanne) and vMyxlac were used as parental strains to create the recombinant viruses vMyxT5$^-$ and vMyxlacT5$^-$, respectively. PCR analysis with M-T5 5′ and S-T5 3′ primers was used to confirm the absence of an intact M-T5 open reading frame, as previously described (35). vMyxlacT5$^-$ was subsequently used to create the revertant vMyxT5rev, in which the complete M-T5 open reading frame was restored to a wild-type myxoma background. Cotransfection of pBamK (described above) and pMyS2a, which contains wild-type myxoma virus sequences surrounding the location of integration of the *lacZ* gene used to create vMyxlac (37), into vMyxlacT5$^-$-infected cells yielded approximately 5% of foci which remained white upon staining with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (37). Within this population, indicative of removal of the *lacZ* gene, approximately 50% of the virus clones now contained an intact M-T5 open reading frame, as confirmed by PCR analysis.

Single-step growth analysis in tissue culture. RK13 or RL5 cells (5×10^5) were infected with either vMyxlac or vMyxlacT5$^-$ at a multiplicity of infection of 5 for 1 h. Unabsorbed free virus was removed, and the cells were washed with serum-free medium three times and then with medium supplemented with serum, and the cultures were harvested at various times postinoculation. Virus titers were determined by a plaque assay on RK13 cells followed by X-Gal staining of fixed monolayers, as outlined previously (37). All growth analyses were performed in triplicate, and data were expressed as $\log_{10}$ PFU per 10^5 cells.

Metabolic labeling of infected cells. Cells (10^6) were infected with virus at a multiplicity of infection of 10 for 1 h, and medium containing serum was added. At the indicated times after viral absorption, the medium was removed and the cells were washed once with cysteine- and methionine-free medium (ICN). The cells were then incubated with 50 μCi of [^{35}S]cysteine-[^{35}S]methionine (ICN) in cysteine- and methionine-free medium for 30 min. The cells were lysed directly in 50 μl of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) buffer, and samples were analyzed by SDS-PAGE followed by autoradiography.

Apoptosis assays. Apoptosis in cultured RL5 and primary rabbit peripheral mononuclear leukocytes was monitored by fluorescence-activated cell sorting analysis based on the TUNEL method of detecting deoxyribonucleoside triphosphate (dNTP) binding to the 3′-hydroxyl ends of fragmented DNA (13). Peripheral blood cells were isolated by layering rabbit blood over an equivalent volume of Ficoll-Paque and centrifuging at $400 \times g$ for 1 h. The mononuclear cell fraction was collected, washed twice with phosphate-buffered saline (PBS), and resuspended in RPMI 1640 supplemented with 10% FCS. Following a 4-h incubation, adherent and nonadherent cell populations were obtained. For the TUNEL reaction, 10^6 cells were mock infected or infected with virus at a multiplicity of infection of 10 for 1 h and harvested 12 to 16 h postinfection. Infected cells were washed once in PBS containing 1% FCS (PBS-FCS), fixed in 200 μl of 2% paraformaldehyde in PBS for 30 min at room temperature with gentle agitation, rewashed in PBS-FCS, and permeabilized with 100 μl of cold 0.1% Triton X-100–0.1% sodium citrate for 2 min on ice. Following two washes with PBS-FCS, the cells were incubated for 1 h at 37°C with 0.6 nmol of fluorescein-12-dUTP (Boehringer Mannheim)–3 nmol of dATP–1 mM $CoCl_2$–25 U of terminal deoxynucleotidyltransferase (Boehringer Mannheim)–terminal deoxynucleotidyltransferase buffer (30 mM Tris [pH 7.2], 140 mM sodium cacodylate) in a total volume of 30 μl. The reaction was stopped by adding 3 μl of 200 mM EDTA, and the cells were washed once in PBS-FCS and resuspended in 0.5 ml of PBS. Flow-cytometric analysis was performed on a FACScan flow cytometer (Becton Dickinson, Mountain View, Calif.) equipped with an argon-ion laser with 15 mW of excitation at 488 nm. Data were acquired on 10,000 cells per sample with light scatter signals at linear gain and fluorescence signals at logarithmic gain.

DNA fragmentation assays were performed on 2×10^6 RL5 cells infected with virus at a multiplicity of infection of 10 for 1 h. At the indicated times postinfection, the cells were lysed in 1% SDS–100 mM NaCl–1 mM EDTA–10 mM Tris (pH 7.5). Lysed cells were treated with 0.5 mg of proteinase K per ml for 1 h at 55°C and then with 0.5 mg of RNase per ml for 1 h at 37°C. DNA was precipitated with 2.5 volumes of 95% ethanol, resuspended in 10 mM Tris (pH 8.0), and subjected to agarose gel electrophoresis in the presence of 0.5 μg of ethidium bromide per ml.

Infection of rabbits with M-T5$^-$ mutant myxoma viruses. Female New Zealand White rabbits (*Oryctolagus cuniculus*) were obtained from a local supplier and housed in C-level biocontainment facilities under the guidelines of the Canadian Council on Animal Care. Injections were performed intradermally in each thigh with 1,000 PFU of virus per site. The rabbits were monitored daily for symptoms of myxomatosis (for comprehensive reviews of myxomatosis, refer to references 11 and 29). Rabbits which became moribund were sacrificed with euthanyl administered intravenously after anesthesia. For gross pathological studies, eight rabbits were inoculated with vMyxT5$^-$ and four were inoculated with the wild-type parental myxoma virus. For histological studies, six rabbits each were inoculated with vMyxlac or vMyxlacT5$^-$ as described above. Two rabbits from each group were sacrificed on days 4, 7, and 10 postinoculation and subjected to a complete postmortem examination. Tissue sections were removed and stored in neutral buffered formalin. Samples were embedded in paraffin, cut

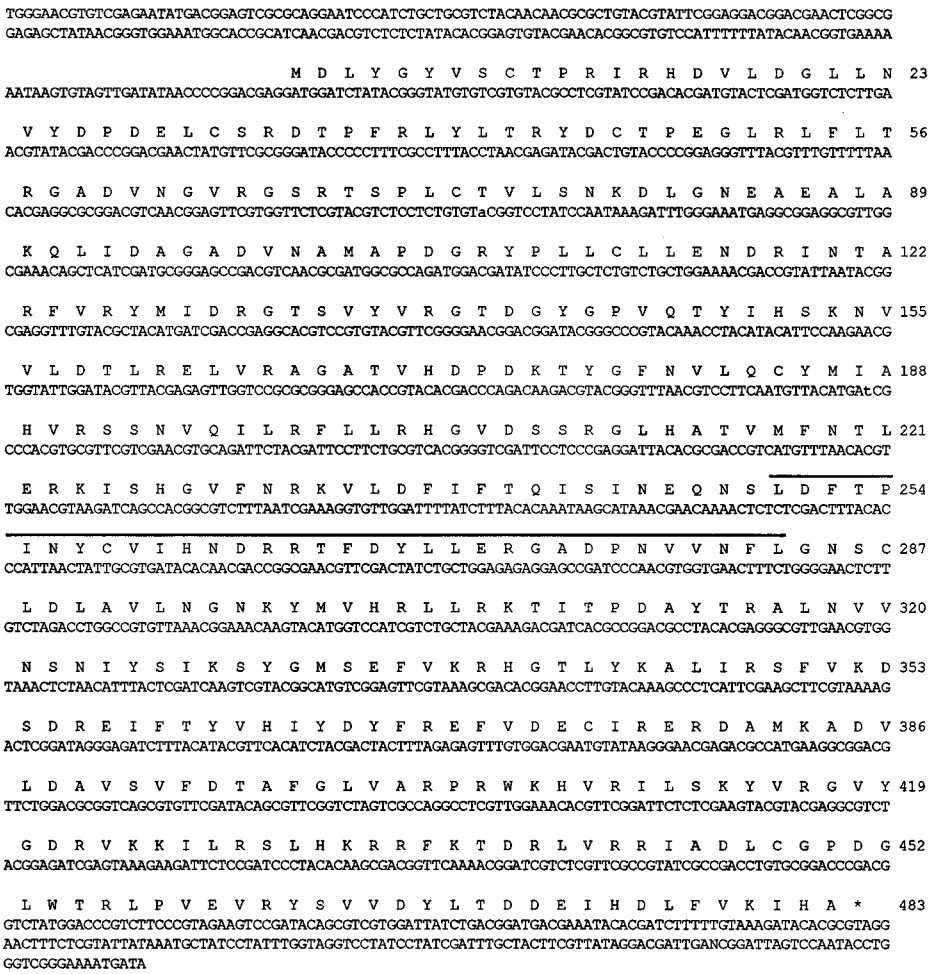


FIG. 1. Nucleotide sequence and corresponding amino acid sequence of the myxoma virus M-T5 gene. The complete M-T5 open reading frame in both orientations from genomic DNA was sequenced with a series of primers, as outlined in Materials and Methods. Nucleotide sequences flanking the M-T5 gene are also shown. The location of an ankyrin-like repeat within the amino acid sequence is highlighted with a solid line.

into 5-μm sections, and stained with hematoxylin and eosin for viewing by light microscopy.

Nucleotide sequence accession number. The myxoma virus M-T5 sequence has been submitted to GenBank and assigned accession number U43201.

RESULTS

Myxomavirus M-T5 possesses homology to poxvirus host range proteins. Because of the overall high homology among members of the *Leporipoxvirus* genus, sequencing of the myxoma virus M-T5 open reading frame from genomic DNA was facilitated by using primers initially directed toward the published Shope fibroma virus S-T5 sequences (49, 51). The myxoma virus M-T5 open reading frame is present as two copies, one within each virus terminal inverted repeat, and consists of 1,452 nucleotides encoding a protein of 483 amino acids (Fig. 1). M-T5, which has a predicted molecular mass of 55 kDa, possesses neither a signal peptide sequence nor sites for N-linked glycosylation but does contain a single ankyrin-like repeat (Fig. 1). Ankyrins bind integral membrane proteins, presumably through ankyrin repeat domains (23, 24), but the significance of the single M-T5 ankyrin-like repeat has yet to be determined.

