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VIRULENCE FACTORS IN LEPORIPOXVIRUSES

BY

ANDREA A. OPGENORTH

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN

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We, the undersigned, give Ms. Andrea Opgenorth permission to use our joint publications as chapters in her PhD thesis. These include the following publications:

1. A. Opgenorth, D. Strayer, C.Upton, and G.McFadden. (1992). Deletion of the growth factor gene related to EGF and TGFα reduces virulence of malignant rabbit fibroma virus. *Virology* 186:175-191.
2. A. Opgenorth, K. Graham, N. Nation, D. Strayer, and G. McFadden. (1992). Deletion analysis of two tandemly arranged genes in myxoma virus, M11L and myxoma growth factor. *Journal of Virology* 66: 4720-4731.
3. A. Opgenorth, K. Graham, N.Nation, and G. McFadden. (1992). Transforming growth factor alpha, Shope fibroma growth factor, and vaccinia growth factor can replace myxoma growth factor in the induction of myxomatosis. *Virology* (In press).

Dr.Grant McFadden ..... Date Dec 11/92

Dr. Chris Upton ..... Date 11/12/92

Dr. Kathryn Graham ..... Date Dec 2/92

Dr. Nick Nation ..... Date 30/11/92

Dr. David Strayer ..... Date 11/25/92

I returned, and saw under the sun, that the race is not to the swift, nor the battle to the strong, neither yet bread to the wise, nor yet riches to men of understanding, nor yet favor to men of skill; but time and chance happeneth to them all.

Ecclesiastes 10:11

Cast thy bread upon the waters:
For thou shalt find it after many days.
Give a portion to seven, and also to eight;
For thou knowest not what evil shall be upon the earth.

Ecclesiastes 11:1

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SUBMITTED BY ANDREA A. OPGENORTH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE

DEGREE OF DOCTOR OF PHILOSOPY.

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To my parents, Joseph and Gloria

ABSTRACT

Malignant rabbit fibroma virus (MRV) and myxoma virus (MYX) are Leporipoxviruses that induce similar lethal syndromes in domestic rabbits characterized by disseminated tumors, generalized immunosuppression, and secondary bacterial infection. The role of the epidermal growth factor (EGF) homologues in MRV and MYX pathogenicity was investigated by constructing viral growth factor deletion mutants (MRV-GF$^-$ and MYX-GF$^-$, respectively). No significant differences between the wild type and growth factor deficient strains were observed in *in vitro* assays of viral growth, cytopathogenicity, or immunosupression. However, a pronounced attenuation was observed after inoculation of rabbits with MRV-GF$^-$ and MYX-GF$^-$. Tumors induced by these strains displayed more prominent inflammatory reactions than their wild type counterparts, contained fewer proliferating cells, and caused less squamous metaplasia and hyperplasia of overlying target epithelia than their wild type counterparts. We conclude that the EGF homologues are virulence factors in MRV and MYX infection and are responsible for at least some of the cellular proliferation observed at tumor sites. The major pathogenic effect of these growth factors likely lies in their ability to alter mucosal epithelial structure, thereby facilitating bacterial colonization.

A novel poxviral pathogenic marker was discovered when DNA sequence analysis of the region upstream of MGF in MYX revealed a partially overlapping open reading frame denoted M11L. Disruption of M11L impaired viral replication in primary rabbit spleen cell cultures and altered plaque morphology *in vitro*, and caused an attenuated phenotype characterized by an enhanced inflammatory response *in vivo*. Simultaneous disruption of M11L and MGF resulted in nearly complete loss of pathogenicity in rabbits.

To determine whether different EGF-like growth factors can perform qualitatively similar functions in the induction of myxomatosis in rabbits the MGF gene in MYX was disrupted and replaced with either vaccinia virus growth factor, SFGF or rat transforming growth factor alpha. The recombinant myxoma virus strains expressing these growth

factors produced infections which were both clinically and histopathologically indistinguishable from wild type myxomatosis. We conclude that these poxviral and cellular EGF-like growth factors, which are diverse with respect to primary structure and origin, have similar biological functions in the context of myxoma virus pathogenesis.

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CAM	chorioallantoic membrane
Con A	concanavalin A
EGF	epidermal growth factor
eIF-2α	eukaryotic initiation factor 2α
EEV	extracellular enveloped virions
kbp	kilobase pairs
hr	host range
i.d.	intradermally
i.v.	intravenously
IFN	interferon
IL-1	interleukin-1
INV	intracellular naked viral particles
LD50	lethal dose of virus for 50% of the test population
MGF	myxoma growth factor
m.o.i.	multiplicity of infection
MRV	malignant rabbit fibroma virus
MYX	myxoma virus
NDF	Neu differentiation factor
nt	nucleotides
ORF	open reading frame
PIR	protein identification resource
pfu	plaque forming units
p.i.	post infection
rr	ribonucleotide reductase
SERP-1	serine protease inhibitor gene product of MRV/MYX

SFGF	Shope fibroma growth factor
SFV	Shope fibroma virus
SH2	*src* homology 2 domain
SPI-1, -2, -3	serine protease inhibitor gene products of orthopoxviruses
TIR	terminal inverted repeat
tk	thymidine kinase
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
TGFα	transforming growth factor alpha
VCP	vaccinia complement binding protein
VGF	vaccinia growth factor

VIRUS MUTANTS

MRV-GF$^-$	growth factor deletion mutant of MRV
vMYX-GF$^-$	growth factor deletion mutant of MYX
vMYX-GF$^-$ΔM11L	strain of MYX in which M11L gene is altered and MGF gene is deleted
vMYXlac	control virus with wild type phenotype
vMYX-M11L$^-$	M11L deletion mutant of myxoma virus
vCΔMGF	growth factor deletion mutant of myxoma virus
vSΔMGF	myxoma virus strain in which SFGF replaces MGF
vVΔMGF	myxoma virus strain in which VGF replaces MGF
vTΔMGF	myxoma virus strain in which rat TGFα replaces MGF

CHAPTER I GENERAL INTRODUCTION

In 1979, the WHO certified the world completely free of one of its most dreaded infectious diseases, smallpox. The global eradication of smallpox by the concerted efforts of individuals and governments worldwide to immunize populations with vaccinia virus remains one of humanity's most outstanding medical health accomplishments. Throughout history, poxviruses have played leading roles in the evolution of science and medicine (Fenner, 1990a and 1992; Henderson, 1988). As long ago as the 10th century, variolation was practiced throughout China to reduce mortality associated with smallpox infection. Jenner's demonstration in 1789 that smallpox could be prevented by previous exposure to cowpox was a pivotal event in the history of immunology. As the largest and most complex animal viruses, poxviruses were the first to be visualized by microscopy and the first to be grown in cell culture. The poxviruses have also played significant roles in the development of molecular biology, being instrumental in the elucidation of the structure of eukaryotic mRNA. Because poxviruses no longer figure as major human pathogens, investigation of poxviral pathogenesis might initially seem superfluous. However, the increasing use of poxviruses as potential vaccine vectors expressing antigens from a wide variety of pathogens for animals and humans demands a fuller understanding of the molecular mechanisms of poxvirus pathogenesis (Paoletti, 1990; Buller and Palumbo, 1992). Perhaps more significantly, poxviruses are endowed with unique characteristics which render them ideal for the study of basic cellular processes such as transcription and DNA replication, and for the molecular dissection of the events that define virus-host interaction (Moss, 1991).

Introduction to poxvirus biology

Classification and physical properties of poxviruses

The poxviruses are a ubiquitous group of DNA viruses that infects virtually every vertebrate species and some insects, producing a diverse spectrum of diseases. The classification, physical properties, gene expression, and replication of the poxviruses have been extensively reviewed (Moss, 1990a, b, c, and 1992; Dales and Pogo, 1982). The two subclasses of poxviruses are the Chordopoxviridae (vertebrate poxviruses) and the Entomopoxviridae (insect poxviruses). The Chordopoxviridae (Table I-1) have been subdivided into eight genera on the basis of antigenic similarity, host range, and genomic cross-hybridization. These include Orthopoxvirus, Suipoxvirus, Leporipoxvirus, Avipoxvirus, Capripoxvirus, Parapoxvirus, Molluscipoxvirus and Yatapoxvirus. All poxviruses possess certain characteristic features. The virion is large (200-400 nm in length), brick shaped, contains a biconcave nucleoprotein core particle with two lateral bodies and is bound by a lipoprotein bilayer membrane. Some strains acquire an additional Golgi-derived lipid bilayer called the envelope upon infection of certain cell types. An impressive array of virus encoded enzymes involved in mRNA synthesis and DNA replication is packaged within the virus particle, which contains 100 or more polypeptides. The poxvirus genome is located within the core and consists of a single double-strand linear DNA molecule of 130-300 kbp containing covalently closed hairpin ends and a terminal inverted repeat of 10-13 kbp.

Summary of poxvirus replication

The entire lifecycle of the poxvirus, including transcription and DNA replication, takes place within the cytoplasm of the infected cell, making it one of the most cellularly autonomous of all animal viruses. The nearly complete independence from the nucleus is possible because the poxviruses encode virtually all of the enzymes and factors required for

their replication (Moyer, 1987). Their large size, the absence of introns within coding sequences, the cytoplasmic site of replication, and relative cellular autonomy has made the poxviruses useful model systems for the study of basic cellular processes such as DNA replication and transcription (Traktman, 1990a).

The infectious cycle (Moss, 1990b and 1992; Traktman, 1990b) is initiated by virus binding and entry into the cell, a poorly understood process that does not appear to involve any specific cellular receptors and is thought to occur by fusion with the cell membrane. Naked cores are released into the cytoplasm and early genes encoding factors required for DNA synthesis are transcribed by core-associated viral RNA polymerase. A second uncoating event follows in which the viral core particle disappears and viral DNA becomes susceptible to treatment with DNAse I. DNA replication then occurs within viral "factories" in the cytoplasm and is followed by intermediate and late gene expression in which virion structural proteins and enzymes required for morphogenesis are synthesized. Virion assembly proceeds within the factories by a complex, poorly understood process in which viral membranes formed *de novo* associate with viral DNA and protein and undergo morphogenesis to mature viral particles. There is no specific lysis function encoded by poxviruses and the majority of mature virions remain cell-associated at the end of the replicative cycle. A variable proportion of progeny virions undergo release from the infected cell, which appears to occur by breakdown of long cytoplasmic extensions resembling microvilli. The number of virions released extracellularly depends on the virus strain and the infected cell type.

Arrangement of essential and nonessential genes

The majority of information regarding the genomic organization of the poxviruses has been obtained from studies of the Orthopoxvirus vaccinia, the prototype poxvirus. Its origin and natural host remain obscure but was likely derived from either cowpox or variola or a hybrid of both during the last century (Fenner, 1992; Baxby, 1981). Comparison of

various strains of Orthopoxvirus showed that whereas the terminal 30 kbp of sequences at both ends of the genome are prone to extreme variability, a core of conserved sequences within the unique central portion of the genome exists (Esposito and Knight, 1985). Analysis of temperature-sensitive mutants and deletion mutants of the Orthopoxviruses (Condit and Niles, 1990) revealed that gene products essential to virus replication, such as the DNA polymerase, topoisomerase, and RNA polymerase subunits, are generally restricted to the central core region, whereas nonessential genes are usually located in the recombinogenic terminal regions of the genome (Turner and Moyer, 1990). This arrangement of essential and non-essential genes has been found to be a general feature of most, if not all, poxvirus genera (Turner and Moyer, 1990; Traktman, 1990a). DNA sequence analysis together with analysis of naturally occurring deletion mutants of these non-essential regions have revealed that the poxviruses encode a multitude of products which, though non-essential for virus replication, have profound effects on viral pathogenesis, virulence, and host range (Buller and Palumbo, 1991 and 1992; Turner and Moyer, 1990). Moreover, many of these gene products have related cellular homologues or interact with host factors whose roles as cytokines, growth factors and inflammatory mediators are currently being elucidated. The entire DNA sequence of the Copenhagen strain of vaccinia virus has been recently determined and was shown to encode at least 263 potential gene products (Goebel *et al.*, 1990). Although the functions of the majority of these products have not yet been determined, the construction of a viable attenuated vaccinia mutant in which 55 open reading frames were deleted attests to the enormous potential for phenotypic and pathogenic variability encoded by these non-essential genes (Perkus *et al.*, 1991).

Poxviral gene products contributing to pathogenicity

In recent years, dissection of molecular pathogenesis of poxviruses has centered on the detection and analysis of specific gene products that may act as virulence factors by subverting host defense systems or creating conditions optimal for viral growth. Tables I-2 and I-3 list known determinants of viral virulence that are non-essential for viral replication. Although the host range, pathological effects, and outcome of the diseases induced by the poxviruses are substantially different from one another, some of these factors (such as the serpins and EGF homologues described below) have been found to exist as families that are distributed across the spectrum of poxvirus genera (Buller and Palumbo, 1991; Fenner, 1990b; Turner and Moyer, 1990). Therefore, it is useful to consider that virulence factors identified in the well-studied Orthopoxviruses may have similar counterparts in other species. The work of this thesis centers around the role of epidermal growth factor homologues in the pathogenesis of two closely related tumorigenic Leporipoxviruses: malignant rabbit fibroma virus and myxoma virus. In the course of these studies, a novel virulence factor, M11L, was also identified in myxoma virus which appears to inhibit the host inflammatory response to infection. The following introductory sections of this thesis will introduce the Leporipoxviruses, describe results of recent studies that examine specific poxviral gene products involved in pathogenesis, and summarize the biology of epidermal growth factor and its homologues.

Leporipoxviruses

Several members of poxvirus family induce distinctively proliferative lesions in their infected hosts and have been described as tumorigenic. These include Yaba tumor virus, which induces proliferation of histiocytes, molluscum contagiosum, which produces wart-like epidermal lesions in humans, and the Leporipoxviruses. The Leporipoxviruses are the best studied tumorigenic poxviruses and constitute the subject of this thesis. It is

important to note that the expression *tumor* denotes swelling (Cotran *et al.*, 1989) and, in the case of the poxviruses, does not imply neoplasia associated with cells transformed to an immortalized phenotype. Permanent cellular transformation by poxviruses, or presence of poxviral DNA in the infected cell nucleus has never been convincingly demonstrated (Buller and Palumbo, 1991; McFadden, 1988). Most evidence indicates that poxvirus-infected cells in tumors do not themselves proliferate (Scherrer, 1968; Kato *et al.*, 1966); rather, the cellular proliferation is considered part of local hyperplastic connective tissue reactions to viral infection (Fenner and Ratcliffe, 1965; Shope, 1932a and b). Nevertheless, the Leporipoxviruses have been studied for many years as systems for investigation of cellular proliferation as a response to viral challenge (Pogo *et al.*, 1989; Febvre, 1962). Members of this group of relevance to this thesis include Shope fibroma virus (SFV), myxoma virus, and malignant rabbit fibroma virus (MRV).