With the exception of the ankyrin-like repeat, database searching revealed no significant homology between the myxoma virus M-T5 protein and any nonviral proteins. However, a low but significant level of homology was observed between M-T5 and a variety of poxvirus proteins, many of which have been defined by their ability to mediate host range (Table 1). Cowpox virus, in contrast to vaccinia virus, is capable of multiplying in Chinese hamster ovary (CHO) cells, and the expression of the cowpox virus CHOhr transgene product is sufficient to allow vaccinia virus replication in CHO cells (9, 43). Vaccinia virus encodes two additional open reading frames, C7L and K1L, which express proteins required for productive replication in human cells and whose deletion can be compensated for by expression of the CHOhr gene product (39). While the C7L and K1L gene products appear to possess overlapping functions in some cell lines, only one gene product is critical for viral replication in other cell lines (36, 45). An ectromelia virus homolog of the vaccinia virus K1L gene product, encoded by the ectromelia virus K1L open reading frame, was found to restrict viral replication in several cell lines but have no effect on in vivo replication and spread in infected mice (5). In addition, rabbitpox virus encodes three serine protease inhibitor homologs, one of which, SPI-1, has been implicated in both the determination of host range and the formation of hemorrhagic lesions (1).

TABLE 1. Summary of the percent identity and similarity between the amino acid sequences of myxoma virus M-T5 and several poxvirus host range superfamily members

Virus	Identity and similarity[a] with:							
	MYX T5	CPV CHOhr	VV B4R	VV K1L	VV C7L	VAR B6R	EV K1L	RPV SPI-1
MYX T5		19.7	23.9	20.9	12.5	25.3	20.5	20.1
CPV CHOhr	47.9		27.1	28.7	23.4	26.4	23.4	17.3
VV B4R	48.3	51.2		23.3	25.5	94.2	23.5	19.2
VV K1L	49.3	55.3	51.6		24.5	22.1	96.5	18.3
VV C7L	45.0	52.5	51.0	46.9		25.2	23.1	21.0
VAR B6R	49.1	50.2	96.2	48.9	51.0		23.5	18.1
EV K1L	48.8	51.4	51.6	97.9	46.9	45.8		18.7
RPV SPI-1	46.6	44.9	44.4	45.4	47.1	45.6	44.0	

[a] Identity is shown at the top right; similarity is shown at the bottom left. Accession numbers for the different poxvirus proteins: M-T5, U43201; CPV CHOhr, A29 887; VV B4R, P24 769; VV K1L and VV C7L, M35027; VAR B6R, P33823; EV K1L, L19 736; RPV SPI-1, U07766. Abbreviations: CPV, cowpox virus; VV, vaccinia virus; VAR, variola virus; EV, ectromelia virus; RPV, rabbitpox virus.

The myxoma virus M-T5 protein contains the highest degree of sequence similarity with the vaccinia virus B4R gene product and its variola virus homolog, the B6R gene product (Table 1) (14, 27). While little is known concerning the function of the vaccinia virus B4R gene product, homology between this protein and the cowpox virus CHOhr encoded protein has been noted (18). Figure 2 illustrates an alignment between the myxoma virus and Shope fibroma virus T5 proteins, the vaccinia virus B4R gene product, and the cowpox virus CHOhr gene product. While the homology among these sequences extends across the entire length of the proteins, both the myxoma virus and Shope fibroma virus T5 proteins contain a substantial deletion within the central region, compared with the other poxviral proteins. The significance of this apparent deletion remains to be determined.

Construction and characterization of M-T5$^-$ recombinant myxoma viruses. Because of the location of the M-T5 open reading frame within the terminal inverted repeats of the myxoma virus genome, it is present as two copies and maps within the *Bam*K restriction fragment (Fig. 3A). To inactivate both copies of the M-T5 gene, a dominant selection method involving the Ecogpt gene was used. This selection method is useful in the construction of poxvirus recombinants (3, 10), including those of myxoma virus (26, 35, 50). Disruption of the M-T5 open reading frame by PCR amplification and subsequent insertion of a cassette containing the Ecogpt gene under the control of the vaccinia virus p7.5 promoter is outlined (Fig. 3B). Recombinant M-T5gpt was transfected into both wild-type (strain Lausanne) myxoma virus- and vMyxlac-infected cells to create two M-T5$^-$ mutants, vMyxT5$^-$ and vMyxlacT5$^-$, respectively, in both the wild-type and vMyxlac backgrounds. Because of the presence of the *E. coli lacZ* gene within the genome of vMyxlac, visualization of viral foci with this background is greatly facilitated by X-Gal staining. We have recently determined that the overall virulence of vMyxlac is slightly lower than that ascribed to the original Lausanne iso-

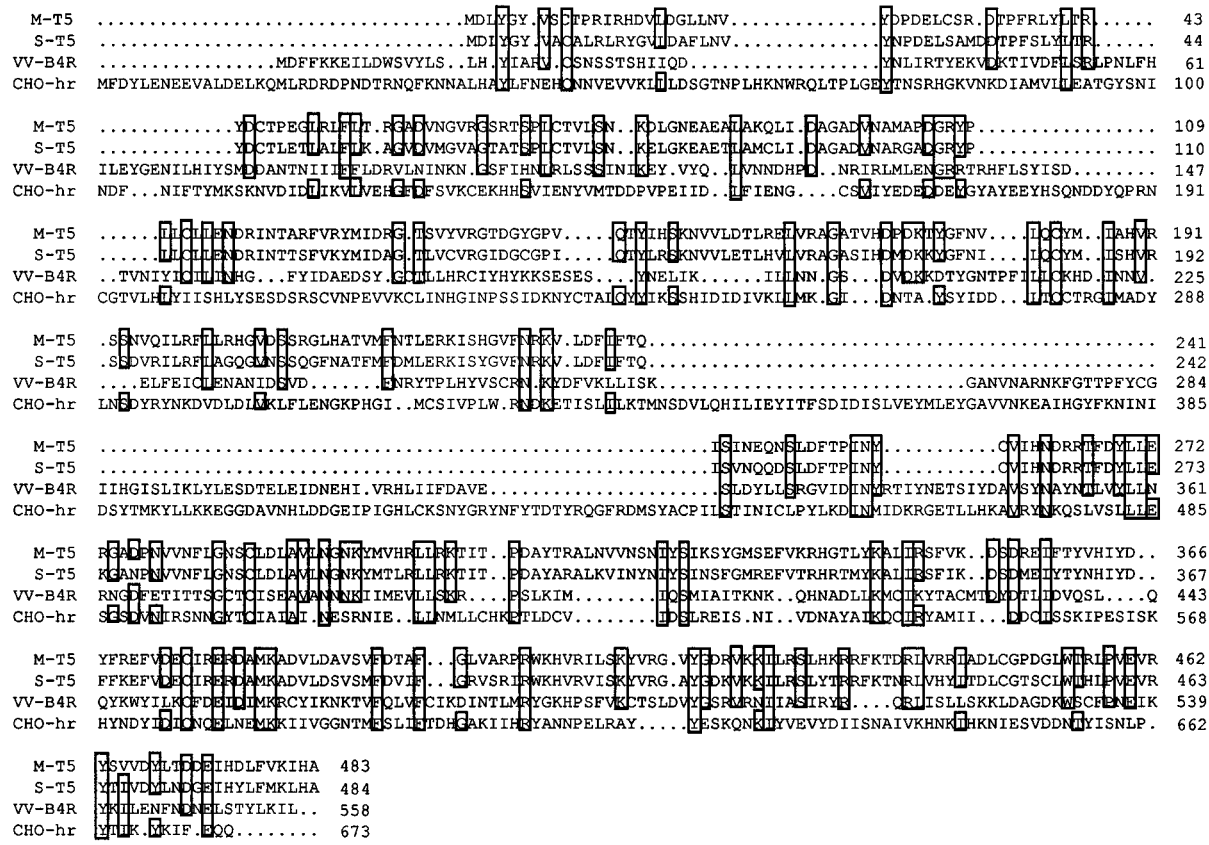


FIG. 2. Amino acid alignment between myxoma virus M-T5 and representative homologous poxvirus proteins. The amino acid comparison between the myxoma virus M-T5, Shope fibroma virus S-T5, vaccinia virus VV-B4R, and cowpox virus CHO-hr proteins is shown. Residues that are identical in at least three of four sequences are highlighted by boxes. Gaps introduced to maintain optimal alignment are indicated by dots.