Shope fibroma virus

SFV was first isolated in 1932 from a subcutaneous fibroma on a wild cottontail rabbit (*Sylvilagus floridanus*) captured in the Eastern United States by R. Shope, who subsequently demonstrated that extracts of the lesion could transmit the tumor to healthy rabbits (Shope, 1932a and b). Transmission in the wild occurs mostly through dermal inoculation by arthropod vectors and both *S. floridanus* and domestic rabbits (*Oryctolagus cuniculus*) are susceptible to infection. The disease course in either host is benign and involves development of a localized, protuberant tumor which, in domestic rabbits, reaches a maximal size of 4-6 cm within two weeks then completely regresses within one month (McFadden, 1988; Febvre, 1962). Tumor regression coincides with development of antibody to SFV but is largely mediated by the cellular immune response to infected cells (Sell and Scott, 1981). Secondary tumors are absent except in newborn or immunocompromised adult rabbits, where SFV infection may cause lethal, disseminated disease. The tumor induced by SFV has been described histologically as a proliferant

fibroma composed largely of connective tissue cells, containing abundant intercellular collagen fibrils and a prominent leukocytic infiltrate (Febvre, 1962; Mare , 1974; Fenner and Ratcliffe, 1965). SFV strains which have lost the ability to induce proliferative lesions have been isolated but are unfortunately no longer available (Andrews and Shope, 1936). Recent work on SFV has focused on the molecular characterization of the virus (Wills *et al.*, 1983; Macaulay and McFadden, 1989; Upton *et al.*, 1990b and 1991b; DeLange and McFadden, 1990; Stuart *et al.*, 1991).

Myxoma virus

Myxoma virus is the causative agent of myxomatosis, which, because of its extreme virulence and narrow host range, was deliberately released in Australia and Europe in the 1950's to facilitate control of the feral rabbit population. In its natural host, which is the tropical forest rabbit (*S. brasiliensis*) or the California brush rabbit (*S. bachmani*), myxoma virus causes only very minor lesions and no systemic disease. In domestic rabbits, myxoma virus causes a lethal disease that is characterized by disseminated tumors and profound immunosuppression resulting in secondary bacterial infection with commensal organisms such as *Pasturella spp.* and *Bortedella spp.* The epidemiology and pathological effects of myxomatosis have been reviewed in great detail (Tripathy *et al.*, 1981; Fenner and Marshall, 1957; Fenner, 1990b; Fenner and Ratcliffe, 1965, McFadden, 1988; Strayer, 1988). Upon intradermal infection with myxoma virus, a proliferative lesion develops at the site of inoculation followed by rapid dissemination of virus throughout the reticuloendothelial system to give rise to a systemic infection. In addition, secondary tumors resembling the initial lesion are induced by viral replication throughout the dermis and are especially prominent on the face. A characteristic feature of this syndrome is severe purulent conjunctivitis and rhinitis caused by opportunistic bacterial infection in the respiratory tract and conjunctiva. Despite development of high titers of antibody to myxoma virus, death inevitably occurs within 14 days. The cause of death is

usually suffocation due to obstruction of the external nares by products of overwhelming bacterial infection. Histologically, the tumors are composed of proliferating mesenchymal cells, often called "myxoma cells" (Hurst, 1937), with large stellate morphology embedded in a seromucinous matrix. Although these lesions resemble those induced by SFV, the former are somewhat less proliferative, with the bulk of the tumor composed of extracellular matrix material .

Although SFV and myxoma virus differ dramatically in their pathogenic effects in the domestic rabbit, they are closely related serologically, with immunity to SFV conferring full protection against infection with myxoma virus. Analysis of the myxoma virus genome (Block *et al.*, 1985; Russell and Robbins, 1989) has revealed substantial similarity with SFV; their genomes cross hybridize under low stringency conditions, and many myxoma virus ORFs sequenced to date have a counterpart in SFV that displays approximately 75% identity at the DNA level (Upton *et al.*, 1987, 1988, and 1991a).

Malignant rabbit fibroma virus

Malignant rabbit fibroma virus (MRV) is a natural recombinant between SFV and myxoma virus that was isolated from an uncloned stock of SFV (Strayer *et al.*, 1983). This virus has been extensively characterized, and produces a lethal syndrome of severe immunological dysfunction and disseminated tumors that is virtually identical to that induced by myxoma virus (Strayer and Sell, 1983; Strayer *et al.*, 1983, 1985, 1987, 1990; Strayer and Leibowitz, 1986 and 1987; Strayer, 1988). Approximately 95% of the MRV genome is identical to that of myxoma virus; the remainder consists of SFV sequences (Upton *et al.*, 1988; Block *et al.*, 1985; Strayer *et al.*, 1983). In the recombinational events that generated MRV, a 7978 bp SFV sequence replaced homologous myxoma sequences at the left terminus of the myxoma genome, followed by copying of a 4674 bp subset of these sequences to the right terminus . These events resulted in replacement of five complete myxoma ORFs with the corresponding SFV homologues, and the generation of two in-

frame fusion ORFs composed of part SFV, part myxoma sequences (Upton *et al.*, 1988). The pathogenesis of MRV infection is highly similar to that of myxoma virus, especially the Lausanne strain (Buller and Palumbo, 1991; Strayer, 1988). Sequence analysis of the recombination junctions of MRV indicate complete sequence identity with myxoma Lausanne (Upton *et al.*, 1988). For these reasons, it is appropriate to consider MRV as another virulent strain of myxoma virus. Among the SFV genes transferred to myxoma virus to generate MRV is the Shope fibroma growth factor gene (SFGF) which encodes a polypeptide belonging to the epidermal growth factor (EGF) family of growth factors (Chang *et al.*, 1987). This gene replaces the endogenous myxoma growth factor (MGF) gene, which encodes a highly similar EGF-like peptide (Upton *et al.*, 1987). Because of the association of EGF with mitogenesis, it has been suggested that these viral growth factors might contribute to the uniquely proliferative nature of the lesions induced by the Leporipoxviruses (McFadden, 1988; Dales, 1990).

One of the outstanding differences between SFV infection on one hand, and MRV and myxoma infection on the other, is that the latter viruses induce immunological dysfunction in their infected hosts whereas SFV does not (Strayer *et al.*, 1983; Sell *et al.*, 1986). SFV has therefore been used as a control in a series of studies that characterized the immunosuppressive effects of MRV on the infected rabbit host (Strayer and Dombrowski, 1988; Strayer *et al.*, 1986a and b, 1988a; Strayer and Leibowitz, 1986 and 1987; Skaletsky *et al.*, 1984). Lymphocytes from rabbits infected with MRV proliferate poorly in response to non-specific B and T cell mitogens compared with those of SFV recipients. The primary cause of this nonspecific immune dysfunction is that MRV and myxoma virus replicate very efficiently in both B and T lymphocytes whereas SFV is incapable of productive infection in lymphoid cells (Strayer *et al.*, 1985 and 1987). The lymphocytotropism of MRV appears to be one of the parameters that prevent the development of a cell-mediated immune response to infection since virus-reactive lymphocytes have not been detected in MRV-infected animals (Skaletsky *et al.*, 1984;

Strayer, 1988). In contrast, a vigorous cell-mediated immune response appears responsible for the regression of SFV-induced tumors (Strayer *et al.*, 1984; Sell and Scott, 1981). Other factors contributing to the immunosuppressive effects of MRV and myxoma virus include downregulation of class I major histocompatability molecules and secretion of a soluble receptor for tumor necrosis factor upon infection of host cells (Boshkov *et al.*, 1992; Upton *et al.*, 1991a).

SFV/MRV recombinants have been created to analyze the genetic components of MRV and, by inference, myxoma virus, critical for replication in lymphocytes. Transfection of portions of the 10.8 kbp Bam HI C fragment of MRV into SFV-infected cells has resulted in the isolation of virus strains capable of replication in lymphocytes (Strayer *et al.*, 1988b and 1990; Heard *et al.*, 1990). Several of these strains are capable of inducing disseminated disease in rabbits that closely resembles MRV infection. Although these putative recombinant viruses have not been extensively characterized and the identity of the gene product(s) allowing growth in lymphocytes is as yet unclear, these experiments reaffirm the notion that the level of lymphocyte infectivity is a major determinant of Leporipoxvirus virulence in rabbits.

Specific gene products implicated as determinants of poxviral pathogenesis

Serpins

The serpins (serine protease inhibitors) are a superfamily of proteins containing members that are important in the regulation of serine proteases (Travis *et al.*, 1990). Since serine proteases are involved in numerous biological regulatory cascades including those that govern immunological and inflammatory responses, the discovery that poxviruses encode serpins was met with considerable excitement. To date, three serpin-like genes have been identified in the Orthopoxviruses and one each in MRV/myxoma, and fowlpox (Smith *et al.*, 1989; Upton *et al.*, 1986 and 1990a; Pickup *et al.*, 1986; Kotwal and Moss, 1989;

Tomley *et al.*, 1988; Boursnell *et al.*, 1988). The first poxviral serpin gene was discovered in cowpox. Wild type cowpox produces bright red, flat lesions on chick chorioallantoic membranes (CAMS). White pock mutants arise from wild type progenitors at a rate of approximately 1% upon passage on CAMs and have a raised, opaque appearance. Upon inoculation of rabbits, mice, non-human primates, and guinea pigs, these mutants were found to be less virulent than wild type cowpox (Buller and Palumbo, 1991 and 1992). Analysis of naturally occurring deletion mutants possessing the white pock phenotype, together with mapping by marker rescue techniques revealed that the wild type red pock phenotype was due to the presence of a gene encoding a 38-kDa protein located between 31 and 32 kbp from the right end of the genome, whereas white pock mutants were lacking the corresponding sequences (Pickup *et al.*, 1986). This gene, known formally as SPI-2 (serine protease inhibitor) or *crm A* (cytokine response modifier gene), was shown, by virtue of its predicted amino acid sequence, to belong to the serpin superfamily and to have significant amino acid homology with antithrombin III. Because of this sequence similarity and the hemorrhagic nature of the red pocks, it was hypothesized that the SPI-2 gene might encode an inhibitor of the blood coagulation pathway. However, comparative histopathological analysis indicated that the primary difference between wild type and white pocks on CAMs was a massive inflammatory response characterized by infiltration of macrophages and neutrophils into the white pock lesions (Chua *et al.*, 1990; Fredrickson *et al.*, 1992; Palumbo *et al.*, 1989). As a consequence of this vigorous inflammatory response, the viral yield and extent of tissue damage were decreased from wild type levels. The hemorrhage observed in the red pocks was attributed to secondary effects stemming from the lack of inflammatory response to the wild type virus. In addition, micropore filter assays using extracts of infected CAMs and cell cultures demonstrated that white pock cowpox infection generated leukocyte chemoattractant activity, whereas wild type infection did not (Chua *et al.*, 1990). Consequently, it was suggested that the 38-kDa product might inhibit the generation or action of chemoattractant molecules necessary for an effective inflammatory

response to viral infection. This hypothesis has recently been substantiated by the demonstration that the cytoplasmic 38-kDa protein specifically inhibits the proteolytic activation of interleukin-1β by the interleukin-1β converting enzyme (Ray *et al.*, 1992). This cytokine is produced by many cell types and is an important component in the generation of inflammatory reactions (Gallin *et al.*, 1988). Therefore, at least *in vitro*, the 38-kDa protein prevents the formation of one of the key mediators of an early host response to viral infection.

Orthopoxviruses contain two additional serpin genes, one designated SPI-1 (Kotwal and Moss, 1989) which is closely related to SPI-2, but of unknown function, and another related gene denoted SPI-3 (Boursnell *et al.*, 1988). The SPI-3 gene product has recently been shown to have a function distinct from that of the 38-kDa protein. SPI-3 controls the ability of infected cells to form syncytia at neutral pH in a manner indistinguishable from that of viral hemagglutinin. That is, cell fusion can be induced in cells infected with vaccinia, cowpox, or rabbitpox strains in which the SPI-3 gene has been rendered inactive (Law and Smith, 1992; Turner and Moyer, 1992; Zhou *et al.*, 1992). Unlike the cytoplasmic 38-kDa protein, SPI-3 contains a hydrophobic C terminus and therefore may be either membrane associated or secreted from infected cells (Turner and Moyer, 1992). Although the precise relationship between fusogenic capacity of poxviruses and pathogenesis remains to be elucidated, fusion of infected cells is almost certainly an important point of control in viral dissemination.

The SERP-1 gene in MRV/myxoma is currently the only serpin-like protein that has clearly been shown to be important for viral virulence in its natural host (Upton *et al.*, 1990a). Of the three Orthopoxvirus serpin genes, SERP-1 is most closely related to SPI-3 at the amino acid level. Upon inoculation of rabbits with an MRV recombinant in which the single copy SERP-1 gene had been rendered inactive (MRV-S1), recipients developed an attenuated syndrome characterized by a decreased extent of overall immunosuppression compared with wild type controls. MRV-S1 was indistinguishable

from wild type MRV with respect to colony morphology and growth characteristics in cell culture. The serpin-like gene in myxoma virus is identical to SERP-1 but is present as two copies, one in each TIR (Upton *et al.*, 1986 and 1990a). Recently, both copies of SERP-1 have been deleted in myxoma virus and the resultant recombinant virus shown to be attenuated in rabbits in a fashion similar to that observed in MRV-S1 (J. Macen, C. Upton, and G. McFadden, manuscript in preparation). Interestingly, the SERP-1 gene in SFV exists as a fragmented pseudogene, a finding which also confirms the correlation between pathogenicity and the presence of an intact SERP-1 gene. The *in vivo* cellular target(s) of the SERP-1 gene product remain unknown but a survey of a spectrum of serine proteases has revealed that *in vitro* SERP-1 will complex to, and inhibit the activity of, plasmin, urokinase, tissue plasminogen activator, and C1s, the first enzyme of the complement cascade (D.A. Lomas, D.L. Evans, C. Upton, G. McFadden, and R.W. Carrell, *J. Biol. Chem*, in press). Since these enzymes participate in blood clotting, fibrinolysis and complement activation cascades, the *in vivo* modulation of these functions could compromise wound healing and inflammatory reactions in the infected host, thereby favoring viral propagation.

"Viroceptors"

The inflammatory response involves a complex series of reactions that are usually initiated as one of the earliest host responses to invasion by foreign pathogens (Gallin *et al.*, 1988; Metcalf, 1991). Compared with bacterial infection, foci of poxviral infection usually display only minimal amounts of inflammatory activity (Buller and Palumbo, 1991). The major factor determining the course and resolution of viral infections is thought to depend predominantly on the capacity of the host to mount an effective cell-mediated immune response, and prevention of reinfection appears to be dependent on humoral immunity (Buller and Palumbo, 1991; Cotran *et al.*, 1989). Since the full activation of an effective immune response is time-dependent, absence of a prominent inflammatory attack

on virus-infected cells is highly favorable for viral replication and dissemination. It is therefore hardly surprising that the poxviruses have developed strategies to circumvent cellular immune mechanisms. For example, several poxviruses have been shown to elaborate receptor-like molecules, called "viroceptors" (Upton *et al.*, 1991a) which bind and sequester key inflammatory mediators as their ligands. Presumably, these virus-encoded products function to inactivate cytokines in the vicinity of viral infection and contribute to the downregulation of a strong antiviral response.