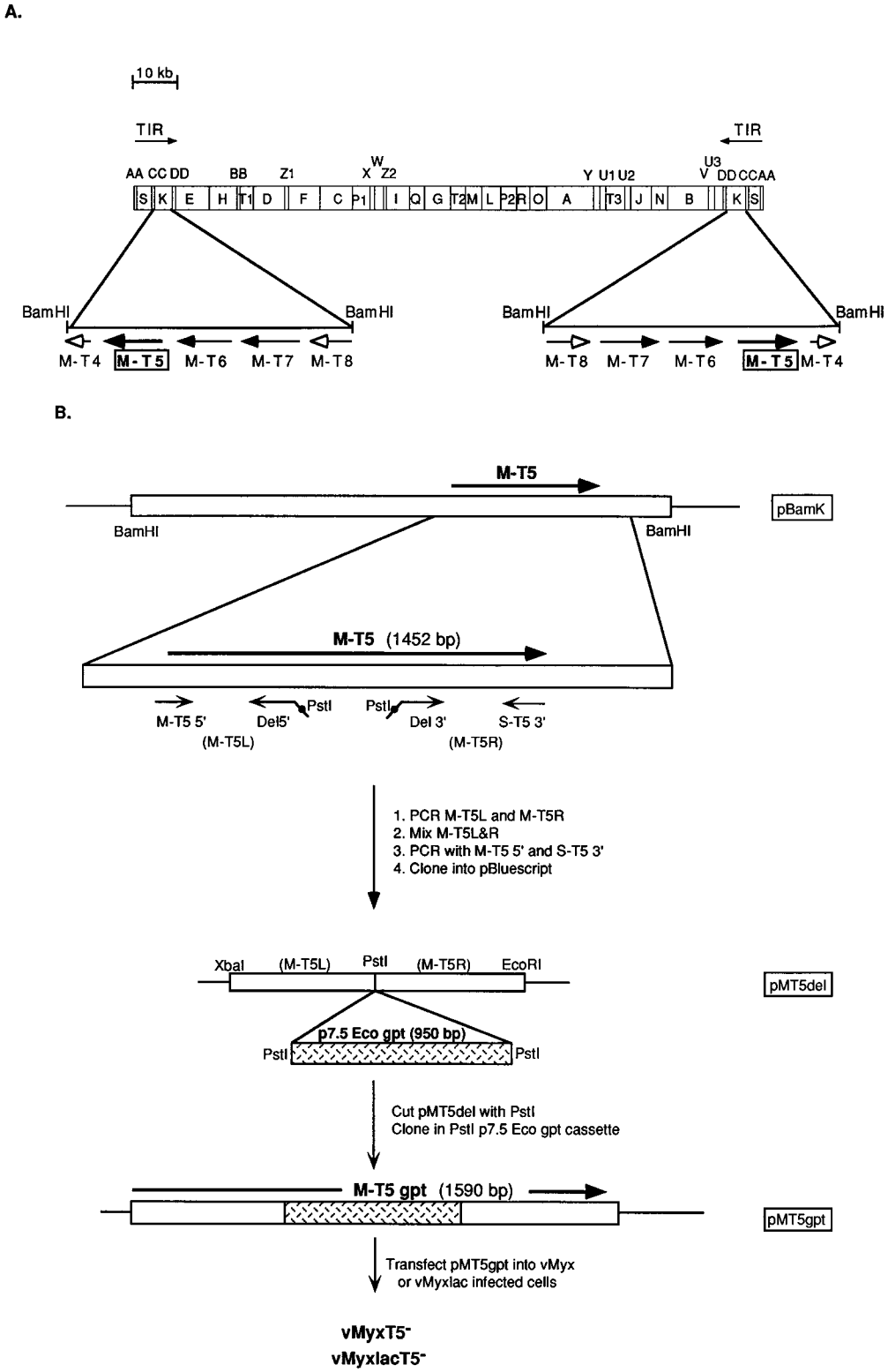


FIG. 3. Construction of a recombinant myxoma virus containing a disrupted M-T5 gene. (A) Location of both copies of the M-T5 open reading frame within the terminal inverted repeat (TIR) region of the myxoma virus genome. The *Bam*HI map of the myxoma virus genome (40) is illustrated. Solid arrows denote complete open reading frames within the *Bam*HI K fragment, whereas open arrows illustrate genes which flank this fragment. (B) Construction of M-T5$^-$ disruption viruses. The *Bam*HI K fragment was cloned into pBluescript, creating the plasmid pBamK. PCR amplification was used to generate the M-T5 terminal fragments M-T5L and M-T5R, which were engineered to contain complementary ends that create an internal *Pst*I site. These fragments were subsequently mixed, amplified, and cloned into pBluescript, creating the plasmid pMT5del. Digestion with *Pst*I and insertion of a *Pst*I p7.5Ecogpt cassette was used to form the M-T5 disruption plasmid pMT5gpt, which was subsequently transfected into vMyx- and vMyxlac-infected cells to create vMyxT5$^-$ and vMyxlacT5$^-$ disruption viruses, respectively.

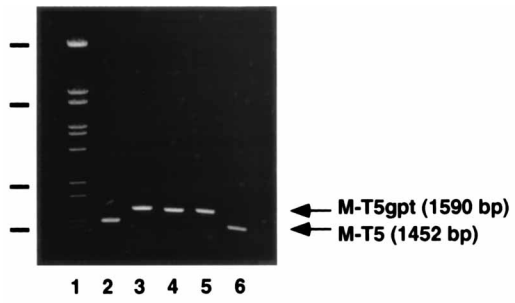


FIG. 4. Characterization of the M-T5 gene from myxoma virus M-T5 recombinants. Agarose gel electrophoresis of PCR products amplified with the M-T5 5′ and S-T5 3′ primers (Fig. 3B) demonstrates the difference in migration between wild-type M-T5 (lane 2) and the disrupted recombinant M-T5gpt (lane 3) derived from pBamK and pMT5gpt plasmid DNA, respectively. PCR analysis of viral DNA harvested from cells infected with vMyxT5$^-$ (lane 4), vMyxlacT5$^-$ (lane 5), and vMyxT5rev (lane 6) demonstrates the presence of either M-T5gpt or M-T5, indicating the purity of the recombinant viral stocks. Molecular size markers of 8,450, 5,686, 2,323, and 1,371 bp corresponding to *Bst*EII-cut λ standards (lane 1) are indicated on the left.

late of myxoma virus (35); thus, the parental strain of myxoma virus used to create recombinant viruses may influence virulence studies in vivo. As a control, therefore, an M-T5 revertant was constructed in which the intact M-T5 open reading frame was restored and it is referred to as vMyxT5rev (see Materials and Methods for details).

To ensure disruption of both copies of the M-T5 gene in vMyxT5$^-$ and vMyxlacT5$^-$ and complete restoration in vMyxT5rev, PCR analysis was performed on DNA isolated from plaque-purified virus. As shown in Fig. 3B, wild-type M-T5 and mutant M-T5gpt genes possess identical sequences at their terminal ends, to which the M-T5 5′ and S-T5 3′ primers hybridize. Wild-type M-T5 (1,452 bp) is easily distinguished from mutant M-T5gpt (1,590 bp) on the basis of size by agarose gel electrophoresis (Fig. 4, lanes 2 and 3, respectively). DNA derived from plaque-purified vMyxT5$^-$ and vMyxlacT5$^-$ demonstrated only the disrupted M-T5gpt sequence (lanes 4 and 5, respectively), whereas DNA derived from plaque-purified vMyxT5rev demonstrated only the wild-type M-T5 sequence (lane 6).

Defective growth of M-T5$^-$ virus in a CD4$^+$ rabbit T-cell line (RL5). No defects in the ability of vMyxlacT5$^-$ to replicate in cultured rabbit RK13 fibroblasts in vitro were noted in a single-step growth curve analysis (Fig. 5A). Similar results were also found in cultured primate BGMK cells (data not shown). However, vMyxlacT5$^-$ was found to be defective for replication in a cultured rabbit RL5 CD4$^+$ T-cell line (Fig. 5B). Initial studies of the host range restriction of vaccinia virus in CHO cells suggested that early and extensive inhibition of host and viral protein synthesis may be responsible for the abortive infection (9, 19). Because of the sequence similarities observed between myxoma virus M-T5 and known poxvirus host range proteins, protein synthesis was analyzed following vMyxlacT5$^-$ infection in replication-permissive RK13 fibroblasts and nonpermissive RL5 lymphocytes. No significant differences in host protein shutoff or viral protein synthesis between vMyxlac- and vMyxlacT5$^-$-infected RK13 cells at early or late times of infection were noted (Fig. 6A). However, the synthesis of both host and viral proteins appeared to be severely inhibited early after vMyxlacT5$^-$ infection of RL5 cells (Fig. 6B). Significant inhibition of protein synthesis was observed as early as 1 h postinfection (Fig. 6B, compare lanes 2 and 3), and appeared virtually complete by 4 h postinfection (compare lanes 6 and 7). In both RK13 and RL5 cells, infection with the control virus, vMyxT5rev, was indistinguishable from that with vMyxlac (Fig. 6A and B, lanes 10). Similar results were found in both cell lines infected with vMyxT5$^-$, indicating that in vitro, the two M-T5$^-$ mutant viruses were indistinguishable (data not shown). Thus, it appears that in RL5 lymphocytes, inhibition of both host and viral protein synthesis directly correlates with the abortive infection observed in the growth curve analysis.

To determine if the extensive early shutoff of host and viral protein synthesis in RL5 cells is unique to the myxoma virus M-T5$^-$ mutant, metabolic labeling of RL5 cells infected with some of the other characterized myxoma virus mutants was performed (Fig. 6C). Mutants of M-T2, a secreted homolog of the mammalian tumor necrosis factor receptor (50), M11L, a receptor-like molecule with no known cellular homolog (16, 37), M-T7, a secreted homolog of the mammalian gamma interferon receptor (35, 52), and SERP-1, a serine proteinase inhibitor (26), were compared with vMyxlac and vMyxlacT5$^-$ at early (Fig. 6C, lanes 2 to 7) and late (lanes 9 to 14) times of

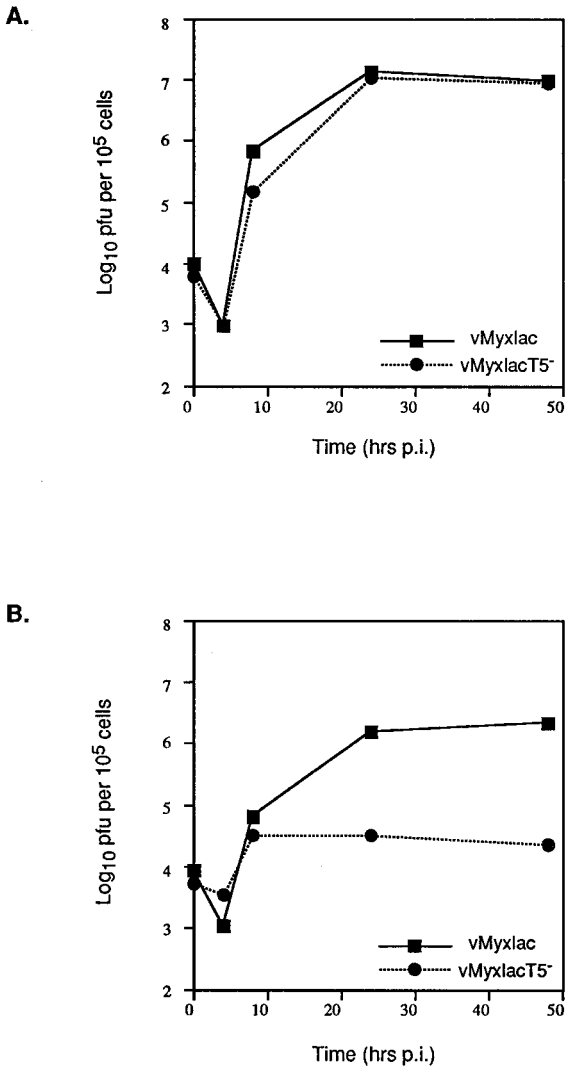


FIG. 5. Single-step growth analysis of wild-type and M-T5$^-$ mutant myxoma viruses. Rabbit kidney fibroblast (RK13) cells (A) and rabbit CD4$^+$ T-lymphocyte (RL5) cells (B) were infected with vMyxlac and vMyxlacT5$^-$ at a multiplicity of infection of 5. The cells were harvested at the indicated times postinfection (p.i.), and infectious virus titers were determined on RK13 cells. Each viral growth analysis was performed in triplicate.

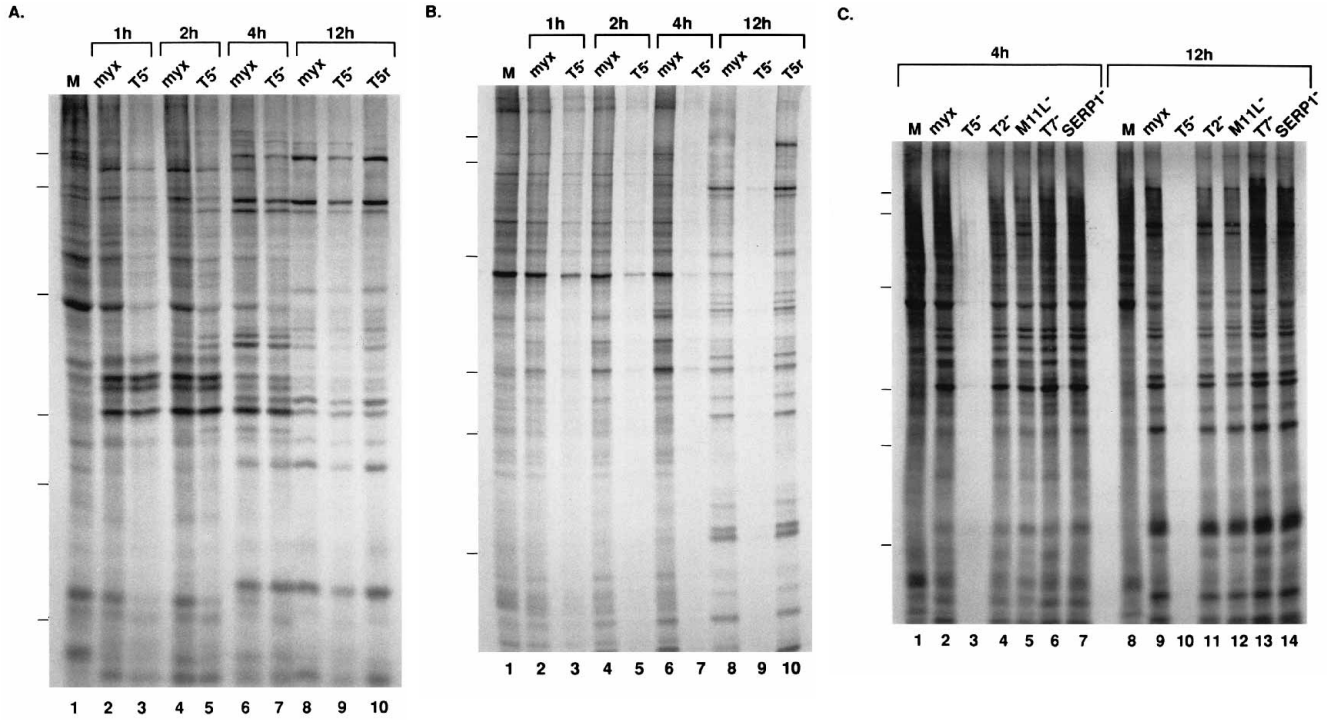


FIG. 6. Metabolic labeling of myxoma virus-infected cells with [^{35}S]cysteine-[^{35}S]methionine. (A and B) RK13 fibroblasts (A) or RL5 lymphocytes (B) were either mock infected (M) or infected with vMyxlac (myx), vMyxlacT5$^-$ (T5$^-$), or vMyxT5rev (T5r) and labeled with 50 μCi of [^{35}S]cysteine-[^{35}S]methionine for 30 min at the indicated times postinfection. Cells were harvested into SDS loading buffer and analyzed by SDS-PAGE followed by autoradiography. (C) RL5 lymphocytes were either mock infected (M) or infected with vMyxlac (myx), vMyxlacT5$^-$ (T5$^-$), vMyx-T2$^-$ (T2$^-$), vMyx-M11L$^-$ (M11L$^-$), vMyx-T7$^-$ (T7$^-$), or vMyx-SERP1$^-$ (SERP1$^-$), harvested at the indicated times postinfection, and treated as in panels A and B. Dashes on the left indicate the positions of molecular mass markers at 101, 83, 50.6, 35.5, 29.1, and 20.9 kDa.

infection. vMyxlacT5$^-$ appeared to be the only characterized myxoma virus mutant to cause inhibition of both host and viral protein synthesis in infected RL5 lymphocytes. This result was relevant since both the T2$^-$ and M11L$^-$ mutant myxoma viruses are known to be defective for replication in RL5 cells (25).

Rabbit lymphocytes undergo apoptosis in response to infection by M-T5$^-$ mutant myxoma viruses. Apoptosis, or programmed cell death, is known to play a physiologically important role in such diverse processes as embryonic development, tumorigenesis, and lysis of virus-infected cells (22, 53). Recently, vaccinia virus infection of CHO cells was shown to induce apoptosis (20). Because of the similarities between infection of CHO cells with vaccinia virus and infection of RL5 cells with M-T5$^-$ mutant myxoma virus, we initiated studies to see if vMyxlacT5$^-$ infection of RL5 cells would lead to apoptosis. A characteristic feature of apoptosis is the degradation of DNA to oligonucleosomal fragments (55), which may be observed as DNA laddering when cell lysates are subjected to agarose gel electrophoresis. DNA laddering was observed in lysates from RL5 cells infected with vMyxlacT5$^-$ but not in lysates from RL5 cells either mock infected or infected with vMyxlac or vMyxT5rev (Fig. 7A). As with the shutoff of host and viral protein synthesis, DNA fragmentation was observed as early as two hours following vMyxlacT5$^-$ infection (Fig. 7A, lane 3). No fragmentation was observed at any time following infection of RK13 cells with these viruses in a parallel study (data not shown).

DNA fragmentation was also measured in RL5 cells by quantitating the terminal deoxynucleotidyltransferase-mediated incorporation of fluorescein-12-dUTP into DNA by flow-cytometric analysis. At 16 h postinfection, mock-, vMyxlac-, and vMyxT5rev-infected RL5 cells did not show any significant apoptosis whereas a significant fraction of RL5 cells infected with vMyxlacT5$^-$ exhibited classic apoptotic features, as seen by the appearance of a new population of fragmented cells with increased fluorescence staining (Fig. 7B). The scatter profiles of vMyxlac- and vMyxT5rev-infected RL5 cells mirrored that of mock-infected RL5 cells, but the light-scattering properties of vMyxlacT5$^-$-infected cells demonstrated the emergence of a population of cells which were smaller, a feature consistent with apoptotic cells, as indicated by a leftward shift in the forward scatter profile (Fig. 7B). Infection of RL5 cells with vMyxlacT5$^-$ for 16 h also resulted in a dramatic reduction in the number of harvested cells, illustrated by the drop in relative cell number. Flow-cytometric analysis of a time course of vMyxlacT5$^-$-infected RL5 cells demonstrated that by 4, 8, and 12 h postinfection, 20, 41, and 50% of cells, respectively, exhibited enhanced fluorescence caused by DNA fragmentation (data not shown). Thus, as a result of the extent of apoptosis in vMyxlacT5$^-$-infected RL5 cells at late times after infection, the number of cells harvested for flow-cytometric analysis dramatically decreased. We conclude that abortive infection of the M-T5$^-$ mutant myxoma virus in RL5 lymphocytes results in the apoptotic death of these cells.

Infection of primary rabbit lymphocytes and monocytes with M-T5$^-$ myxoma virus. While the amount of apoptosis seen in RL5 cells infected with vMyxlacT5$^-$ is striking, RL5 is a T-lymphoma cell line derived from a herpesvirus ateles-induced rabbit tumor (21). Thus, to ascertain whether apoptosis is a feature of vMyxlacT5$^-$-infected primary rabbit leukocytes in vivo, peripheral blood mononuclear leukocytes were har-

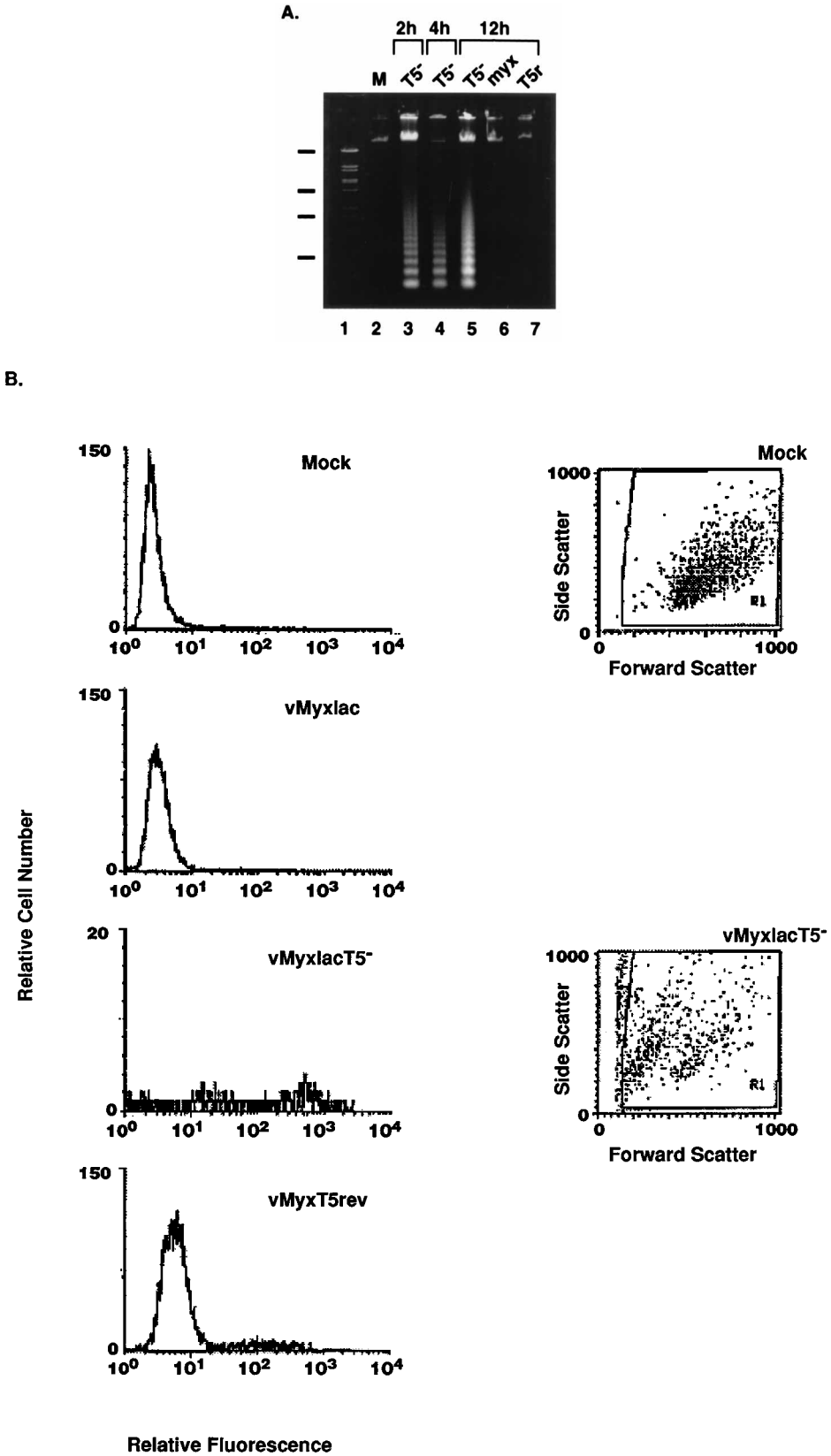


FIG. 7. Apoptosis in RL5 lymphocytes infected with the M-T5$^-$ mutant myxoma virus. (A) Agarose gel electrophoresis of RL5 cells either mock infected (M) or infected with vMyxlacT5$^-$ (T5$^-$), vMyxlac (myx), or vMyxT5rev (T5r). At the indicated times postinfection, cells were harvested, digested with proteinase K and RNase, ethanol precipitated, and subjected to agarose gel electrophoresis, as described in Materials and Methods. Molecular size markers of 8,450, 3,675, 1,929, and 702 bp corresponding to *Bst*EII-cut λ standards (lane 1) are indicated on the left. (B) Flow-cytometric analysis of infected RL5 lymphocytes. RL5 cells were infected with the indicated viruses, harvested 12 h postinfection, and subjected to the TUNEL method of detecting dNTP binding to 3′-hydroxyl ends of fragmented DNA, as described in Materials and Methods. The cell scatter profiles of RL5 cells either mock infected or infected with vMyxlacT5$^-$ are shown beside their corresponding fluorescence profiles. The scatter profile of vMyxlac- and vMyxT5rev-infected cells was indistinguishable from that of mock-infected cells.