Soluble TNF receptors

The first example of a viral homologue to a host cytokine receptor was detected by computer homology searches which showed that the SFV T2 ORF, mapping in the TIR near the end of the genome, showed significant amino acid homology with the ligand binding domain of the human type I tumor necrosis factor receptor (TNFR) (Smith *et al.*, 1990). Tumor necrosis factor (TNF) α and β are cytokines which have multiple activities mediating inflammatory and immune responses to a variety of bacterial and viral pathogens, neoplasia, and wounding (Beutler and Cerami, 1989; Piguet *et al.*, 1991; Vassali, 1992). The TNFs are also known for their ability to directly kill virally infected and tumor cells by incompletely understood mechanisms. When expressed in COS cells, recombinant T2 protein from SFV was shown to be secreted and to specifically bind human TNF α and β (Smith *et al.*, 1991). Myxoma and MRV also encode a T2 homologue within the TIR which is 76% identical to that of SFV and is also secreted from infected cells (Upton *et al.*, 1991a). Deletion of both copies of the T2 ORF from myxoma resulted in a virus (vMYX-T2gpt) which grew normally in cell culture but which was attenuated upon intradermal inoculation of rabbits. The attenuation was reflected by smaller tumor sizes, decreased bacterial superinfection, and 65% survival rate upon infection of susceptible rabbits (Upton *et al.*, 1991a). Although the precise cause of attenuation remains to be established, it is likely that deletion of T2 allowed a more vigorous antiviral host response

by either permitting endogenous TNF to act on infected cells in an unimpeded fashion, or else short-circuiting TNF signalling within the local cytokine network that regulates the inflammatory response. Two ORFs described in vaccinia virus corresponding to T2 are both interrupted by frameshift mutations and termination codons and therefore both are unlikely to produce an active TNF binding protein (Howard *et al.*,1991; Upton *et al.*,1991a). It is likely that these vaccinia genes represent ancestral remnants of virulence genes in the progenitor virus from which vaccinia virus evolved. Recently, it has been shown that the T2 protein of myxoma virus/MRV is a potent inhibitor of rabbit TNF in a cytolysis bioassay, suggesting that the ability to inhibit a specific cytokine family may exhibit a species specificity that directly reflects the host animal in which the virus has evolved (M. Schreiber and G. McFadden, manuscript in preparation).

IL-1 viroceptors

A viral analogue for another cytokine receptor has been detected in vaccinia virus based on computer-assisted analysis of amino-acid sequence similarities with proteins in the SWISSPROT database (Smith and Chan, 1991). ORFs B15R and B18R encode closely related products that are also related to the immunoglobulin superfamily, and more specifically, the interleukin-1 (IL-1) receptor. The B18R protein is known to be expressed on the cell surface at early times after infection (Ueda *et al.*,1990). IL-1 is a lymphokine produced by monocytes /macrophages which potentiates the immunological and inflammatory activities of T cells, B cells, neutrophils, and macrophages. If these putative viroceptors are indeed capable of binding IL-1, they could prevent this cytokine from reaching its target cells, thereby providing another means of diminishing host defenses against infection.

Gene products that interfere with interferon action.

Interferon production is one of the earliest antiviral defense mechanisms that can initiated upon viral infection (Joklik, 1990; Billiau and Dijkmans, 1990; Samuel, 1991; Trinchieri and Perussia, 1985). These cytokines interfere with viral replication by inhibiting translation initiation via at least two pathways, both of which are also induced by double stranded RNA. One pathway involves induction of a 2'-5'A oligoisoadenylate synthetase, which in turn activates a latent endoribonuclease, which then inhibits protein synthesis by cleaving mRNA and rRNA. In the other pathway, double stranded RNA activates a protein kinase (DAI kinase) which phosphorylates eukaryotic initiation factor 2α (eIF-2α), leading to inhibition of translation initiation. An important facet of interferon action is its ability to protect neighboring uninfected cells from productive viral replication. Poxviruses are potent inducers of interferon due to the production of large amounts of double stranded RNA during late gene expression (Boone *et al.*, 1979). Nevertheless, anti-interferon properties of vaccinia virus have been well documented (Buller and Palumbo, 1991). At least one specific gene product from vaccinia virus has been found to hinder interferon action within the infected cell. Computer-assisted analysis of the sequenced Copenhagen strain of vaccinia virus (Goebel *et al.*, 1990) revealed that the product encoded by the K3L ORF is 28% identical to eIF-2α. Deletion of this ORF produced a strain with significantly increased sensitivity to interferon (Beattie *et al.*, 1991). K3L has recently been found to inhibit phosphorylation of eIF-2α by preventing autophosphorylation and activation of the DAI kinase, possibly by acting as a substrate decoy to protect eIF-2α from phosphorylation (Davies *et al.*, 1992).

A novel viral anti-interferon strategy has been discovered in myxoma virus (Upton *et al.*, 1992). Amino acid sequence determination of the major secreted virally encoded protein from myxoma infected cells revealed that the corresponding viral ORF was highly similar to the SFV T7 ORF (Upton and McFadden, 1986). After cloning and sequencing

the corresponding myxoma ORF, computer-assisted sequence analysis revealed that both T7 ORFS, and a vaccinia ORF of unknown function (B8R) (Goebel *et al.*, 1990), showed significant amino acid sequence similarity with the ligand-binding domain of the human and mouse γ-interferon receptor (Upton *et al.*, 1992). Cross-linking experiments demonstrated that the T7 product specifically binds rabbit γ-IFN and a γ-IFN bioassay indicated that T7 inhibits rabbit γ-IFN in a stoichiometric fashion. Myxoma T7 thus appears to be the first virally encoded protein that can specifically sequester an interferon ligand, and may provide another level of protection to the virus from the effects of interferon, either by directly protecting from the induced anti-viral state in target cells, or by preventing the activation of macrophages by γ-IFN.

The 13.8-kDa secreted protein of vaccinia virus

The vaccinia virus spontaneous deletion mutant denoted 6/2 is a highly attenuated strain in which a 12.5 kbp segment of the genome near the left terminus was deleted (Kotwal and Moss, 1988b). Two polypeptides that were absent in culture supernatants of cells infected with the 6/2 mutant but present in the culture medium of cells infected with wild type vaccinia virus were identified (Kotwal and Moss, 1988b). One of these secreted proteins was found to be structurally similar to a family of complement-binding proteins and is described below (Kotwal and Moss, 1988a). Antibody to the other secreted protein immunoprecipitated an *in vitro* translation product whose mRNA hybridized to the N1L ORF. Disruption of this ORF alone resulted in a reduction of virulence by a factor of 1.6×10^4 compared with wild type virus, as measured by intracranial LD_{50} in mice (Kotwal *et al.*, 1989). An N1L homologue has been detected in rabbitpox virus and is also one of the ORFs deleted in a naturally occurring attenuated mutant (Bloom *et al.*, 1991). Together, these observations show that, although the function of the N1L product is unknown, this secreted protein is a powerful determinant of viral virulence.

The complement proteins are a group of factors present in plasma which, upon activation, participate in a cascade-like series of reactions whose products are critical for host defense against microorganisms (Cotran *et al.*, 1989). These reactions cause alterations in vascular permeability, generate membrane attack complexes which cause cell lysis, produce opsonins, and generate molecules which are chemotactic for leukocytes. Therefore, complement proteins can act as mediators of inflammation as well as enhancers of immunological reactions. Two pathways for complement activation have been elucidated (Fearon and Wong, 1983) The classical pathway requires either a soluble or cell-surface antigen-antibody complex to initiate the reaction sequence; the alternative pathway can bypass this step and be triggered by a variety of non-immunological stimuli. Several mechanisms by which complement components could interfere with successful poxvirus infection have been proposed (Buller and Palumbo, 1991; Hirsch, 1982). These include lysis of the virion or virus-infected cells by membrane-attack complexes, opsonization of virions by complement components to enhance phagocytosis, generation of C5a to recruit neutrophils, and enhancement of antibody-mediated neutralization of virion infectivity in the absence of virolysis by buildup of proteins on the virus. Although the precise roles played by complement proteins in determining the course of poxvirus infection are presently unclear, several categories of viral proteins have been discovered that may interfere with complement action.

Vaccinia complement binding protein (VCP) was initially detected as the major polypeptide secreted from cells infected with vaccinia virus (Kotwal and Moss, 1988a) and is also secreted by cowpox and ectromelia viruses (Kotwal *et al.*, 1990). It is encoded by ORF C21L and displays amino acid homology with a family of eukaryotic proteins that control complement activation, which includes C4b binding protein. As measured by a hemolysis assay, VCP can inhibit the classical pathway of complement activation and binds specifically to complement component C4b (Kotwal *et al.*, 1990). Deletion of C21L does

not affect virus replication in cell culture. VCP was shown to inhibit complement-enhanced neutralization of vaccinia virus infectivity in the presence of antibody (Isaacs *et al.*, 1992). Unexpectedly, this effect did not require the presence of C4, a component required for activity of the classical pathway, as VCP also inhibited neutralization of virus infectivity when complement from genetically C4-deficient guinea pigs was used. Therefore, VCP appears to interfere with the alternative pathway of complement activation in addition to the classical pathway. The involvement of the alternative pathway was confirmed by the demonstration that serum depleted of factor B, a component of the alternative pathway, was unable to enhance antibody-mediated neutralization of infectivity (Isaacs *et al.*, 1992). VCP was shown to play a significant role *in vivo* in moderating the host response to infection; intradermal inoculation of both normal and C4-deficient guinea pigs resulted in smaller lesions when a VCP-deficient vaccinia strain was used compared with lesions induced by wild type vaccinia virus. Rabbitpox virus encodes a VCP homologue and is deleted from rabbitpox strains with reduced virulence (Bloom *et al.*, 1991).

A sensitive method for the analysis of genomic regions contributing to virulence using a weight loss assay in mice has been developed by Moyer and colleagues (Bloom *et al.*, 1991). An avirulent deletion mutant of rabbitpox was used as a parent virus for the construction of a series of strains bearing systematically reintroduced overlapping portions of the deleted sequences. Among the virulence factors identified by this method were two novel rabbitpox gene products encoded by ORFs M2L and B20L resembling complement components C4 and C5, respectively. The possibility that these proteins might act through conserved binding domains to prevent activation or activity of complement reactions remains to be tested.

The product encoded by vaccinia ORF B5R represents another class of poxviral products that bears structural homology with complement control proteins (Smith *et al.*, 1991; Takahashi-Nishimaki *et al.*, 1991). In contrast to VCP which is secreted from infected cells, B5R of vaccinia strain WR is a 35-kDa membrane-associated glycoprotein

that is found in multiple forms in extracellular enveloped virions (EEV), but is absent from intracellular naked particles (INV). It is presently unknown whether BR5 can interfere with complement function during viral infection.

Envelope proteins

Several viral proteins located in the outer envelope have demonstrable effects on pathogenesis. Vaccinia virus produces a hemagglutinin which is detectable on the infected cell surface and allows adsorption of erythrocytes to virally infected tissue culture cells (Buller and Palumbo, 1991; Turner and Moyer, 1990). Although the relevance of this protein to viral propagation is unclear (Oie *et al.*, 1990; Brown *et al.*, 1991), absence of functional hemagglutinin reduces viral virulence as measured by intracranial lethality assays in mice (Flexner *et al.*, 1987) and reduces the size of skin lesions upon intradermal inoculation of rabbits (Shida *et al.*, 1988).

The 32-kDa envelope protein of vaccinia virus has been shown to be involved in attachment of virions to cells. It binds specifically to the surface of cultured cells but is non-essential for virus growth in cell culture (Lai *et al.*, 1990 and 1991). Deletion of this gene in vaccinia virus produced a strain in which gene expression as measured by luciferase assays was reduced by 50-60% in target tissues. Intracranial inoculation of mice with the 32-kDa deletion mutant resulted in an 80% reduction in mortality over the parental wild type strain, which suggests an important role for the 32-kDa protein in pathogenesis (Rodriguez *et al.*, 1992).

Biosynthetic enzymes

Poxviruses encode several non-essential enzymes involved in synthesis of precursors for DNA synthesis, such as a thymidine kinase (*tk*) and a ribonucleotide reductase (*rr*) (Buller and Palumbo, 1991). Although these enzymes are thought to maintain cytoplasmic pools of biosynthetic precursors at high levels, deletion of the *tk* and *rr* genes

have no detrimental effect on viral growth in cell culture, even in serum starved cells (Child *et al.*, 1990). In contrast, a substantial attenuation *in vivo* is observed when the *tk* gene is disrupted in vaccinia virus, and the LD50 of *tk* minus vaccinia is 40,000 times higher than that of wild type vaccinia virus (Buller *et al.*, 1985). Deletion of the *rr* gene also reduces vaccinia virus pathogenicity in the mouse LD50 assay, but to a lesser extent (Child *et al.*, 1990).

Host range genes

Whereas the host species tropism of most individual poxviruses is limited to a narrow range of species, several Orthopoxviruses, such as vaccinia and cowpox, can productively infect a broad range of mammalian hosts (Buller and Palumbo, 1991; Turner and Moyer, 1990). The natural host of vaccinia virus is unknown and cowpox has been isolated from rodents, cattle, cats, and humans. In contrast, the only natural host for the Orthopoxvirus ectromelia is the mouse. The basis for the host specificity of the poxviruses is thought to reside in viral genes, termed host range (hr) genes, that are cell type specific. Although the mechanism of action of these genes remains unknown, several distinct genes have been isolated that confer upon an individual virus the ability to productively infect certain cell types. These include the CHO hr gene from cowpox (Spehner *et al.*, 1988), and two vaccinia hr genes, C7L (Perkus *et al.*, 1990) and K1L (Gillard *et al.*, 1986). Homologues for cowpox hr genes have recently been detected in rabbitpox virus, the species in which host range mutants were originally described and analyzed (Turner and Moyer, 1990; Bloom *et al.*, 1991).

Chinese hamster ovary (CHO) cells are permissive for cowpox replication but do not support productive vaccinia virus infection (Spehner *et al.*, 1988). Transfer of the CHO hr gene from cowpox to vaccinia virus (Spehner *et al.*, 1988) or to ectromelia (Chen *et al.*, 1992) overcomes the block to viral replication in CHO cells. The mechanism by which this occurs is unclear but is thought to allow the continuation of viral protein synthesis

during the period of virus-induced shutoff of host protein synthesis. The K1L gene of vaccinia virus was found to relieve the host restriction of a vaccinia virus mutant to replication in several human cell lines, one rabbit, and one pig cell line (Drillien *et al.*, 1981; Gillard *et al.*, 1986; Perkus *et al.*, 1990). Interestingly, both the 77-kDa CHO hr protein and the predicted product encoded by K1L contain an ankyrin repeat unit (Lux *et al.*, 1990). Ankyrins are thought to mediate interactions between integral membrane proteins and the cytoskeleton. The C7L gene of vaccinia virus also allows replication on human and pig cells, but shows no homology with K1L or the ankyrin repeat (Perkus *et al.*, 1990). C7L, K1L, and CHO hr all provide functions that allow vaccinia virus replication in certain human cell lines. C7L and K1L are additionally capable of conferring host permissiveness for growth in pig kidney cells, whereas only K1L allows growth on rabbit kidney cells. Deletion of both K1L and C7L greatly reduced the ability of vaccinia virus to replicate in human cells, though replication was not completely abrogated (Perkus *et al.*, 1990). It is important to note that host range genes may function with varying effectiveness in different cell types within a single species. For example, the Copenhagen strain of vaccinia virus grows well in lung cells of the Chinese hamster, but poorly in ovary or peritoneal cells from the same species (Chen *et al.*, 1992). Further experiments are required to determine the extent to which host range functions alter tissue tropisms during natural infections. It is possible that, in addition to the species tropism conferred by host range genes, subtle alterations in tissue and cell tropism may be effected by these factors which could influence the pathogenic profile of viral infection.