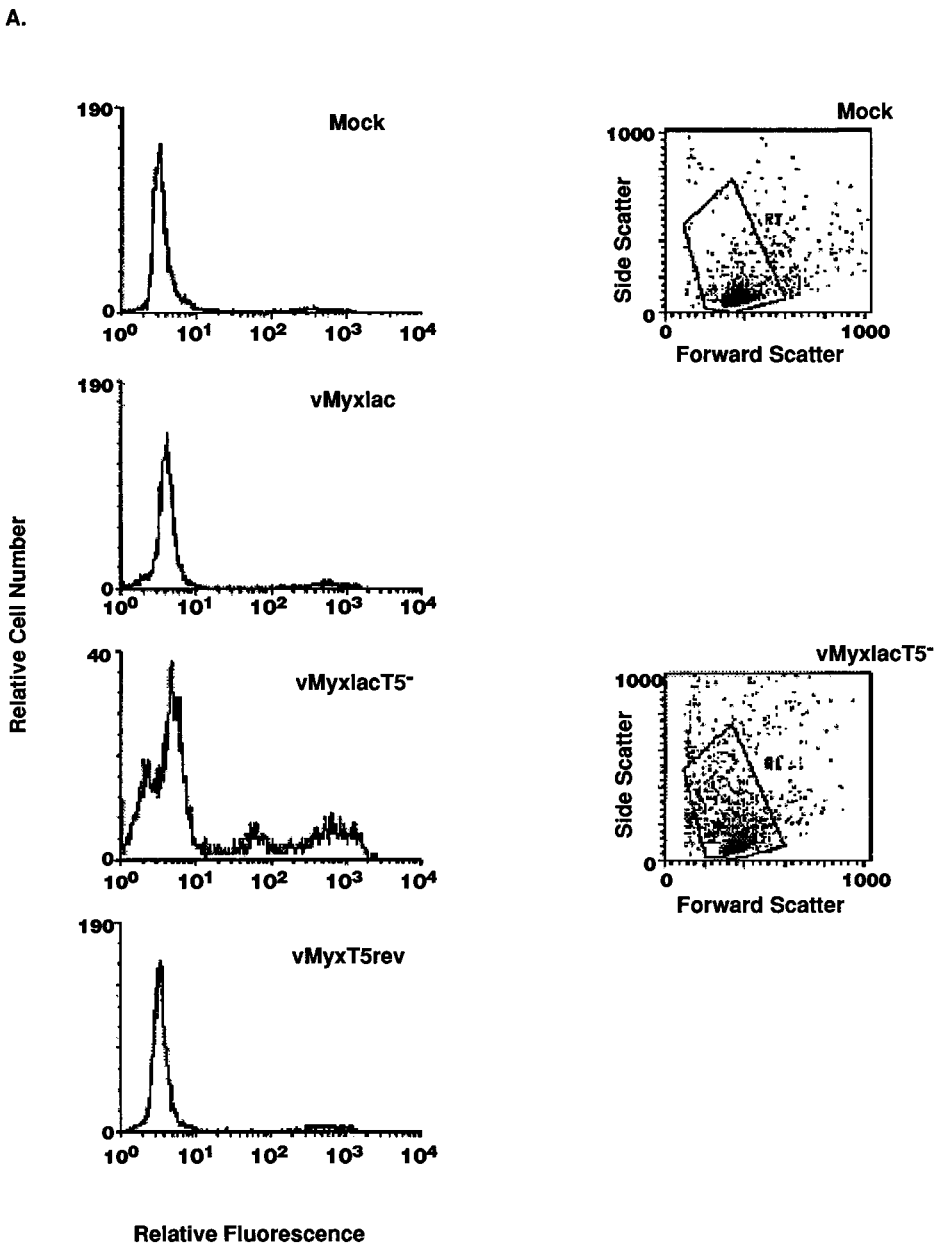


FIG. 8. Flow-cytometric analysis of infected rabbit peripheral blood mononuclear cells. Rabbit peripheral blood mononuclear leukocytes were separated into nonadherent (A) and adherent (B) fractions, enriching for lymphocytes and monocytes, respectively. The cells were then infected with the indicated viruses, harvested 12 h postinfection, and subjected to the TUNEL method of detecting dNTP binding to 3′-hydroxyl ends of fragmented DNA, as described in Materials and Methods. The cell scatter profiles of peripheral blood cells either mock infected or infected with vMyxlacT5$^-$ are shown beside their corresponding fluorescence profiles. The scatter profile of vMyxlac- and vMyxT5rev-infected cells was indistinguishable from that of mock-infected cells.

vested, fractionated into adherent and nonadherent cells, and subjected to flow-cytometric analysis following infection with the various myxoma virus recombinants (Fig. 8). In peripheral blood lymphocytes infected with vMyxlacT5$^-$, a distinct population of cells demonstrated an increase in relative fluorescence as a result of DNA fragmentation, which routinely corresponded to 20 to 30% apoptosis (Fig. 8A). The scatter profile of vMyxlacT5$^-$-infected lymphocytes also differed from that of mock-, vMyxlac-, and vMyxT5rev-infected lymphocytes, consistent with the appearance of a population of smaller, apoptotic cells. Similar to the case of RL5 cells, primary lymphocytes infected with vMyxlacT5$^-$ also routinely exhibited a decrease in the total number of cells harvested. In contrast, adherent cells, enriched for circulating monocytes, showed no significant increase in relative fluorescence following infection with vMyxlac, vMyxlacT5$^-$, or vMyxT5rev, as compared with mock infected cells (Fig. 8B). In all of these experiments, no differences were found in the levels of DNA fragmentation when wild-type myxoma virus and vMyxT5$^-$ were used in place of vMyxlac and vMyxlacT5$^-$, respectively (data not shown). Thus, we conclude that infection of primary rabbit peripheral blood lymphocytes but not cells of the monocyte/macrophage lineage with an M-T5$^-$ mutant myxoma virus results in apoptosis and abortive infection.

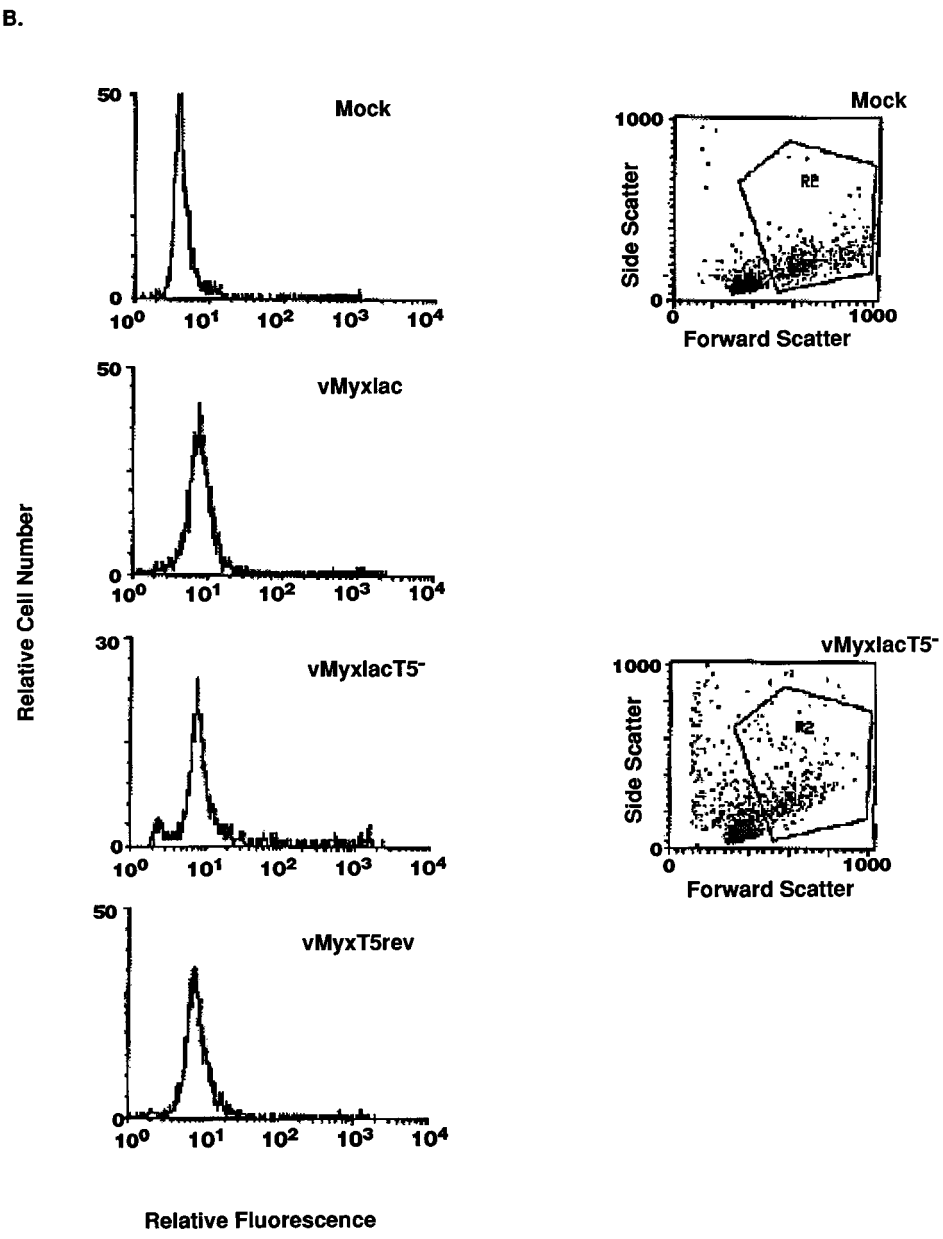


FIG. 8—*Continued.*

Myxoma virus M-T5 is a potent virulence factor in European rabbits. Myxoma virus infection of European rabbits induces a rapidly progressive and lethal disease known as myxomatosis (11). Infection of European rabbits with wild-type myxoma virus (strain Lausanne) is routinely 100% fatal, whereas infection of European rabbits with the vMyxlac derivative strain expressing the *lacZ* cassette results in approximately 95% mortality with a slight attenuation of disease symptoms (35). Since no significant differences were observed between in vitro infections with vMyxT5$^-$ (wild-type background) and vMyxlacT5$^-$ (vMyxlac background), vMyxT5$^-$ was chosen for comparison with wild-type myxoma virus to determine the effect of an M-T5 disruption on myxoma virus virulence in vivo.