Downregulation of the ability to replicate in human cells has provided another molecular approach to the attenuation of vaccinia virus. A dramatic example of the role of host range genes in pathogenesis is provided by the recent construction of profoundly attenuated strains of vaccinia virus (Tartaglia *et al.*, 1992; Lee *et al.*, 1992) which were specifically designed for potential use as vaccine vectors. By removing 18 ORFs involved in virulence, including host range functions, a vaccinia deletion mutant denoted NYVAC

was generated that retained immunogenicity while causing minimal pathogenic effects in infected mice. Upon intradermal inoculation of rabbits, no detectable induration or ulceration was observed and virus was rapidly cleared from the inoculation site. The ability to replicate in a number of human cell lines was greatly reduced. Despite poor replication in rabbit tissues, NYVAC was nonetheless capable of eliciting a strong immune response when used as a vector for the rabies glycoprotein (Tartaglia *et al.*, 1992).

Epidermal growth factor

Epidermal growth factor (EGF) and its receptor (EGF receptor) are key regulators of cellular proliferation and differentiation in a variety of cell types (Carpenter and Cohen, 1990; Peppelenbosch *et al.*, 1992; Plata-Salaman, 1991; Iwashita and Kobayashi, 1992; Carpenter and Wahl, 1991). The recent realization that a family of distinct EGF-like growth factors exists, together with continued progress in elucidating the mechanisms of signal transduction upon stimulation of the EGF receptor, have provided a valuable framework for improved understanding of growth factor biology.

Structure of EGF and related polypeptides

The list of EGF family proteins is growing and presently includes transforming growth factor alpha (TGFα) (Salomon *et al.*, 1990), the poxviral growth factors (Buller and Palumbo, 1991), amphiregulin (Plowman *et al.*, 1990; Kimura *et al.*, 1990), and a heparin-binding EGF-like growth factor (Naglich *et al.*, 1992; Higashiyama *et al.*, 1992). The basis of the structural relationship between EGF family proteins is that they all contain an "EGF unit", a sequence of residues that includes six characteristically spaced cysteines in the following general sequence: $CX_7CX_{4-5}CX_{10-13}CXCX_8CX$. These cysteine residues are linked in a defined configuration forming three disulfide bridges. Numerous structure-function studies using native and recombinant EGF and TGFα have shown that all six

cysteine residues within the EGF unit that form the three intramolecular disulfide bonds are essential for biological activity and that all three peptide loops defined by the disulfide bonds are required for EGF receptor binding.(Defeo-Jones *et al.*, 1988 and 1989; Derynck, 1992). Nuclear magnetic resonance, circular dichroism, and Fourier transform infrared spectroscopic studies of EGF and TGFα have demonstrated that the secondary and tertiary structures of EGF and TGFα are extremely similar (Montelione *et al.*,1992; Prestrelski *et al.*,1992; Mayo *et al.*,1989). Both proteins contain N and C terminal domains that consist of antiparallel β-sheets, with primarily irregular secondary stucture in the remaining regions. The extreme N and C terminal "tails" of mature EGF and TGFα are relatively unrestrained compared with the remaining portions of these molecules, and TGFα displays greater overall flexibility than EGF. High resolution crystal structures of EGF-like proteins are not yet available.

All EGF family members bind the EGF receptor with high affinity, induce tyrosine kinase autophosphorylation of the EGF receptor, and trigger mitogenesis in responsive cells (Derynck, 1992). All known members of this group of polypeptides, with the exception of two Leporipoxviral growth factors (see below), are proteolytically cleaved from the extracellular portion of larger membrane-bound glycosylated protein precursors (Derynck, 1992). Interestingly, the membrane-bound precursor forms of EGF and TGFα have recently been shown to possess the biological activity of the final secreted products, including induction of tyrosine autophosphorylation and mitogenesis in cells expressing the EGF receptor (Brachmann *et al.*,1989; Wong *et al.*,1989; Mroczkowski *et al.*,1989).

Many proteins have been identified which contain EGF-like domains within their sequences, but are not themselves mitogenic and therefore not considered EGF homologues. These include several blood coagulation factors, cell surface receptors such as the receptor for low density lipoproteins, extracellular matrix components, and invertebrate products involved in development. The functions of the EGF-like domains in these proteins remain unclear (Guterson and Laurence, 1990), but the fact that membrane-bound TGFα

and EGF precursor proteins are biologically active suggests a role for these domains in cell-cell or cell-matrix interactions that may involve the EGF receptor (Steele, 1989).

It is important to note that activity of EGF family members as EGF receptor ligands does not necessarily preclude interaction of these polypeptides with other, yet unidentified, receptors. A case in point is the *neu*/HER-2/*erbB-2* proto-oncogene product (Yarden, 1990). The HER-2 gene encodes a receptor-like transmembrane tyrosine kinase which is structurally closely related to the EGF receptor and was first identified as an amplified gene in a human mammary carcinoma. Recently, an EGF-like molecule denoted heregulin or Neu differentiation factor (NDF) has been identified as a specific ligand for HER-2 (Holmes *et al.*,1992; Lupu *et al.*,1992; Wen *et al.*,1992). Like EGF family proteins, heregulin/NDF retains the conserved cysteine-containing EGF structural unit, is synthesized as a glycosylated membrane-bound precursor which undergoes cleavage to release a smaller molecule into the medium, and induces tyrosine autophosphorylation upon binding to its cognate receptor, the HER-2 protein. EGF is not a ligand for the HER-2 protein, nor does heregulin/NDF bind the EGF receptor. However, given the structural similarity between heregulin and EGF, it remains possible that certain EGF-like molecules might be capable of cross-recognition of related but distinct receptors such as HER-2 and the EGF receptor.

The EGF receptor

The human EGF receptor is composed of a single glycosylated polypeptide chain of 1186 amino acid residues. It consists of an extracellular ligand binding domain, a single hydrophobic membrane anchor sequence, and a cytoplasmic domain which contains a ligand-regulated tyrosine kinase activity (Carpenter and Cohen, 1990; Hernandez-Sotomayor and Carpenter, 1992). Binding of EGF to the receptor results in transmission of a mitogenic signal via a cascade of poorly understood intracellular responses. These include receptor oligomerization, tyrosine kinase activation, receptor tyrosine autophosphorylation.

tyrosine phosphorylation and activation of cellular substrates, activation of second messenger systems, receptor internalization, eventual dephosphorylization of the tyrosine residues, degradation and recycling.

Activation of the tyrosine kinase activity of the EGF receptor is an early event that occurs upon EGF binding and is essential for all cellular responses to EGF (Iwashita and Kobayashi, 1992). Two models have been proposed for the mechanism of activation of the EGF receptor kinase. One model proposes that binding of EGF to the extracellular domain of the receptor induces a conformational change that is transmitted across the transmembrane segment to produce an activated conformation of the cytoplasmic kinase. An alternative model suggests that the kinase is activated through intersubunit interactions that occur upon receptor dimerization (Ullrich and Schlessinger, 1990). In this model, binding of EGF to the receptor shifts an equilibrium between inactive monomer receptors and active dimer receptors towards the dimers. Recent studies examining the kinetics of EGF receptor binding, activation, and dimerization support the latter model (Canals, 1992; Felder *et al.*, 1992; Kwatra *et al.*, 1992).

Several substrates for the EGF receptor tyrosine kinase have been identified and include both cellular proteins and tyrosine residues within the EGF receptor itself. Cellular substrates include phospholipase C-γ1, the GTPase activating protein of *ras* (GAP), phosphoinositide 3-kinase, the *vav* proto-oncogene product, two serine kinases, microtubule associated protein (MAP) kinase and the product of the *raf* proto-oncogene (Carpenter and Cohen, 1990; Margolis *et al.*, 1992; Bustelo *et al.*, 1992). Putative cellular substrates have been identified by immunoaffinity chromatography using anti-phosphotyrosine antibodies (Fazioli *et al.*, 1992). Five tyrosine residues located near the C terminus of the receptor are substrates for EGF-dependent autophosphorylation (Carpenter and Cohen, 1990; Vega *et al.*, 1992; Sorkin *et al.*, 1992). It is thought that C terminal autophosphorylation sites compete with cellular substrates for binding at the kinase active site, thereby inhibiting cellular tyrosine phosphorylation. This inhibition can be relieved by

autophosphorylation or deletion of the intrinsic substrate sites (Ullrich and Schlessinger, 1990). Simultaneous autophosphorylation of three of these tyrosines (Y1173, Y1148, Y1068) positively regulates biological and tyrosine kinase activity of the receptor, and allows rapid internalization and downregulation of the receptor. Simultaneous point mutation of these three tyrosines reduced receptor phosphorylation of a cellular substrate, phospholipase C-γ1, and reduced receptor internalization rate significantly (Sorkin *et al.*, 1992). Two additional tyrosine regulatory sites have been identified (Y1086 and Y992) and simultaneous mutation of all five tyrosines further decreased the internalization rate to a minimal level and reduced kinase activity toward phospholipase C-γ1 to 10% of wild type receptor activity (Sorkin *et al.*, 1992). Several cellular substrates of the EGF receptor kinase, including phospholipase C-γ, GAP, and the product of the protooncogene *vav*, contain a common sequence motif consisting of a stretch of approximately 100 amino acids called the *src* homology 2 domain (SH2). These SH2 domains bind specifically to protein phosphotyrosine sequences and are involved in complex formation with the EGF receptor. It has been recently demonstrated that the Y992 phosphorylation site represents a high affinity binding site for the SH2 domain of phospholipase C-γ (Rotin *et al.*, 1992). Both tyrosine phosphorylation of phospholipase C-γ and binding via its SH2 domain to the activated EGF receptor at Y992 are required for stimulation of phospholipase C-γ activity (Vega *et al.*, 1992). Furthermore, it appears that a tyrosine phosphatase and the SH2 domain of phospholipase C-γ compete for the same Y992 binding site of the EGF receptor (Rotin *et al.*, 1992). Dephosphorylation of activated EGF receptors by phosphatases constitutes a mechanism for downregulation of EGF tyrosine kinase activity (Faure *et al.*, 1992) and modulation by SH2 domains may be a physiologically important route for the control of dephosphorylation.

The cascade of events that occurs upon activation of the EGF receptor is complex and incompletely understood (Iwashita and Kobayashi, 1992). In addition, it must be remembered that the EGF receptor is nearly ubiquitously expressed over a spectrum of diverse cell types and may activate different second messenger systems depending on the cell type and milieu. However, several pathways that may be involved in EGF receptor signal transduction have been identified (Ullrich and Schlessinger, 1990; Iwashita and Kobayashi, 1992; Peppelenbosch *et al.*, 1992). Activation of phospholipase C-γ1 by the EGF receptor kinase results in increased hydrolysis of phosphoinositides to produce the second messengers 1,2 diacylglycerol and inositol 1,4,5-triphosphate, which cause release of Ca^{2+} from intracellular stores and activation of protein kinase C. Subsequent phosphorylation of cellular substrates by activated protein kinase C and other kinases in a poorly defined chain of events leads to generation of a mitogenic signal. Depending on the cell type, additional signal transduction pathways triggered by EGF receptor ligand binding include activation of *ras* via GAP proteins and activation of cAMP production.

The EGF-induced rise in intracellular Ca^{2+} concentrations is only partially derived from intracellular stores; the majority originates from Ca^{2+} influx through the plasma membrane. Phospholipase A_2 activity is stimulated by EGF in many cell types and arachidonic acid metabolites are known to act as second messengers in regulation of a variety of ion channels. Studies examining regulation of Ca^{2+} channels involved in EGF-mediated Ca^{2+} influx revealed that leukotriene C_4 produced by the phospholipase A_2-mediated release of arachidonic acid followed by conversion to leukotriene C_4 by 5-lipoxygenase was the effector responsible for activation of these Ca^{2+} channels. Thus, phospholipase A_2/5-lipoxygenase activity constitutes a novel pathway for growth factor signal transduction (Peppelenbosch *et al.*, 1992).

The product of the *vav* oncogene is the first example of a tyrosine kinase substrate that may act as a transcription factor (Margolis *et al.*, 1992; Bustelo *et al.*, 1992). $p95^{vav}$ is a

proto-oncogene product specifically expressed in hematopoietic cells. It contains sequence motifs characteristically found in transcription factors, including zinc finger, helix-loop-helix, leucine zipper motifs, and nuclear localization signals. It also contains an SH2 domain, which is common to many protein tyrosine kinase substrates. When NIH 3T3 cells ectopically expressing $p95^{vav}$ or embryonic human kidney cells transiently expressing $p95^{vav}$ are treated with EGF, the *vav* product becomes phosphorylated on tyrosine residues, and associates with the EGF receptor through its SH2 domain. Although its activity as a transcription factor has not been demonstrated, $p95^{vav}$ represents a novel class of products that may directly link events at the cell surface to transcriptional control.

Functions of EGF and related polypeptides

The effects of EGF and related polypeptides on various biological systems have been extensively reviewed (Fisher and Lakshmanan, 1990; Plata-Salaman, 1991; Guterson and Laurence, 1990; Salomon *et al.*,1990; Derynck, 1992; Carpenter and Wahl, 1991) Despite the wealth of literature on the subject, the roles of these growth factors in normal physiology remains unclear. EGF receptors have been identified in most cell types with the exception of hematopoietic cells. The predominant biological response to EGF in target cells is enhancement of cellular proliferation. A partial list of actions in the intact animal include stimulation of precocious eyelid opening and tooth eruption in newborn mice, stimulation of lung, gastrointestinal, liver, and pancreatic maturation, enhancement of wound healing and angiogenesis, inhibition of gastric acid secretion, and stimulation of pituitary hormone secretion. TGFα shares most of the same biological properties, but can be distinguished from EGF in several assays. For example, TGFα is much more potent than EGF in a neovascularization assay and in its ability to promote calcium release from bone (Derynck, 1992).

Inappropriate overexpression of TGFα, EGF, or the EGF receptor are associated with neoplasia, and the roles of EGF family proteins in tumorigenesis has been intensively

studied (Salomon *et al.*,1990; Maruo *et al.*,1992; Stromberg *et al.*,1992; Wong *et al.*,1992). Autocrine stimulation of malignant cells overexpressing the EGF receptor with EGF or TGFα has been invoked as a mechanism by which growth control could be abrogated. In addition, since EGF and TGFα are mitogens, these factors may act as classical tumor promoters by amplifying a population of premalignant cells.

Poxviral homologues of EGF

Vaccinia growth factor

Vaccinia growth factor (VGF) is encoded by an early gene (19K) of vaccinia virus located near the unique sequence /TIR border as one or two copies, depending on the strain of the virus (Venkatesan *et al.*,1982; Lai and Pogo, 1989). Its relationship to EGF and TGFα was discovered fortuitously when computer-assisted searches of protein sequences revealed that the predicted polypeptide product of 140 a.a. encoded by the 19K gene showed strong sequence homology with these growth factors (Brown *et al.*,1985; Reisner, 1985; Blomquist *et al.*,1984). The six cysteine residues critical for the characteristic folding pattern of this growth factor family are fully conserved in VGF and sequence homologies of 45% and 37% exists with human EGF and mouse TGFα, respectively, as calculated from residues 45 to 89 of VGF. VGF is secreted into the medium of cells infected with vaccinia virus, competes with EGF for binding to the EGF receptor, and induces EGF receptor tyrosine autophosphorylation (Stroobant *et al.*,1985; Twardzik *et al.*,1985; King *et al.*,1986). It is serologically distinct from EGF, showing minimal cross-reactivity with EGF and none with TGFα (Twardzik *et al.*,1985; Stroobant *et al.*,1985). The VGF primary sequence contains a hydrophobic signal sequence near the amino terminus and a transmembrane-like hydrophobic region near the carboxyl terminus. Amino acid analysis of the secreted VGF peptide, together with pulse-chase analysis of infected cells using an antipeptide antiserum, revealed that VGF is synthesized as a 140 amino acid glycosylated

transmembrane precursor, which is cleaved in two locations to release a 77 amino acid, 22-kDa polypeptide into the medium of infected cells (Chang *et al.*,1988; Stroobant *et al.*,1985). Like EGF and TGFα, VGF is mitogenic, can stimulate anchorage-independent growth of of normal rat kidney (NRK) cells in the presence of TGFβ, and enhances epithelial wound healing (Stroobant *et al.*,1985; Twardzik *et al.*,1985; King *et al.*,1986; Schultz *et al.*,1987).