As outlined in Table 2, a dramatic reduction in virulence was observed in rabbits infected with vMyxT5$^-$ with respect to rabbits infected with wild-type myxoma virus. On day 4 postinoculation, primary-site lesions in rabbits infected with vMyxT5$^-$ were consistently smaller and firmer than those in rabbits infected with wild-type myxoma virus. By day 7 postinoculation, when rabbits infected with wild-type myxoma virus demonstrated all of the classical symptoms of myxomatosis including gram-negative bacterial infection of nasal and conjunctival mucosa and multiple secondary skin lesions, rabbits infected with vMyxT5$^-$ remained essentially asymptomatic, with no evidence of viral spread to secondary sites. By day 10 postinoculation, all four rabbits infected with wild-type myxoma virus had to be sacrificed (two rabbits each on days 9 and 10) whereas all rabbits infected with vMyxT5$^-$ demonstrated no overt signs of myxomatosis, save for one minute secondary lesion on the ear of one rabbit. The primary lesions of vMyxT5$^-$-infected rabbits were fully resolved by 14 days

TABLE 2. Pathogenicity of M-T5$^-$ mutant myxoma virus in European rabbits

Day	Virulence of:	
	vMyx (wild type)	vMyxT5$^-$ (M-T5$^-$ mutant)
0	Inoculation of four rabbits intradermally with 1,000 PFU of vMyx (Lausanne)	Inoculation of eight rabbits intradermally with 1,000 PFU of vMyxT5$^-$
4	Primary lesions at inoculation sites, ca. 2 cm, raised, soft, and red	Primary lesions at inoculation sites, ca. 1 cm, hard
7	Gram-negative bacterial infections of nasal and conjunctival mucosa; multiple secondary skin lesions on face and ears	No gram-negative infections or secondary lesions detected
10	Severe gram-negative bacterial infections in conjunctiva and respiratory tract; secondary skin lesions turning necrotic; all rabbits sacrificed because of increasing severity of symptoms	No gram-negative infections detected; single minute secondary lesion detected in one ear of one rabbit
14		Full regression of primary lesions; rabbits immune to subsequent challenge with vMyx (wild-type) infection

postinoculation, and these rabbits were immune to subsequent challenge by wild-type myxoma virus. Rabbits infected with the control virus, vMyxT5rev, in which the T5gpt disruption was restored to the wild-type situation, displayed a disease state indistinguishable from that of rabbits infected with wild-type myxoma virus (data not shown). Thus, we conclude that M-T5 is a potent virulence factor which plays a critical role in the pathogenesis of myxoma virus in European rabbits and that in the M-T5$^-$ deletion mutant, the virus spreads poorly, if at all, to secondary sites.

Histological analysis of rabbits infected with wild-type and M-T5$^-$ mutant myxoma viruses. Because of the significant difference in the pathogenic profiles of the M-T5$^-$ mutant virus and the M-T5$^+$ parent or M-T5rev myxoma viruses, a more detailed histological analysis was performed on tissue sections harvested from vMyxlac and vMyxlacT5$^-$ mutant virus-infected rabbits on days 4, 7, and 10 postinoculation (summarized in Table 3). The most striking difference between wild-type myxoma virus and M-T5$^-$ mutant virus infection was observed at the primary site of inoculation (Fig. 9). On day 4 postinoculation, primary lesions from rabbits infected with vMyxlac (Fig. 9A) demonstrated considerable edema with a minor accumulation of heterophils. In contrast, although primary lesions from rabbits infected with vMyxlacT5$^-$ (Fig. 9B) displayed little edema, a significant infiltration of heterophils was observed throughout the dermis. Heterophils function as the primary line of cellular defense early within an inflammatory reaction and are subsequently replaced with mononuclear lymphocytes as the inflammatory reaction progresses. Also, whereas parental vMyxlac-infected primary lesions showed a slight disorganization of the epithelium, vMyxlacT5$^-$-infected primary lesions demonstrated a zone of actively mitotic basal keratinocytes within the inner epithelium, which was effectively replacing degenerating cells of the outer epithelium with a new epithelial layer.

On day 7 postinoculation, primary lesions from rabbits infected with parental vMyxlac demonstrated considerable edema and hemorrhage within the dermis, necrosis and degranulation of accumulating heterophils, and a necrotic epithelium that was beginning to lift off the basement membrane (Fig. 9C). Primary lesions from rabbits infected with vMyxlacT5$^-$, however, demonstrated a normal epidermal surface, replacement of heterophils with mononuclear lymphocytes, and neither edema nor hemorrhage within the dermis (Fig. 9D). By day 10 postinoculation, when vMyxlac-infected primary lesions continued to display extensive edema, hemorrhage, and necrosis of heterophils, along with a completely disorganized epithelial layer (Fig. 9E), the site of inoculation with vMyxlacT5$^-$ (Fig. 9F) essentially resembled a parallel section harvested from an uninfected control rabbit (data not shown). Thus, it appears that within primary lesions of rabbits infected with parental M-T5$^+$ myxoma virus, an inflammatory reaction capable of controlling viral infection is not established, whereas in primary lesions of rabbits infected with the M-T5$^-$ mutant myxoma virus, a very rapid and effective inflammatory reaction results in complete resolution of the infection by day 10 postinoculation, with no development of secondary lesions.

Since complete resolution of M-T5$^-$ mutant myxoma virus infection appeared to occur by 7 to 10 days at the primary site of inoculation, it is useful to consider whether the lymph nodes and spleen may have played a critical role in the resolution process. Infection of rabbits with vMyxlac led to mild reactivity within the lymph nodes and spleen by day 4 postinoculation (Table 3), as immature lymphocytes replaced mature lymphocytes that began to migrate out of the lymphoid organs. By day 7 postinoculation, very few mature lymphocytes were found within the lymphoid organs of rabbits infected with vMyxlac, and it appeared that the replication of stem cells was insufficient to replace the mature lymphocytes, resulting in the net outward migration of immature cells. On day 10 postinoculation, even fewer lymphocytes were found within the germinal centers of the lymphoid organs of vMyxlac-infected rabbits, and within the spleen, a significant level of lymphocyte necrosis was observed, perhaps corresponding to productive replication of virus in this organ. In stark contrast, on days 4 and 7 postinoculation, no cellular reactivity was observed within the lymph nodes and spleen of rabbits infected with vMyxlacT5$^-$. On day 10 postinoculation, however, mild reactivity characterized by increased mitotic activity within the germinal centers of both lymphoid organs was observed, along with the appearance of macrophages and a high level of mature lymphocytes. Given the histological and pathogenic profile of the M-T5$^-$ mutant myxoma virus on day 10 postinoculation, this reactivity is consistent with the recognition and formation of memory T lymphocytes in response to viral antigen. Thus, the early resolution of the M-T5$^-$ mutant myxoma virus within the primary site of infection appeared not to be accompanied by the development of antigen-activated lymphocytes within the secondary immune system organs.

Consistent with the absence of gross lesions at peripheral locations in rabbits infected with M-T5$^-$ mutant myxoma virus and the rapid resolution of infection at the primary site of inoculation, no lesions were found upon histological analysis of tissues harvested from outside the primary inoculation site in vMyxlacT5$^-$-infected rabbits (Table 3). In contrast, by day 7 postinoculation, rabbits infected with vMyxlac demonstrated extensive secondary lesions, established by virtue of the dissemination of infectious virus within productively infected lymphocytes. Therefore, we conclude that wild-type myxoma virus

TABLE 3. Major histological differences between vMyxlac (M-T5$^+$) and vMyxlacT5$^-$ (M-T5$^-$) infection in European rabbits

Day	Histological effects of[a]:	
	vMyxlac (wild-type)	vMyxlacT5$^-$ (M-T5$^-$ mutant)
4	Primary sites	Primary sites
	Inflammatory reaction with enhanced edema, presence of scattered heterophils	Inflammatory reaction with little to no edema, accumulation of heterophils, regeneration of damaged epithelium
	Damaged epithelium	
	Secondary sites	Secondary sites
	No detectable reaction	No detectable reaction
	Lymph nodes mildly reactive	Lymph nodes normal
	Spleen mildly reactive	Spleen normal
7	Primary sites	Primary sites
	Acute necrosis of epidermis	Normal epithelium
	Inflammation with edema and hemorrhage; necrosis of heterophils	Inflammation without edema and hemorrhage; heterophils replaced by mononuclear cells
	Secondary sites	Secondary sites
	Thickened, disorganized epidermis	No detectable reaction
	Inflammatory reaction in dermis and subcutis; heterophils predominate	
	Lymph nodes show loss of mature lymphocytes	Lymph nodes normal
	Spleen shows loss of mature lymphocytes	Spleen normal
10	Primary sites	Primary sites
	Inflammatory reaction with necrosis, edema, and hemorrhage; necrotic heterophils	Resolution of infection
	Degenerated epithelium	Tissue sections normal
	Secondary sites	Secondary sites
	Thickened, disorganized epidermis	No detectable reaction
	Inflammation with edema; heterophils and macrophages predominate	Lymph nodes show mild reaction
	Lymph nodes show loss of mature lymphocytes	Spleen shows mild reaction
	Spleen shows necrosis of lymphocytes	

[a] Primary-site samples taken from the site of inoculation in the thigh. Secondary-site samples taken from the ear, lip, nose, and eyelid.