Two hypotheses regarding the biological function of EGF homologues in poxviruses have been proposed. Several authors have suggested that secreted VGF may serve to stimulate the metabolism of neighboring uninfected cells, thereby increasing the capacity of these cells to support viral replication (Blomquist *et al.*,1984; Brown *et al.*,1985; Reisner, 1985). An alternative hypothesis, that VGF, if present on the surface of the virus particle, may exploit the cell-surface EGF receptor as one pathway for infection, has been put forth as a result of experiments designed to demonstrate that occupancy of the EGF receptor reduces the ability of vaccinia virus to infect cells (Marsh and Eppstein, 1987; Eppstein *et al.*,1985). Of the several vaccinia virus mutants described which lack the VGF gene (Lai and Pogo, 1989; Kotwal and Moss, 1988b), the most useful information regarding the biological activity of VGF has been obtained from analysis of vSC20, a vaccinia WR strain in which both copies of VGF have been specifically inactivated (Buller *et al.*,1988a). In order to determine whether VGF mediates binding to cells via the EGF receptor, infection initiation efficiencies of wild type vaccinia virus and vSC20 were compared by examining viral growth in several cell lines bearing different concentrations of surface EGF receptors. vSC20 and wild type vaccinia virus demonstrated similar particle to pfu ratios, and the relative infectivities of vSC20 and wild type vaccinia virus did not correlate with the EGF receptor densities on the infected cell lines. Furthermore, pretreatment of A431 cells with a monoclonal antibody to the EGF receptor reduced vSC20 and wild type plaque forming abilities to the same extent. These results, together with the

observation that VGF is not detectable on purified vaccinia virions (Buller *et al.*, 1988a), suggest that VGF does not play a prominent role in mediating virus entry into cells.

The effect of VGF on virus yields in cell culture was minimal (Buller *et al.*, 1988a; Buller and Palumbo, 1991). Plaque morphology was unchanged by deletion of VGF and infection of several cell lines with vSC20 and wild type vaccinia virus yielded similar amounts of progeny virus. Only Swiss 3T3 cells that had been rendered quiescent by serum starvation showed slightly reduced yields of vSC20 progeny compared with wild type virus. In contrast, deletion of VGF resulted in substantial attenuation of virulence in live hosts (Buller *et al.*, 1988a and b). Infection of inbred mice by the intracranial route to determine the lethal dose of virus for 50% of the population (LD_{50}) has been shown to be a sensitive method for monitoring the effects of mutations on vaccinia virus replication *in vivo* (Buller *et al.*, 1985). The LD_{50} of the VGF minus mutant was increased from that of wild type virus by a factor of 20,000 (Twardzik *et al.*, 1985), but histological examination of infected tissues revealed similar pathological profiles. Intradermal inoculation of the backs of outbred rabbits with serial dilutions of vSC20 and wild type virus showed that approximately 10 to 100 fold more vSC20 than wild type virus was required to produce a visible lesion, and that the lesions induced by vSC20 were less severe than those caused by wild type virus. Infection of the chorioallantoic membrane (CAM) of fertilized chicken eggs with vaccinia virus produces individual pocks which manifest discrete areas of cellular necrosis and hyperplasia (Goodpasture *et al.*, 1932). Infection of CAMs with vSC20 produced pocks that contained fewer proliferating cells, decreased amounts of infectious virus, less inflammatory infiltrate, and less virus antigen than those induced by wild type virus. Although it is difficult to differentiate between direct effects of VGF and induction of proliferative responses by increased cell damage and inflammation (Cotran *et al.*, 1989), the results of the experiments described above are consistent with the notion that VGF secreted by infected cells stimulates hyperplasia of neighboring cells, which results in additional metabolically active targets for further viral infection.

Two distinct growth factors have been identified in the Leporipoxviruses; the Shope fibroma growth factor gene (SFGF) is present in SFV and MRV, whereas myxoma virus contains the myxoma growth factor gene (MGF) (Upton *et al.*, 1987 and 1988; Chang *et al.*, 1987). These genes are present as single copies located near the TIR/unique border at the left end of the viral genomes. The SFGF gene was initially detected in SFV using degenerate oligonucleotide probes corresponding to a well-conserved stretch of seven amino acids common to human EGF, rat TGFα, and VGF (Chang *et al.*, 1987). The SFGF gene was then used as a hybridization probe to detect the corresponding growth factor sequence in myxoma virus (Upton *et al.*, 1987). Fine analysis of the recombination junctions in MRV revealed that MGF has been replaced with sequences identical to those of SFGF in SFV, and is located immediately 3' of the SFV/myxoma recombination junction at the left end of the genome (Upton *et al.*, 1988). The SFGF gene encodes an 80 amino acid polypeptide which shows 37% identity with VGF and TGFα as measured in the highly conserved region of amino acids 33 to 77 of SFGF. MGF is 85 residues long and 80% identical to SFGF. The spacing between the six cysteine residues critical for proper folding and biological activity of EGF and TGFα has been conserved in both SFGF and MGF. SFGF and MGF each contain a stretch of N-terminal hydrophobic residues resembling a signal sequence for transport to the endoplasmic reticulum. In contrast to EGF, VGF and TGFα precursors, neither SFGF nor MGF contain hydrophobic sequences near their C termini that could serve as membrane anchor sequences.

SFGF is transcribed as an early gene product from a promoter typical of other early poxvirus promoters (Chang *et al.*, 1990). The expression of SFGF has been examined in SFV-infected cells using an antiserum raised against a non-glycosylated synthetic peptide containing residues 26-80 of the predicted primary translation product of SFGF ($SFGF_{26-80}$). Pulse chase analysis of infected cell lysates indicated that the most abundant intracellular form of SFGF is a highly glycosylated 16-kDa species (Chang *et al.*, 1990).

This antiserum, which appeared to have been raised against a denatured form of $SFGF_{26-80}$, had a relatively poor affinity against native SFGF in immunoprecipitation assays and was unable to specifically recognize a product in conditioned medium from SFV infected cells. However, when SFGF was over-expressed in a VGF-minus vaccinia recombinant, conditioned medium from infected cells was able to compete the binding of [I^{125}]EGF for the EGF receptor, indicating that at least some biologically active SFGF is secreted (A. Opgenorth, M. O'Connor, G. McFadden, unpublished data). $SFGF_{26-80}$ and a non-glycosylated synthetic peptide spanning residues 30 to 83 of MGF have been shown to have specific EGF-like activity (Lin *et al.*, 1988 and 1991). These peptides bind to the EGF receptor on A431 cells and stimulate [^{3}H] incorporation into NRK cells. Furthermore, $SFGF_{26-80}$ was shown to induce colony formation of NRK cells in soft agar assays, and accelerate precocious incisor eruption and eyelid opening in newborn mice (Ye *et al.*, 1988). Taken together, these results suggest that viral SFGF and MGF are biologically active ligands and are *bona fide* members of the EGF family of growth factors.

THESIS OBJECTIVE

The purpose of the work of this thesis was to ascertain whether SFGF and MGF are virulence factors in MRV/myxoma virus infection and to investigate their roles in viral pathogenesis. In particular, we wished to determine whether SFGF/MGF contribute to the proliferative nature of the lesions induced by these viruses, and whether SFGF/MGF possess any unique features that make them biologically distinct from other EGF-like molecules in the context of myxoma virus infection. In the course of these investigations we discovered a novel gene product, M11L, which also contributes to viral virulence. We therefore examined the role of M11L in myxoma virus infection and pathogenesis.

TABLE I-1

Classification of Poxviruses of Vertebrates

Family: Poxviridae; Subfamily: Chordopoxviridae

Genus	Species
Orthopoxvirus	Vaccinia, variola, ectromelia, cowpox, camelpox, monkeypox, buffalopox, rabbitpox, raccoon pox, tatera pox, vole pox viruses
Suipoxvirus	Swinepox virus
Leporipoxvirus	Shope fibroma, myxoma, malignant rabbit fibroma, squirrel fibroma, hare fibroma viruses
Avipoxvirus	Fowlpox, canary pox, junco pox, pigeon pox, quail pox, turkey pox, starling pox, sparrow pox viruses
Capripoxvirus	Goatpox, sheeppox, lumpy skin disease viruses
Parapoxvirus	Orf, pseudocowpox, chamois contagious ecthyma, stomatitis papulosa viruses
Molluscipoxvirus	Molluscum contagiosum virus
Yatapoxvirus	Tanapox, Yaba tumor viruses

Table I-2

Individual Orthopoxviral Gene Products Shown to Contribute to Virulence by Targeted Deletion Analysis

Name	Synonyms	Virus	Test Animal	Function	Reference
SPI-2	*crmA*, 38-kDa protein	Cowpox	Chick embryo	38-kDa serpin; inhibitor of Interleukin 1-β processing	Fredrickson *et al.* 1992
N1L	13.8 k-Da secreted protein	Vaccinia	Mouse	Unknown	Kotwal *et al.* 1989
VCP	VCBP, C21L C4b binding protein	Vaccinia	Mouse	Interferes with complement activity	Isaacs *et al.* 1992
32-kDa protein	32K gene	Vaccinia	Mouse	Viral attachment to cell membrane	Rodriguez *et al.* 1992
Thymidine kinase	TK	Vaccinia	Mouse	Ribonucleotide biosynthesis	Buller *et al.* 1985
Ribonucleotide reductase	rr	Vaccinia	Mouse	Ribonucleotide biosynthesis	Child *et al.* 1990
SalF7L		Vaccinia	Mouse	Steroid dehydrogenase	Moore *et al.* 1992
Hemagglutinin	HA	Vaccinia	Mouse	May promote cell to cell spread	Flexner *et al* 1987 Shida *et al* 1988
VGF	VVGF	Vaccinia	Mouse, Chick embyro Rabbit	Growth factor	Buller *et al.* 1988

Table I-3

Individual Leporipoxviral Gene Products Shown to Contribute to Virulence by Targeted Deletion Analysis

Name	Synonyms	Virus	Test Animal	Function	Reference
SERP-1		Myxoma MRV	Rabbit	Secreted and glycosylated serpin	Upton *et al.* 1990a
T2	M-T2	Myxoma MRV	Rabbit	Soluble receptor for TNFα	Upton *et al* 1991a
SFGF		MRV	Rabbit	Growth factor	This thesis
MGF		Myxoma	Rabbit	Growth factor	This thesis
M11L		Myxoma	Rabbit	Downregulates inflammmatory response	This thesis

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CHAPTER II- DELETION OF THE GROWTH FACTOR GENE RELATED TO EGF AND TGFα REDUCES VIRULENCE OF MRV [1]

INTRODUCTION

The poxviruses are a diverse family whose members infect a wide range of species and produce remarkably different pathologies (Fenner *et al.*,1989; Fenner, 1990b; Moss, 1990). Despite heterogeneity in host range and diversity of the diseases caused by members of the various poxvirus genera, DNA sequence analysis has revealed that many of the gene products encoded by any particular poxvirus have homologous counterparts in other poxvirus species. Conserved genes are not only restricted to those essential for viability, but include dispensable sequences that may play a role in determining host range, pathogenicity, and degree of virulence (Traktman, 1990; Goebel *et al.*,1990; Turner and Moyer, 1990; Buller and Palumbo, 1991). Many poxviruses are known to encode a group of growth factors that bear significant amino acid homology to the epidermal growth factor (EGF) family of growth factors (Brown *et al.*,1985; Twardzik *et al.*,1985; Porter and Archard, 1987; Chang *et al.*,1987; Upton *et al.*,1988; Porter *et al.*,1988). Poxviral members of this family include vaccinia growth factor (VGF), myxoma growth factor (MGF), Shope fibroma growth factor (SFGF), and a related gene in molluscum contagiosum virus. Both viral and cellular members of the EGF family, including transforming growth factor α (TGFα) and VGF, have been shown to compete with EGF for binding to the EGF receptor, induce autophosphorylation of this receptor, and are mitogenic for responsive cell types that bear the receptor (Stroobant *et al.*,1985; King *et al.*,1986; Twardzik *et al.*,1985).

[1] A version of this chapter has been published. A. Opgenorth, C. Upton, D. Strayer, and G. McFadden 1992. Virology 186: 175-191.

Although it remains to be shown that the native SFGF and MGF gene products are secreted and bind to the EGF receptor, synthetic peptides comprising the carboxyl portions of the MGF and SFGF gene products have been shown to interact with the EGF receptor and have *bona fide* EGF-like biological activities (Ye *et al.*, 1988; Lin *et al.*, 1988 and 1991). The studies of Buller and colleagues have indicated that VGF is an important pathogenic marker for vaccinia virus growth in mice and rabbits (Buller *et al.*, 1988a and b; Buller and Palumbo, 1991). However, since the true animal host for vaccinia virus is unknown and the disease profiles caused by different poxviruses vary substantially, it is of interest to determine the role(s) of the viral growth factors in members of other genera which are associated with defined disease syndromes involving cellular hyperplasia.

Some of the poxviruses are referred to as "tumorigenic" because they cause extensive cellular proliferation at or near sites of viral replication (McFadden, 1988; Fenner, 1990a and b), and the presence of EGF-like genes in these viruses suggests a possible role for these growth factors in the associated cellular hyperplasia. Malignant rabbit fibroma virus (MRV) is a *Leporipoxvirus* that induces a uniformly lethal syndrome of disseminated fibromyxosarcoma accompanied by profound immunosuppression (Strayer *et al.*, 1983a and b; Strayer and Sell, 1983; Strayer, 1988). MRV is a natural recombinant between Shope fibroma virus (SFV) and myxoma virus in which approximately 8 kb of myxoma sequences, including the MGF gene, are deleted and replaced by related SFV sequences, including the intact SFGF gene (Block *et al.*, 1985; Upton *et al.*, 1988). The expression of SFGF has been examined in SFV (Chang *et al.*, 1990) and the product was shown to be a 16 kilodalton glycosylated protein synthesized early in the infectious cycle. MRV-expressed SFGF is identical to that of SFV, but MRV has the experimental advantage that the role(s) of this peptide growth factor can be evaluated at both primary and secondary sites of viral infection (McFadden, 1988). Whereas SFV produces a localized and self-limited proliferative lesion, MRV rapidly disseminates via infected lymphocytes through the reticuloendothelial system and causes additional lesions throughout the internal

tissues of the rabbit. In addition, the dermis and subcutis undergo viral infections at secondary sites and fibroblastic tumors become especially prominent on the ears and face (Strayer *et al.*, 1983a; Strayer and Sell, 1983). In order to directly determine the role of SFGF in MRV tumorigenesis and pathogenicity, we constructed a MRV growth factor deletion mutant and examined its properties *in vivo* and *in vitro*.

MATERIALS AND METHODS

Cells and Virus

Rabbit SIRC cells were obtained from the American Type Culture Collection (ATCC) and maintained in Dulbecco modified Eagle's medium (DME) supplemented with 10% fetal bovine serum (FBS). RK-13 cells (provided by S. Sell) and BGMK cells (provided by S. Dales) were grown in DME supplemented with 10% newborn calf serum (NCS). Isolation and biological characteristics of MRV have been described elsewhere (Strayer *et al.*, 1983a).

Enzymes and Chemicals

Restriction enzymes were supplied by Bethesda Research Laboratories, Boehringer Mannheim, and New England Biolabs. T4 DNA polymerase, T4 DNA ligase, calf intestinal phosphatase (CIP), exonucleases III and VII, and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) were purchased from Boehringer Mannheim. Bgl II linkers were obtained from the Regional DNA Synthesis Laboratory, University of Calgary.

Plasmid Constructs

A 3.5 kb Sma I fragment containing the SFGF gene was isolated from MRV subclone A^A, a 9.5 kb Bgl II-Bam HI fragment (derived from what is now defined as the left end of the viral genome) flanking the terminal inverted repeat with the left unique internal sequences (Block *et al.*, 1985), and ligated into the Sma I site of pUC 13. The resulting plasmid, pMS-2a, was unstable in standard recA bacterial strains (e.g. HB101), but could be propagated in *E. coli* DB1256 containing *recA*, *recBC*, and *sbcB* loci (DeLange *et al.*, 1986). SFGF coding sequences were removed by digestion of pMS-2a with Cla I, whose single recognition site maps near the center of the SFGF open reading frame (Chang *et al.*, 1987), followed by bidirectional deletion with exonucleases III and VII. The DNA was blunt-ended, Bgl II linkers were added, the deletion construct was circularized with T4 DNA ligase, and used to transform DB1256. A plasmid (pMCD-3) was isolated in which approximately 50 bp from the 5' side and 80 bp from the 3' side of the Cla I site had been removed and the deletion site tagged with a novel Bgl II site. A 3.15 kb fragment containing the *E. coli* lac Z gene driven by the vaccinia p11 promoter (β-gal cassette) was isolated from pSC20 (Buller *et al.*, 1988a), and ligated into Bgl II - cleaved pMCD-3. The DNA was used to transform DB1256 and a plasmid (pMPL-16) was isolated in which the β-galactosidase cassette had been inserted into the disrupted coding region of the SFGF gene (see Fig. II-2). G. McFadden performed some of these procedures.

Construction of MRV Growth Factor Deletion Mutant (MRV-GF$^-$)

MRV-GF$^-$ was constructed by modified standard procedures used to generate recombinant poxviruses (Chakrabarti *et al.*, 1985). In these procedures, cells infected with wild type virus are transfected with a cloned DNA fragment containing a disrupted copy of the gene to be targeted for deletion in the virus. A double recombination event between the transfected DNA and the viral DNA leads to mixed virus progeny consisting of wild type virus as well as recombinant virus containing the disrupted gene. Plating of the progeny

virus at low dilutions followed by screening or selection procedures allows identification of putative recombinants. A 35 mm dish of 70 % confluent BGMK cells was infected with MRV at a multiplicity of infection (m.o.i.) of 0.05 at t=0 hrs; after 2 hrs, a calcium phosphate/DNA precipitate containing 5 µg carrier MRV DNA and 500 ng Hind III-linearized pMPL-16 was added. At t=6 hrs, the medium was replenished and at t=48 hrs, the virus was harvested and replated on BGMK cells at low m.o.i. After 4 days, recombinant viruses (blue plaques) were identified by overlaying the monolayers with 1% low melting point (LMP) agarose in DME plus 5% NCS containing 500 µg/ml X-gal. After 3 cycles of plaque purification under 1% LMP agarose, stocks were grown to high titer in RK-13 cells. The genomic structures of candidate recombinant viruses were confirmed by Southern blot analysis (not shown) as described previously (Wills *et al.*, 1983), and a recombinant virus containing a partially deleted growth factor gene interrupted with the β-gal cassette (MRV-GF$^-$) was identified and the stock was expanded in RK-13 cells.

Virus Replication Analysis in Cell Culture

RK-13 or SIRC cells were infected in 35 mm dishes in triplicate at a m.o.i. of 5 for single step growth analysis, or 0.002 to generate low multiplicity growth curves, for 1 hr at 37 ^{o} C in 0.3 ml DME. The inoculum was removed and replaced with 2 ml per well of DME containing the appropriate amount of serum, and the cultures were harvested at various times post infection (p.i.). When viral growth was studied in growth arrested cells, they were rendered quiescent by reducing serum content to 0% (RK-13) or 0.2% (SIRC) for 4 days prior to infection. Virus titers were determined by plaque assay on RK-13 cells in duplicate.

Rabbit Spleen Cell Cultures

Spleen cell suspensions from normal and virus-infected rabbits were prepared as described previously (Strayer and Leibowitz, 1987). Briefly, cells were suspended by

passage through a steel mesh and cultured in 96-well flat-bottomed microtiter plates (Costar) at a concentration of 10^5 per well in 200 µl of RPMI 1640 (GIBCO) supplemented with 10 % FCS, L-glutamine (2mM), 50 µM 2-mercaptoethanol, and antibiotics. Where present, concanavalin A (Con A) and virus were added to the cultures at the time of culture initiation. To measure the extent of lymphocyte proliferation in response to Con A activation, twenty-four hour incorporation of ^{3}H-thymidine was measured at time of maximal proliferative responsiveness to Con A, which was 3 days after culture initiation. The data represent averages of experiments performed in triplicate.

Infection of Rabbits with MRV-GF$^-$

Adult female New Zealand White rabbits (2.5-3 kg) were purchased from local suppliers. They were housed and maintained according to standard procedures and sacrificed with euthanyl administered intravenously after anaesthesia. Rabbits were inoculated intradermally (i.d.) in the thigh with 10^2, 10^3, or 10^4 plaque forming units (pfu) in 1 ml normal saline and monitored daily for external signs of the developing MRV syndrome, including appearance of primary and secondary tumors, febrility, and secondary bacterial infections in the respiratory tract and conjunctiva. The dose of the inoculating virus within this range had no effect on the outcome of the disease. Tissues from infected rabbits were isolated after necropsy on days 7 and 11, and were fixed in neutral buffered 10% formalin, paraffin embedded, and sectioned at 5 µm. Sections were stained with hematoxylin and eosin and viewed by light microscopy. D. Strayer interpreted the histological sections.

RESULTS

Construction of MRV with deleted growth factor (MRV-GF$^-$)

Because MRV is a recombinant virus produced by an accidental cocultivation of SFV and myxoma (Strayer *et al.*, 1983a), some of the viral genes near the termini are from SFV, some are from myxoma, and three are in-frame fusions which created hybrid open reading frames (Block *et al.*, 1985; Upton *et al.*, 1988). Figure II-1 shows the arrangement of viral ORFs, including SFGF, in the left terminal area of MRV which resulted from the replacement of 8 kb of myxoma sequences with homologous DNA from SFV (Upton *et al.*, 1988). A 5 kb subset of these sequences, including one fusion protein (MT5) and three SFV ORFs (T6, T7, and T8) is also located in the right TIR of MRV (Upton *et al* 1988).We constructed a recombinant MRV virus, designated MRV-GF$^-$, in which a substantial portion of the SFGF coding sequences were deleted and replaced with the β-gal cassette under the control of the vaccinia p11 late promoter (Bertholet *et al.*, 1985). This promoter, as well as the vaccinia p7.5 early/late promoter, was previously shown to function correctly in the *Leporipoxviruses* (Venkatesan *et al.*, 1981; Macaulay and McFadden, 1989). In order to confirm the genomic structure of MRV-GF$^-$, Southern blots with wild type and recombinant MRV-GF$^-$ DNA were performed using the β-gal cassette and the SFGF-containing 1.1 kb Hinc II fragment from pMS2a as probes (see Fig. II-2). Digestion with diagnostic restriction enzymes such as Bgl I, Bgl II, Hinc II, and Sma I yielded the expected products (not shown). These results indicate that the MRV-GF$^-$ genome does not contain an intact SFGF gene and that the loss of this gene does not affect the viability of the virus in cell culture.

Effects of SFGF deletion on MRV virulence

In order to assess the role of SFGF in the pathogenesis of MRV-induced disease, we infected rabbits with MRV (15 animals) or MRV-GF$^-$ (18 animals) and followed them

clinically. Our observations of the disease course caused by each virus are summarized in Table II-1. As expected, the control animals that received the parental MRV invariably developed large, protuberant tumors at the site of inoculation, disseminated secondary tumors, and contracted severe purulent conjunctivitis and rhinitis during the terminal stages of the disease. All wild type MRV recipients were moribund by 11 days after infection. In contrast, 75% of rabbits infected with MRV-GF$^-$ developed symptoms of reduced severity and, after a crisis stage during which the remaining 25% became moribund, were able to clear the viral infection completely and survived. Although MRV-GF$^-$ recipients initially developed symptoms resembling those of wild type infections, most of the animals never became severely ill. Tumor regression began at about 17 days p.i. and the rabbits were completely recovered by about day 30. Upon challenge with wild type MRV, these animals occasionally developed a small primary tumor which regressed (not shown), but otherwise remained healthy.

The overall syndromes induced by MRV and MRV-GF$^-$ progressed from primary to secondary sites in a qualitatively similar fashion. At the gross level, there were no obvious differences in the size or general appearance of the primary lesions induced by MRV and MRV-GF$^-$, although secondary dermal tumors were more nodular in MRV-GF$^-$ recipients. The most striking difference between the two types of infection was in the extent and severity of the secondary Gram negative bacterial infections. MRV-GF$^-$ recipients rarely (25% of cases) developed symptoms such as breathing difficulty and conjunctivitis that were comparable in severity to those in MRV recipients. The milder forms of conjunctivitis and rhinitis that resulted from MRV-GF$^-$ infection appeared to allow the majority of animals to survive past the stage at which MRV recipients uniformly succumb. We conclude from this experiment that deletion of SFGF from MRV substantially attenuates the course of the viral infection and allows the animals to mount an effective immune response, but that the ability of the recombinant virus to migrate to secondary sites is largely unaffected by the mutation.

Comparative histological analysis of tissues from MRV and MRV-GF$^-$ recipients

The histological characteristics of wild type MRV infection have been presented previously (Strayer and Sell, 1983). We examined tissue sections of primary and secondary tumors, spleen, liver, kidney, heart, large and small intestine, lung, conjunctiva, and nasal mucosa from MRV and MRV-GF$^-$ recipients. A summary of the major histological differences between MRV and MRV-GF$^-$ infection is presented in Table II-2.

MRV Recipients, Day 7

In these animals, the primary tumors were highly myxoid, with abundant connective tissue matrix proteins and mucopolysaccharides surrounding scattered and clustered atypical tumor cells (see Fig.II-3 A). The inflammatory response to the tumor was minimal, both peripherally and within the tumor mass. Skin overlying the tumor was not obviously affected. In the spleen, a great expansion of the red pulp was observed and lymphoid areas of the spleen were reduced (Fig.II-3 B). Considerable edema was seen within the alveolar spaces of the lung, though both bronchial and pulmonary epithelia were normal. Other organs examined were found to be within normal limits.

MRV-GF$^-$ Recipients, Day 7

The primary tumors in these animals resembled those of wild type MRV recipients except that they contained fewer mitotically active cells and instead possessed a considerable infiltrate of heterophile leukocytes (Fig. II-3 C). This infiltrate was accompanied by substantial necrosis of individual tumor cells. The extracellular matrix and overlying skin were identical to those of wild type MRV tumors. The spleen shows perivascular and interfollicular lymphoid proliferation (Fig.II-3 D). Tissues from normal uninfected rabbits are shown in Fig.II-3 E-G.

MRV recipients, Day 11

Cells comprising the tumors 11 days p.i. were more atypical, and showed greater nuclear and cytoplasmic pleiomorphism (see Fig. II-4. C). Nuclear membrane and nucleoli were more prominent in these cells than in the cells of tumors 7 days p.i.. Extensive extracellular matrix material was observed surrounding skin appendage structures, especially hair follicles, accompanied by a mild to moderate lymphocytic infiltrate. The epithelium of these appendages showed acantholytic changes and architectural disruption. Lymphocytes were not, however, seen within the tumor or along its periphery. An inflammatory infiltrate within the tumor was present, and consisted principally of heterophiles. By this time, the spleen showed considerable reactive changes, especially an increase in the white pulp: increased germinal center activity as well as perifollicular and perivascular lymphoid hyperplasia (Fig.II-4 A). The former indicates increased B lymphocytic activity and the latter T lymphocytic activity. Pulmonary edema had greatly increased by 11 days p.i., compared with 7 days p.i.. Extensive proliferation of conjunctival epithelium was observed,.taking the form of squamous metaplasia and hyperplasia simultaneously (Fig.II-4 B). It was accompanied by acute inflammation in the superficial conjunctival epithelium. Underlying secondary tumor in the conjunctival area resembled the primary tumor (Fig.II-4 B).

MRV-GF$^-$ Recipients, Day 11

Primary tumors in animals receiving MRV-GF$^-$ 11 days previously resembled those of wild type MRV recipients except that the acute inflammatory infiltrate within the tumor was much more pronounced, with heterophiles surrounding individual tumor cells and considerable necrosis of individual tumor cells (Fig.II-5 A). The lymphoid infiltrate that was seen in and around the cutaneous appendages in day 11 MRV recipients was absent in MRV-GF$^-$ recipients. Lymphoid hyperplasia detected in spleens from these animals was

much more prominent than that observed in recipients of wild type MRV and consisted of both B and T lymphocyte zone proliferative activity (Fig.II-5 B). Conjunctival epithelium showed less proliferation (3-4 cell layers thick) than in wild type MRV recipients (8-10 cell layers thick) (Fig.II-5 C). Inflammatory reaction within secondary tumors (heterophiles mostly) was much more pronounced and was attended by greater individual tumor cell necrosis (Fig.II-5 C). Lungs of these animals resembled those of wild type MRV recipients. The other internal organs examined were not affected in any obvious fashion that was distinguishable from the parent virus infection.

Growth of MRV-GF$^-$ *in vitro*

We were unable to detect any differences between MRV and MRV-GF$^-$ in terms of plaque morphology (not shown) or growth properties in RK-13, SIRC, or BGMK cells. Rendering the cells quiescent by serum depletion likewise had no effect, at least at the m.o.i.'s used. High multiplicity (m.o.i.=5) single step productive infections of RK-13 cells yielded comparable growth curves for MRV and MRV-GF$^-$ (not shown). In order to examine propagation of virus through cultured cells at low multiplicity, we infected BGMK (not shown), SIRC, or RK-13 cells at m.o.i. of 0.002, harvested the cells at various times after infection, then titered virus progeny on RK-13 cells. As shown in Fig. II-6, deletion of the SFGF gene did not affect virus yields, whether or not the cells were actively growing at the time of infection.

The ability to grow in resting and mitogenically stimulated lymphocytes is crucial to the pathogenesis of MRV because it provides a means for compromising immune function as well as facilitating dissemination to secondary sites via the lymphatic channels in the host (Strayer *et al.*, 1985 and 1987). The very closely related SFV produces tumors that resemble those induced by MRV at early stages of infection but generalized systemic infection with SFV does not occur, largely due to its inability to replicate in lymphocytes (Strayer *et al.*, 1990; Heard *et al.*, 1990). Therefore, any impediment to growth of MRV in

lymphocytes would likely result in attenuation. To address this question we examined growth of MRV-GF$^-$ in spleen cell cultures from uninfected rabbits in the presence or absence of Con A stimulation at m.o.i.'s of 0.001 (see Fig. II-7), 0.01 (not shown), and 0.1 (not shown). We observed no major differences in MRV and MRV-GF$^-$ growth in this assay, indicating that the growth factor deletion did not result in impaired ability of the virus to propagate in a mixed lymphocyte population *in vitro*.