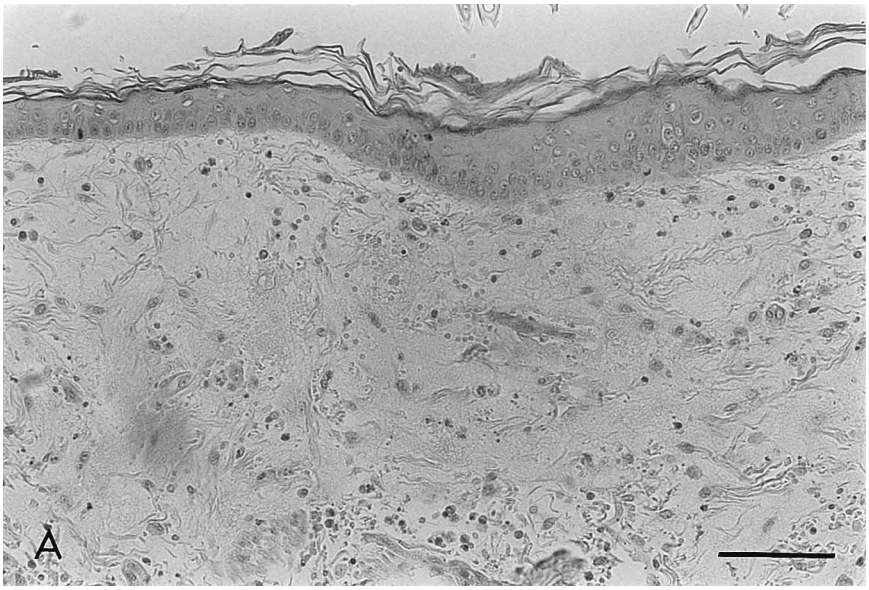
is capable of disseminating via infected lymphocytes to peripheral areas and establishing secondary sites of infection, whereas M-T5$^-$ mutant myxoma virus is incapable of disseminating from the primary inoculation site because a rapid supervening apoptotic response in infected lymphocytes prevents the transit of infectious virus through the lymphoreticular system to secondary sites.

DISCUSSION

To efficiently replicate and establish a productive infection, myxoma virus produces a wide range of immunomodulatory proteins which perform diverse functions (31, 32). In this study, we report on the role of the myxoma virus M-T5 protein, which, along with the S-T5 protein from the antigenically related Shope fibroma virus (49), contains significant homology to a larger family of poxvirus proteins implicated in the determination of host range (1, 5, 39, 43). Interestingly, both the myxoma virus and Shope fibroma virus T5 proteins have internal deletions of 106 amino acids when compared with the cowpox virus CHOhr-encoded protein, the most extensively studied of the poxvirus host range proteins. The myxoma virus M-T5$^-$ mutant failed to replicate on cultured rabbit T lymphocytes while displaying wild-type replication on cultured rabbit kidney fibroblasts, and it is likely that one of the roles of M-T5 is to extend the tissue range of myxoma virus to cells of lymphoid origin. Myxoma virus displays a very restricted "host" range in nature, because only members of the family *Leporidae* are infected by myxoma virus and only members of the genus *Oryctolagus* are susceptible to myxomatosis (11). As such, the myxoma virus M-T5 protein defines cell restriction within its natural host, as opposed to the classical definition of host range, meaning the ability to infect a heterologous species. Thus, a more accurate description would be that M-T5 imparts tissue or cell type specificity, rather than host range.

An important factor in the extreme virulence of myxomatosis is the ability of myxoma virus to replicate efficiently in lymphoid cells, allowing for dissemination of the virus and establishment of internal and external secondary sites of infection. The myxoma virus M-T5 protein appears to be involved in allowing productive replication of myxoma virus in rabbit lymphocytes. In cultured rabbit CD4$^+$ T cells (RL5 cells) or primary lymphocytes, infection with an M-T5$^-$ mutant virus results in rapid and extensive programmed cell death, or apoptosis (22), coupled with a rapid inhibition of both host and

FIG. 9. Primary inoculation site tissue sections. Shown are tissue sections from the primary site of infection with vMyxlac or vMyxlacT5$^-$ harvested on day 4 (A and B, respectively), day 7 (C and D, respectively), and day 10 (E and F, respectively) postinoculation. The tissue sections were harvested and stained with hematoxylin and eosin as outlined in Materials and Methods. Bar, 100 μm.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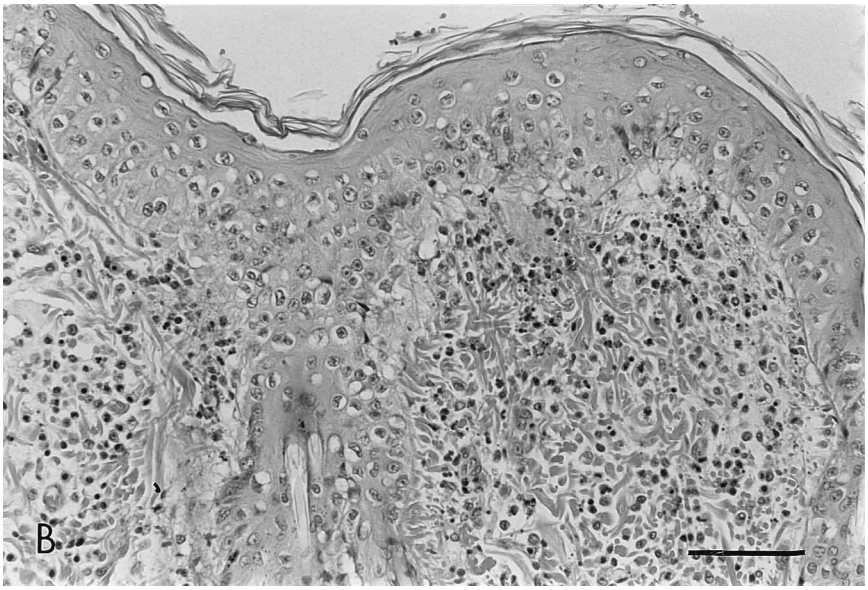
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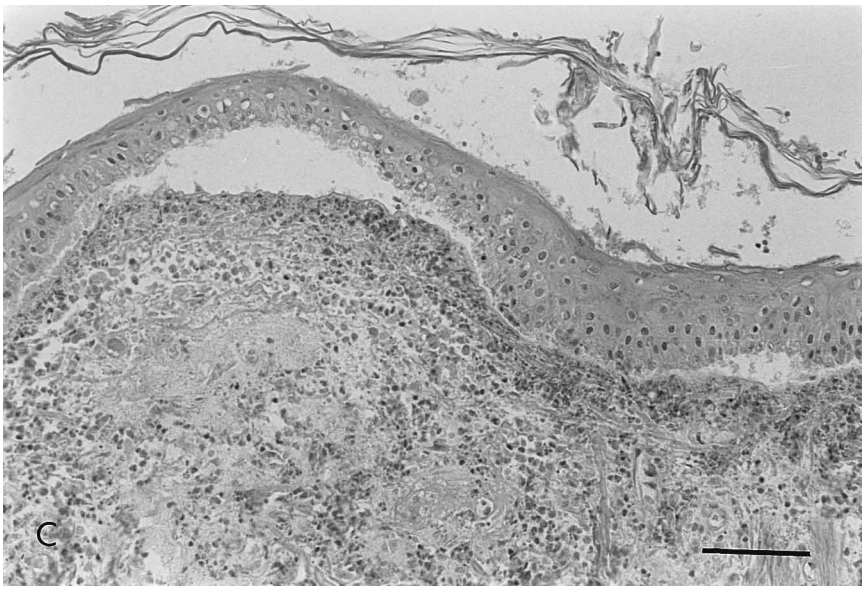
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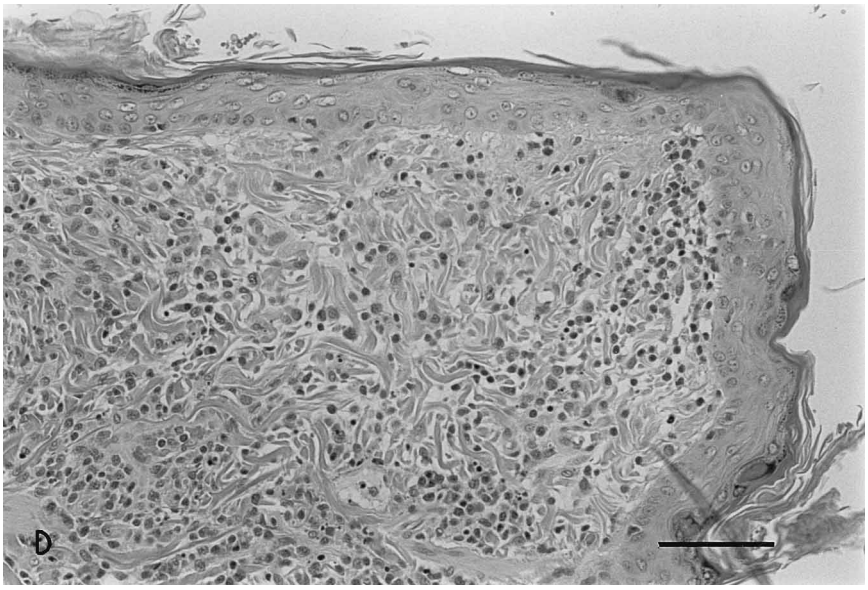