Inhibition of lymphocyte proliferation *in vitro*

One aspect of the immunosuppression caused by MRV involves direct inhibition of normal lymphocyte activation in response to antigens or mitogens (Strayer *et al.*, 1983b and 1985). To investigate whether deleting the growth factor gene altered the capacity of MRV to inhibit proliferation of lymphocytes cultured *in vitro*, we measured 24 hour incorporation of ^{3}H-thymidine into splenic lymphocytes in response to Con A at three days after infection with MRV or MRV-GF$^-$ *in vitro* (Table II-3). These data demonstrate a small but consistent reduction in the ability of MRV-GF$^-$ to prevent Con A-induced proliferation. This suggests that at the lower multiplicities of infection, mitogenic stimulation of infected lymphocytes is less strongly inhibited in MRV-GF$^-$ than in MRV infection, and hence this aspect of the immune response might be slightly less dampened by the mutant. The slight decrease in suppressive ability seen in this assay is not simply a function of slower growth in spleen cell cultures since the data in Fig. II-7 indicate that MRV and MRV-GF$^-$ grow at similar rates in these cultures.

Recovery of virus from spleens of infected animals

It has been shown previously (Strayer and Leibowitz, 1987) that MRV can be recovered from lymphocytes at 7 days p.i. but is undetectable by 11 days p.i.. Clearance of virus from the circulation coincides with recovery of Con A responsiveness by splenic lymphocytes (Strayer and Leibowitz, 1986 and 1987). Therefore, one possible explanation

for the observed attenuation of MRV-GF$^-$ is that more efficient elimination of virus by the animal could facilitate recovery from infection. To determine whether MRV-GF$^-$ is present in lower amounts at secondary sites or is cleared more rapidly than MRV, we assayed infectious virus in spleens from rabbits that had been infected 7, 9, or 11 days prior to sacrifice. Spleen cells removed from infected animals were cultured for various time periods with or without Con A, then virus titers were determined by plaque assay on RK-13 cells. As shown in Fig. II-8, comparable titers for the two viruses were recovered at day 7 from animals either in the absence (panel a) or presence (panel b) of mitogenic stimulation. No infectious virus was detectable at day 9 or day 11 from spleens of either MRV or MRV-GF$^-$ recipients (not shown). These results suggest that comparable amounts of virus are found in spleens of rabbits infected with either MRV or MRV-GF$^-$ during the latter course of the infection, and that more efficient elimination of MRV-GF$^-$ by the animal is not a likely cause of attenuation.

Immunosuppression by MRV and MRV-GF$^-$ *in vivo*

The profound immunosuppression that accompanies tumor development in MRV infection is complex and appears to involve direct compromise of host lymphocyte functions by virus (Strayer, 1988). As measured by the ability of splenic lymphocytes from infected animals to proliferate in response to mitogens, the responsiveness of these cells gradually declines until it is virtually abrogated by 7 days p.i., although it shows substantial recovery by 11 days p.i. In order to determine whether MRV-GF$^-$ debilitates this particular parameter to the same extent as MRV, we cultured spleen cells from animals that had been infected with MRV or MRV-GF$^-$ 7 or 11 days previously, and monitored Con A-induced proliferation as a function of ^{3}H-thymidine incorporation. The data in Table II-4 indicate that MRV-GF$^-$ does indeed suppress Con A-induced lymphocyte proliferation in a fashion indistinguishable from that of the parent MRV. At first, these results seem counter-intuitive in the light of the *in vitro* proliferation data presented in Table II-3.

However, the expe resented in Table II-3 examined solely *in vitro* depression of Con A responsiveness after direct addition of virus to spleen cell cultures. The experiments described in Table II-4 were performed after 7 days of viral incubation in the rabbit host during which the virus first multiplies in the skin at the initial site of inoculation, followed by dissemination via the lymphatics and multiplication in lymphocytes. The modest effect seen *in vitro* in Table II-3 appears to be biologically insignificant to the overall course of the viral disease when the complexities of virus multiplication and spread, and host responses are considered, since the MRV-GF$^-$ virus is indeed able to migrate to the spleen and downregulate lymphocyte function to the same extent as the parental virus. Thus, the marked difference in outcomes of the viral diseases cannot be readily explained in terms of extent of immune compromise at the level of total lymphocyte activation. We suggest that the attenuation observed by deletion of SFGF in MRV is probably due to differences in the effects of MRV and MRV-GF$^-$ on conjunctival and nasal epithelia. The squamous metaplasia and hyperplasia in target epithelia of MRV recipients is thought to impair clearance ability and renders them more susceptible to bacterial invasion (Strayer *et al.*, 1983; Strayer and Sell, 1983). Since MRV-GF$^-$ has much less deleterious effects on the overall architecture of these tissues, it is likely that the ability of the host to mechanically clear the bacterial infections is less compromised and results in an attenuated disease syndrome.

DISCUSSION

Virally encoded growth factors analogous to EGF may very well occur in all poxviruses (Turner and Moyer, 1990). VGF, the prototype of this family, is expressed as an early gene, processed by proteolytic cleavages, glycosylated, and secreted from vaccinia virus-infected cells, after which the mature 22-26 kd ligand can bind to EGF receptors on secondary target cells, activating tyrosine phosphorylation in a fashion similar to that described for EGF and TGFα (Twardzik *et al.*, 1985; King *et al.*, 1986; Stroobant *et al.*, 1985; Chang *et al.*, 1988; Lin *et al.*, 1990). It has therefore been suggested that viral EGF-like growth factors may function to metabolically stimulate cells surrounding the focus of infection, enhancing their ability to sustain high levels of viral replication (Twardzik *et al.*, 1985; Stroobant *et al.*, 1985; Buller *et al.*, 1988a; Buller and Palumbo, 1991). In the case of a poxvirus capable of generalized systemic infection, achieving greater virus titers at the initial site of replication could facilitate dissemination throughout the host as well as potentially affect the extent of viral replication at secondary sites. The speculation that poxvirus growth factors can activate neighboring uninfected cells has been substantiated by analysis of VGF-minus vaccinia virus. These studies indicate that VGF largely mediates the localized cellular proliferation along the advancing edge of lesions induced by vaccinia virus in both chick chorioallantoic membrane and rabbit skin (Buller *et al.*, 1988a and b).

SFGF was the second poxviral growth factor gene to be identified and shown to possess the three pairs of conserved cysteine residues common to all members of the EGF/TGFα superfamily (Chang *et al.*, 1987). An identical SFGF gene was later shown to exist in the genome of MRV, a natural recombinant between SFV and myxoma that induces fibroblastic tumors similar to those of SFV but which spreads to secondary sites of infection in a fashion highly similar to that found in myxomatosis (Upton *et al.*, 1988).

Thus, MRV provides a useful model to study the biological role of SFGF in a broader spectrum of target tissues than is normally encountered in an SFV infection.

Studies using anti-SFGF antibody indicated that SFGF is an early viral gene product that is post-translationally glycosylated to give a final 16 kd form as the most abundant form within the infected cell (Chang *et al.*, 1990). Unlike other members of the EGF family, SFGF does not contain an obvious C-terminal putative membrane spanning hydrophobic domain (Chang *et al.*, 1987), and it is as yet unclear whether SFGF is secreted in large amounts from SFV or MRV-infected cells. However, when SFGF is overexpressed in a VGF-minus vaccinia recombinant, conditioned medium from infected cells can efficiently compete for binding of I^{125}-EGF for the EGF receptor, indicating that at least some active SFGF can indeed be detected extracellularly (A.Opgenorth, M.O'Connor, and G.McFadden, unpublished). Tam and colleagues (Lin *et al.*, 1988; Ye *et al.*, 1988) have shown that a non-glycosylated synthetic peptide comprising the carboxyl terminal 55 residues of SFGF has specific EGF-like activity. This peptide stimulates ^3H-thymidine incorporation into DNA of NRK cells, binds to the EGF receptor on A431 cells, induces colony formation in soft-agar assays in the presence of TGF-β, and accelerates precocious incisor eruption and eyelid opening in newborn mice. Similarly, synthetic myxoma growth factor (residues 30-83) also binds to the EGF receptor and stimulates mitogenesis (Lin *et al.*, 1991). The question arises: do these different viral EGF analogues perform qualitatively similar functions *in vivo* or do they have different roles? At least in the case of VGF, there is evidence that the glycosylation state of the protein can dramatically alter the mitogenicity towards some target cells (Lin *et al.*, 1990), suggesting that the post-translational processing of these factors is important for proper function. In addition, differential effects of EGF, TGF-α, and VGF in the positive regulation of IFN-γ production in the C57BL/6 mouse system have been reported (Abdullah *et al.*, 1989).

The tumors and disease course caused by the SFV, myxoma virus, and MRV are substantially different from the localized cytopathology caused by vaccinia virus

(McFadden, 1988). Furthermore, since the host and disease profile of vaccinia virus in the wild are unknown, the *Leporipoxviruses* provide a good model system to study systemic poxviral pathogenesis in a natural host. SFGF is expressed identically in both SFV and MRV, but since MRV induces a more complex and virulent disease involving disseminated tumor and impaired immune function, we sought to determine the function of SFGF in MRV infection by construction of MRV-GF$^-$.

As in the case of VGF-minus vaccinia virus (Buller *et al.*, 1988a), the most obvious effects of deleting the growth factor in MRV were observed *in vivo*, not *in vitro*. MRV-GF$^-$ was found to be markedly attenuated in infected rabbits since 75% of recipients survived the viral disease and completely recovered from the infection whereas wild type MRV infection is 100% lethal. The clinical course of MRV-GF$^-$ is characteristic of a virus with attenuated virulence; fewer mit fibroblasts were observed, but the much more prominent inflammatory response together with extensive cellular necrosis seen in MRV-GF$^-$ primary and secondary tumors suggest that the host is capable of mounting a more effective response to MRV-GF$^-$ than to wild type MRV. The increase of the perifollicular/perivascular lymphocyte population in MRV-GF$^-$ recipients by day 7 p.i. also supports this hypothesis. In addition, the lymphoid proliferation in the spleen by day 11 when the MRV disease syndrome reaches its most severe state is much greater in MRV-GF$^-$ than in MRV recipients. This difference likely represents increased ability of splenic lymphocytes to mount a more vigorous response to MRV-GF$^-$. The most pronounced disease symptom that distinguished the infections by MRV and MRV-GF$^-$ was that secondary bacterial infections in the respiratory tract and conjunctiva resulting from viral infection were considerably less extensive in MRV-GF$^-$ recipients. The reason for this could in theory involve a decrease in immunosuppressive ability of MRV-GF$^-$. However, our experiments summarized in Table II-3 indicate that MRV-GF$^-$ is only slightly less immunosuppressive in an *in vitro* assay than wild type MRV, and the data in Table II-4 indicate that this modest effect may be insignificant *in vivo* by day 7 when the virus has

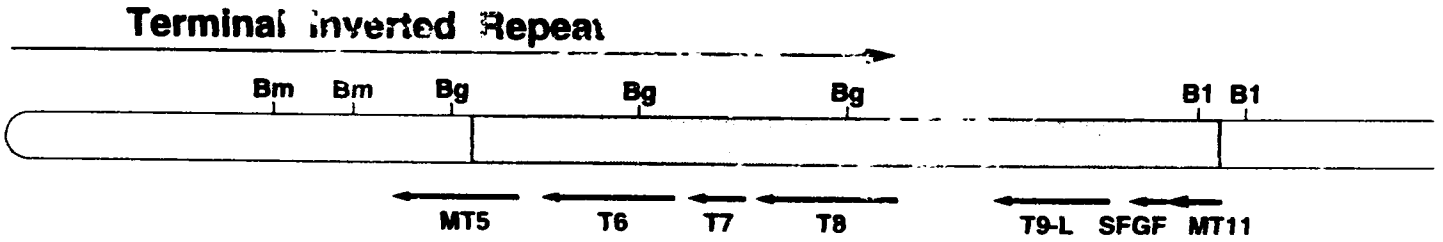
migrated to the spleen. Accelerated clearance of virus from the lymphatic circulation *per se* also does not appear to explain the attenuation of MRV-GF$^-$ infection. On the other hand, the nasal and conjunctival epithelia which are targets for bacterial infection (Strayer *et al.*, 1983a; Strayer and Sell, 1983) are differently affected by MRV and MRV-GF$^-$ since the latter causes dramatically less hyperplasia in these tissues. This difference may be important because, at least in the case of influenza and parainfluenza virus infection, epithelial changes result in loss of clearance ability and can predispose the affected tissue to excessive bacterial superinfection (Heath, 1979; Jakab *et al.*, 1979). Therefore, the reduced ability of MRV-GF$^-$ to cause proliferation of cells in epithelial tissue of the conjunctival and nasal mucosa might be responsible for moderating the normally fatal infection with such adventitious pathogens as *Pasturella sp.* and *Bordetella sp.*. Although our data show that MRV and MRV-GF$^-$ replicate comparably in fibroblasts and spleen [illegible] possibility that MRV-GF$^-$ replication could be impaired in a small subset of [illegible]s *in vivo*, thereby precluding a lethal infection, cannot be formally excluded.

Other EGF-like growth factors may play a similar role in pathogenicity when introduced into the appropriate genetic background. Using the Lausanne strain of myxoma virus (Fenner and Burnet, 1957; Bouvier, 1954), which is closely related to MRV, we inactivated the MGF gene in a fashion similar to that described for the SFGF gene. This mutant myxoma construct was attenuated similarly to MRV-GF$^-$, produced tumors which were considerably less hyperplastic than those caused by the parental wild type virus, and the moderated disease was accompanied by less Gram negative bacterial infections. In addition, when the deleted MGF gene was replaced with rat TGFα, SFGF, or VGF, the diseases produced by these recombinant viruses were indistinguishable at the gross level from that caused by wild type MGF-plus myxoma virus (this thesis, chapter IV). Therefore, it is likely that these EGF-like peptides have similar cellular targets when conveyed by comparable viral vehicles.

SFGF produced by MRV appears to contribute to the proliferative response of both fibroblasts and epithelial cells. Primary and secondary tumors produced in MRV-GF$^-$ infected rabbits exhibited a reduced degree of fibroblastic proliferation compared to wild type lesions, and the epithelia overlying MRV-GF$^-$ secondary tumors were much less hyperplastic than the MRV counterparts. However, because of the extensive infiltration and necrosis seen in MRV-GF$^-$ induced tumors, it is difficult to unequivocally determine how much of the decreased cellularity in the bulk of the tumor represents cell lysis and how much is due to decreased cellular proliferation. We conclude that SFGF is an important pathogenic marker in MRV, and that cells responsive to this ligand play a critical role in determining the disease course, especially at late times of infection when the balance between viral invasion and immune defense is at its most critical stage.

Fig. II-1. Diagram of left end of MRV genome showing location of SFGF gene. Arrows denote open reading frames. The SFV sequences are shown in the stippled box and SFV open reading frames denoted T6, T7, T8, T9-L, and SFGF. The designations MT5 and MT11 denote myxoma/SFV fusion genes. Abbreviations are Bm, Bam HI; Bg, Bgl II; B1, Bgl I.

MRV Left End



SFV Sequences

MYX Sequences

Fig. II-2. Structures of wild type MRV and MRV-GF$^-$ genomes. The construction of the plasmid used to produce MRV-GF$^-$ (pMPL-16) is described in Materials and Methods. The viral terminus is towards the right in this illustration. The SFGF coding sequence is shown as a black region and the p11-βgal cassette is indicated as a stippled box. Abbreviations are B1, Bgl I; Bg, Bgl II; C, Cla I; H, Hinc II; Sm, Sma I.