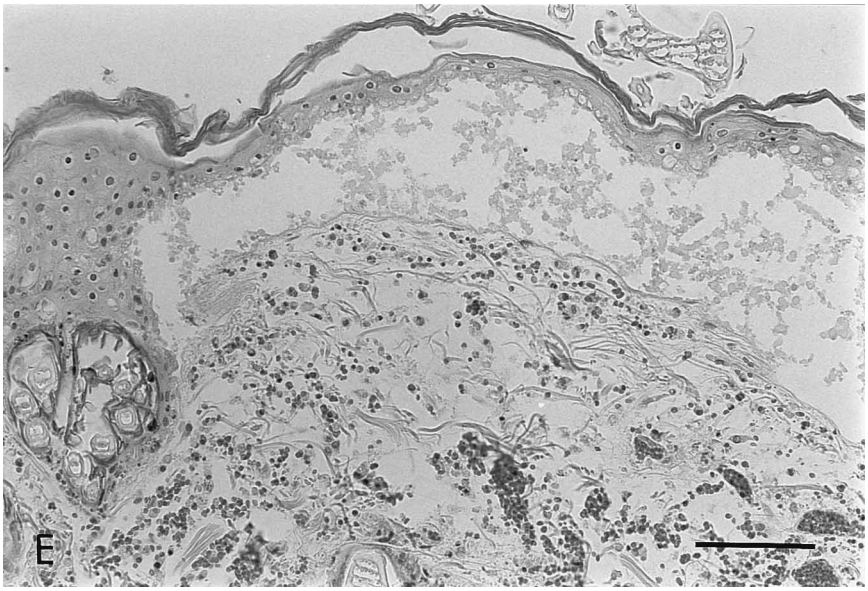

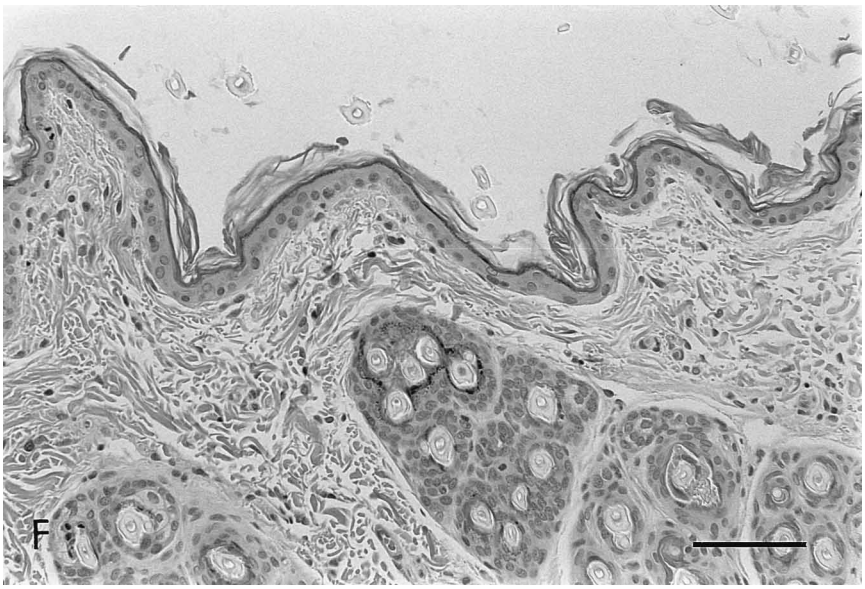

FIG. 9—*Continued.*

virus protein synthesis. Since viral infection is capable of inducing apoptosis in certain circumstances (22, 53), many viruses, including adenoviruses, herpesviruses, baculoviruses, and poxviruses, are known to produce specific viral gene products responsible for blocking the apoptotic response (7, 8, 17, 46, 54). While some viral proteins, such as the cowpox virus CrmA protein, inhibit cell death by directly blocking specific apoptotic pathways (46), the myxoma virus M-T5 protein appears instead to ensure that lymphocytes do not undergo an abortive infection due to a nonspecific shutoff of protein synthesis, which is probably the stimulus that leads to programmed cell death. Infection of nonpermissive CHO cells with vaccinia virus, insect SF-21 cells with baculovirus, or SK-N-SH neuronal cells with certain herpes simplex virus type 1 mutants, also results in the global inhibition of all protein synthesis and subsequent apoptotic cell death (7, 8, 43). For the herpes simplex virus type 1 $\gamma_1 34.5^-$ mutant, it has been shown that the protein kinase PKR complexes with a 90-kDa phosphoprotein, leading to overall protein synthesis shutoff via phosphorylation of eukaryotic initiation factor 2α (eIF-2α) (6).

The myxoma virus M-T5 protein is not unique in influencing viral replication in rabbit lymphocytes, since at least two additional myxoma virus proteins appear to play a role in allowing a fully productive infection. Infection of RL5 lymphocytes with mutants of either the M-T2 protein, a soluble homolog of the mammalian tumor necrosis factor receptor (50), or the M11L protein, a membrane-associated protein of unknown function (16, 37), also results in the inhibition of viral replication and subsequent death of infected cells by apoptosis (25). However, a critical difference is observed upon infection of lymphocytes with these three myxoma virus mutants: the M-T5$^-$ mutant virus is the only mutant which results in the global inhibition of both host and virus protein synthesis. As demonstrated by flow-cytometric analysis, infection of primary peripheral blood lymphocytes with the M-T5$^-$ mutant virus resulted in the apoptotic death of a population of lymphocytes while causing no detectable apoptosis in infected monocytes. Thus, it is reasonable to propose that unlike M-T2 and M11L, the M-T5 protein ensures the continued operation of the translational machinery specifically in infected lymphocytes and that the failure to maintain translation is itself an apoptosis inducer in these cells. Indeed, we have observed that cycloheximide treatment alone in uninfected RL5 lymphocytes induces apoptosis (unpublished data).

While the rapid inhibition of host and virus protein synthesis and subsequent programmed cell death observed upon infection of rabbit lymphocytes with an M-T5$^-$ mutant virus suggest a mechanism of M-T5 function, the biochemical properties of this protein have yet to be determined. However, an interesting correlation exists between M-T5 and the cowpox virus CHOhr, vaccinia virus K1L, and ectromelia virus K1L proteins implicated in the poxvirus host range determination. Infections of rabbit RL5 cells with an M-T5$^-$ mutant virus, rabbit kidney RK13 cells with K1L$^-$ mutants of vaccinia virus and ectromelia virus, and CHO cells with vaccinia virus lacking the addition of the cowpox CHOhr protein all induce the rapid shutoff of both host and virus protein synthesis. Notably, each of these poxvirus proteins contains at least one ankyrin-like repeat. Ankyrins constitute a family of proteins believed responsible for coordinating the interactions between integral membrane proteins and cytoskeletal elements, and a variety of cellular proteins involved in protein-protein interactions contain ankyrin-like repeats (24). A number of orthopoxvirus proteins contain ankyrin-like repeats, and it has been suggested that these proteins may interact with either cellular cytoskeletal proteins or host or virus membranes in the formation of structures necessary for protein synthesis and assembly of virions, thus regulating the virus growth cycle (18, 41). It will be relevant to determine whether the ankyrin-like repeat is important in the localization of the myxoma virus M-T5 protein in rabbit lymphocytes.

Myxoma virus utilizes a diverse range of proteins to circumvent the host immune system in the establishment of a lethal myxomatosis infection (31, 32). To date, deletion analysis of M-T2 (the tumor necrosis factor receptor homolog), M11L (unknown function), M-T7 (the gamma interferon receptor homolog), SERP1 (the serine proteinase inhibitor) and MGF (the epidermal growth factor homolog) has shown that these myxoma virus proteins function as virulence factors which influence the pathogenicity of myxoma virus within the European rabbit (26, 35, 38, 52). Like M-T2 and M-T7, the M-T5 open reading frame is located within the terminal region of the myxoma virus genome, where genes nonessential for viral replication in tissue culture but critical for pathogenesis and host-virus interactions in vivo tend to cluster (47). While M-T5 is the third viral gene shown to be necessary for the productive infection of rabbit lymphocytes (along with M-T2 and M11L), an event which is believed to be critical for the dissemination of myxoma virus within a susceptible host, it is the first viral gene shown to be required for protein synthesis in infected lymphocytes.

Infection of European rabbits with the M-T5$^-$ mutant virus demonstrated that M-T5 is one of the most potent virulence factors yet characterized. While the mortality rate of rabbits infected with wild-type virus was 100%, all of the rabbits infected with the M-T5$^-$ mutant virus displayed virtually none of the disease symptoms of myxomatosis and fully recovered from the infection. To date, the only other myxoma virus mutant which demonstrated a recovery rate of 100% is the M11L$^-$ mutant; the other virulence factor mutant viruses demonstrated intermediate recovery rates ranging from 60% with the SERP1$^-$ mutant to over 90% with the M-T7$^-$ mutant (26, 35, 37, 50). Of importance is the observation that rabbits infected with the M-T5$^-$ mutant virus remained essentially asymptomatic, demonstrating neither lesions at secondary sites nor establishment of a gram-negative bacterial infection, two of the hallmarks of a myxomatosis infection. Rabbits infected with any of the other characterized myxoma virus mutants, regardless of the recovery rate, demonstrated mild to severe symptoms of myxomatosis. It should also be noted that the M-T5 protein is currently the only intracellular, non-membrane-associated protein which functions as a virulence factor in myxoma virus pathogenesis. Aside from the M11L protein, which is membrane associated, all the other characterized virulence factors are secreted proteins.

The dramatic attenuation of the M-T5$^-$ mutant virus appears to reflect an increased ability of the host to rapidly control the infection at the primary site of inoculation. During an effective inflammatory reaction to tissue damage, heterophils function as the main early line of cellular defense and are progressively replaced by infiltrating mononuclear cells. Heterophils in rabbits are functionally analogous to neutrophils in humans and possess the properties of chemotaxis, adherence to immune complexes, and phagocytosis, whereas the functions of mononuclear cells include phagocytosis, killing of ingested microorganisms, antigen presentation, and secretion of enzymes, complement components, and cytokines. Within the primary lesions of rabbits infected with the M-T5$^-$ mutant virus, the early accumulation of heterophils was rapidly replaced by the infiltration of mononuclear cells, suggesting that a well-controlled and effective inflammatory reaction to the virus infection was under way. In fact, by day 10 postin-

oculation, it was difficult to differentiate between the primary site of inoculation with the M-T5$^-$ mutant virus and a parallel section of skin tissue harvested from an uninfected control rabbit. Furthermore, this inflammatory response at the site of inoculation against the M-T5$^-$ mutant virus was not accompanied by noticeable increases in activation of lymphocytes in either the lymph nodes or spleen, suggesting that activation of acquired cellular immunity was not a major component of virus clearance. Only on day 10 postinoculation, when infection with the M-T5$^-$ mutant virus had been effectively controlled, was mild activation of lymphocytes in the lymphoid organs detected. This observation differs substantially from that reported for the M-T7$^-$ myxoma virus mutant, which is disrupted for the soluble gamma interferon receptor homolog, which demonstrated extensive activation of lymphocytes within the spleen and regional lymph nodes of infected animals by day 4 postinoculation (35). Furthermore, M-T7 functions early in infection to retard inflammatory-cell migration into infected tissues, and the M-T7$^-$ myxoma virus mutant did not elicit as effective an inflammatory response within the primary site of inoculation as that observed upon infection with the M-T5$^-$ mutant virus.

Thus, we conclude that the M-T5 protein of myxoma virus functions as a critical virulence factor by preventing the abortive infection of rabbit lymphocytes, allowing for the replication and dissemination of the virus throughout the lymphoreticular system. Given the importance of how lymphotropism is regulated by viruses in general and the observation that the M-T5 protein appears highly specific for lymphocytes, further biochemical analysis of this protein is warranted. Since the natural history of myxoma virus and its host has been well established (11, 28, 29), the myxomatosis model should prove useful to further our understanding of virus-host interactions, particularly in terms of how immune system cells themselves respond to virus infection.

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