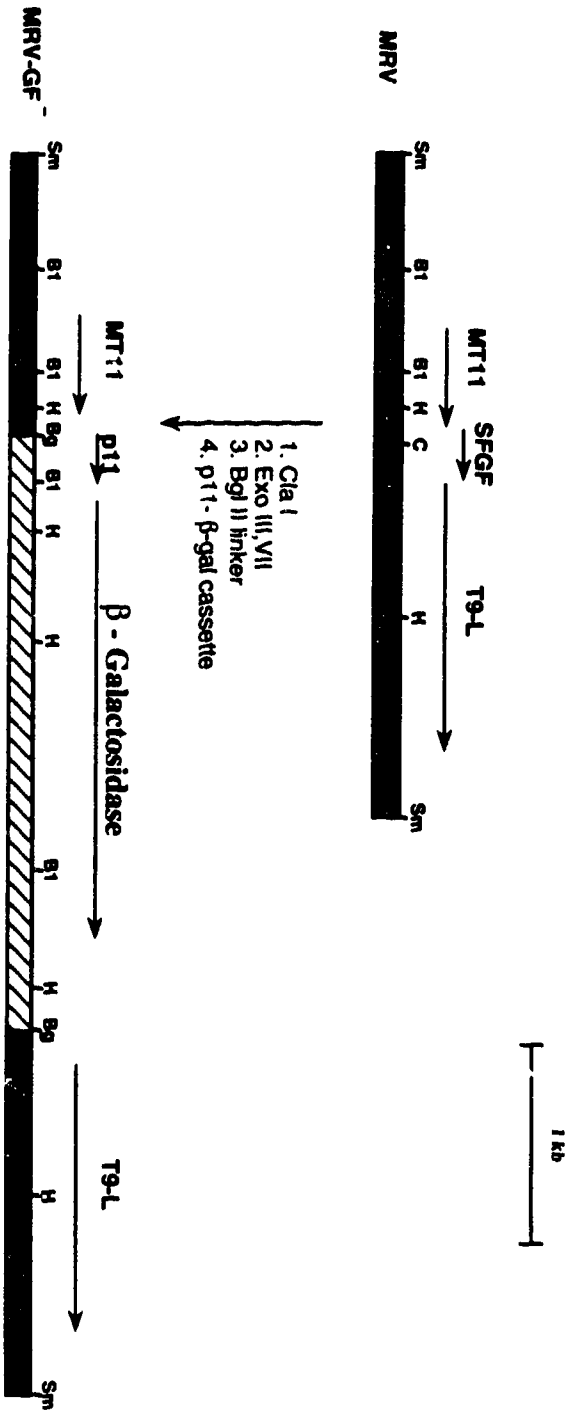

MRV
Sm
B1
MT11
SFGF
H
C
T9-L
1 kb
MRV-GF⁻
p11
β - Galactosidase
Bg
1. Cla I
2. Exo III, VII
3. Bgl II linker
4. p11 - β-gal cassette

Fig. II-3. Panel A) MRV primary tumor, day 7. The tumor consists of large cells in a highly myxoid connective tissue matrix. Inflammatory cells are absent. Typical tumor cells are shown (arrows). H & E, original magnification x 400.

Panel B) MRV spleen, day 7. Typical lymphoid aggregates in the spleen are shown. While the cores of the follicles are evident (arrows), there is no evidence of follicular proliferative activity. In particular, there is no division of germinal center cells and no evidence of perifollicular lymphocyte accumulation. The red pulp surrounding the follicles is greatly expanded. H&E, original magnification x 100.

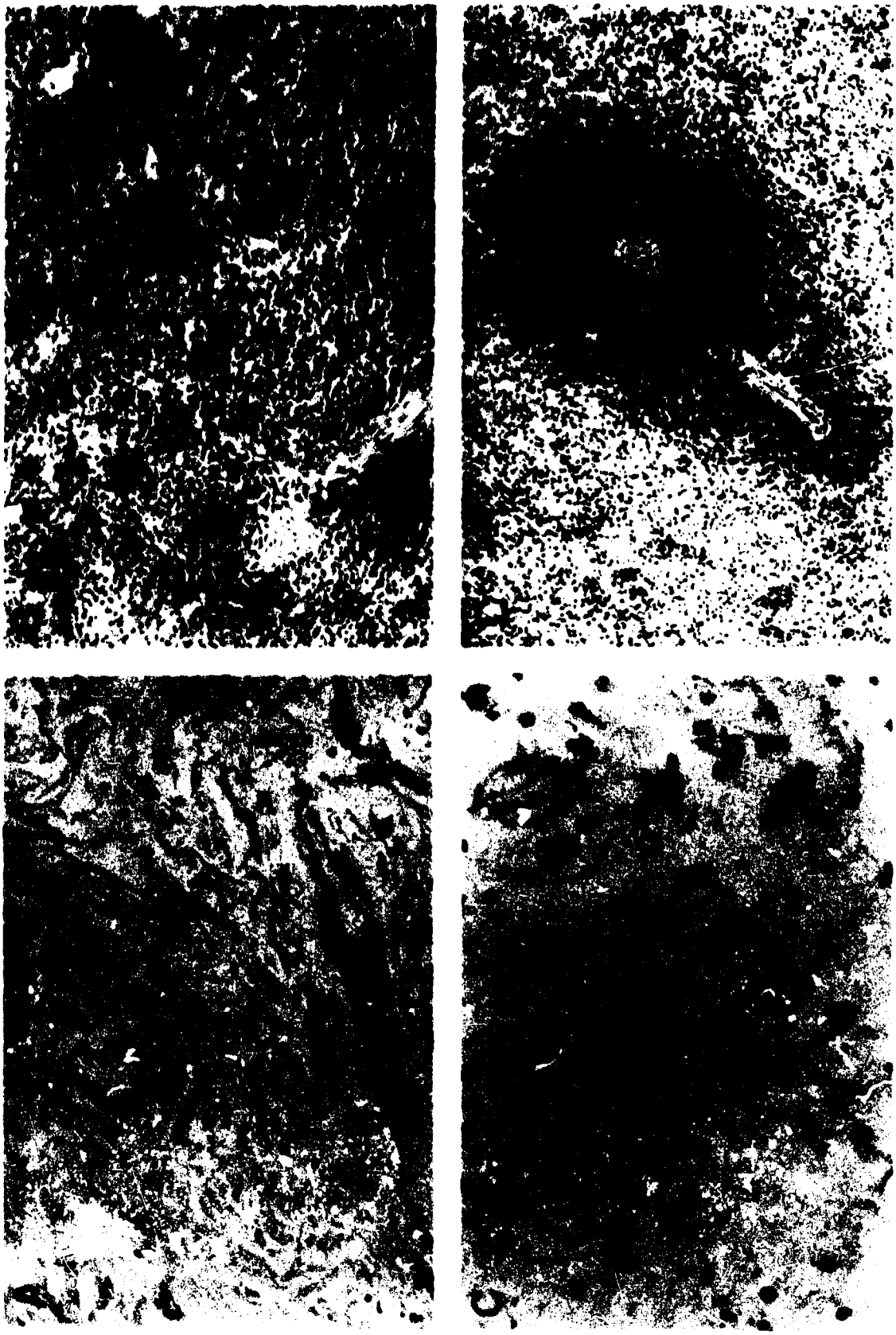
Panel C) MRV-GF$^-$ primary tumor, day 7. Tumor cells are large and surrounded by matrix very similar to that observed in wild type MRV tumors. However, nuclei of these cells often show karyorrhexis. That is, nuclear structure shows signs of dissolving: chromatin is clumped and scattered, without clear nuclear boundaries from cytoplasm (closed arrows). In addition, an acute inflammatory infiltrate (open arrows) is present here that is absent from wild type MRV tumors at this time point. H&E, original magnification, x 400.

Panel D) MRV-GF$^-$ spleen, day 7. Spleen morphology in MRV-GF$^-$ infected animals is characterized by proliferation of lymphocytes in the perivascular areas, as shown here. H&E, original magnification, x 100.

Panel E) Normal connective tissue from uninfected rabbit at usual site of inoculation. H&E, original magnification, x 160.

Panel F) Spleen section from uninfected rabbit. H&E, original magnification x 160.

Panel G) Conjunctiva from uninfected rabbit, H&E, original magnification, x 400.



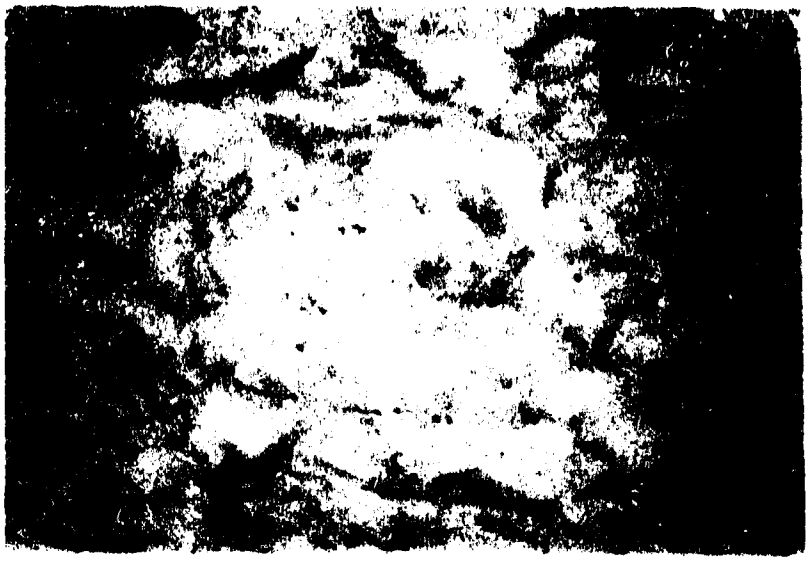


Fig. II-4. Panel A) MRV spleen, day 11. Florid proliferation of germinal centers is present by 11 days p.i. in MRV recipients. H&E, original magnification, x 100.

Panel B) MRV conjunctiva and secondary tumor, day 11. Extensive proliferation of conjunctival epithelium overlying secondary tumor is present. Normal conjunctival epithelium is 2-3 cell layers thick. Average thickness of the conjunctival epithelium in such wild type MRV-infected rabbits is approximately 8-10 cell layers. The secondary tumor seen beneath the epithelium resembles the primary tumor. H&E, original magnification, x 400.

Panel C) MRV primary tumor, day 11. Tumors from wild type MRV recipients 11 days p.i. resemble those earlier in infection, except that a slight inflammatory infiltrate is noted. Otherwise tumor cell growth and arrangement are unchanged. H&E, original magnification, x 100.

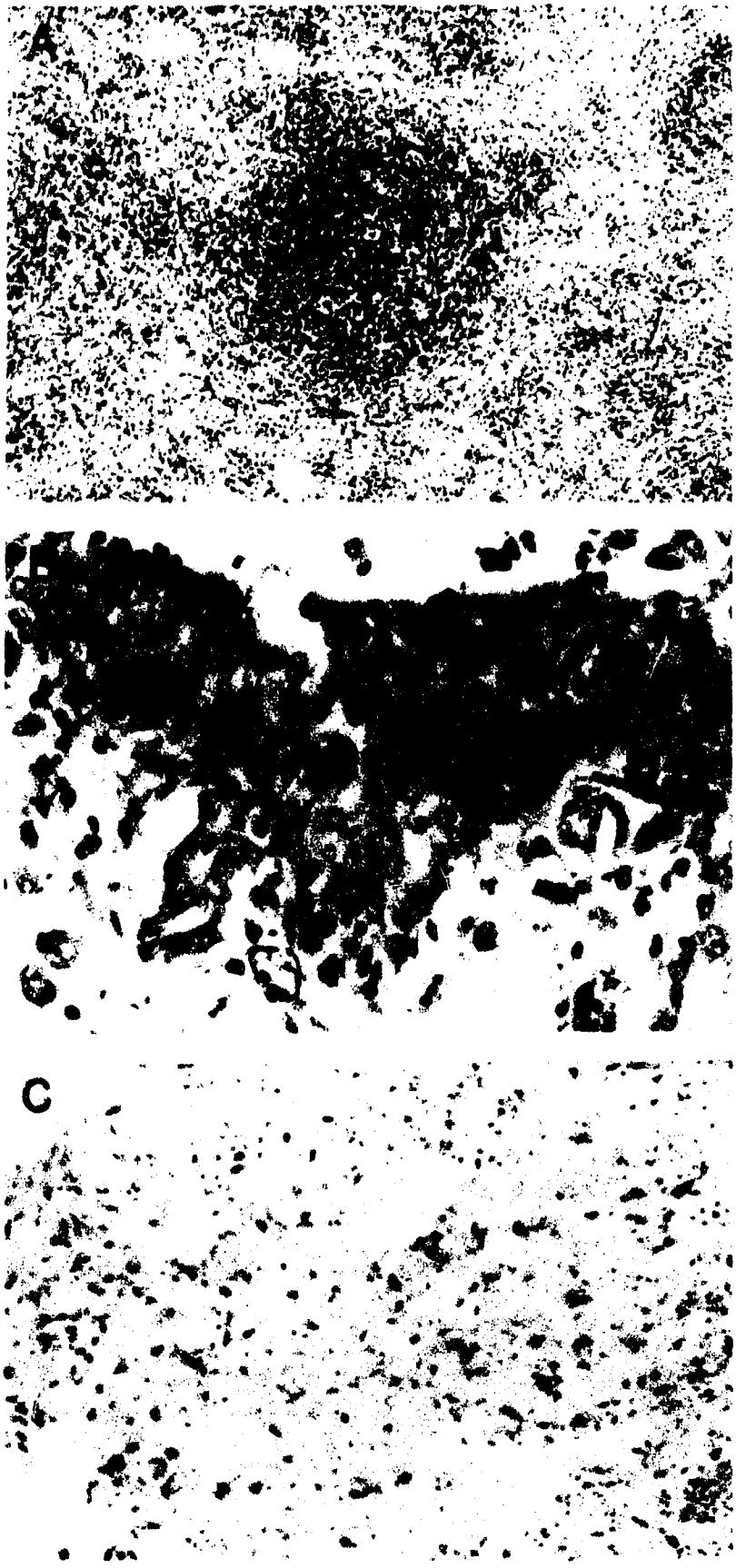

A
C

Fig. II-5. Panel A) MRV-GF$^-$ primary tumor, day 11. Tumor cells show evidence of dissolution. Only occasional tumor cells are not surrounded by clusters of heterophiles. Typical clusters of heterophiles around a tumor cell are seen at the end of the open arrows. Other heterophiles infiltrating the tumor are shown by the closed arrows. H&E, original magnification, x 400.

Panel B) MRV-GF$^-$ spleen, day 11. Tremendous lymphoid proliferation is seen in spleens from MRV-GF$^-$ recipients at this time point. A germinal center is shown here, with follicular proliferation as well as perifollicular accumulation of lymphocytes. H&E, original magnification, x 100.

Panel C) MRV-GF$^-$ conjunctiva and secondary tumor, day 11. Conjunctiva from rabbits receiving MRV-GF$^-$ 11 days earlier show some mild increase in epithelial thickness, but much less than that seen in wild type MRV recipients at the same time point. In this case, the conjunctival epithelium is approximately 3-4 cell layers thick. Note the prominent plasma cellular infiltrate (arrows) in the underlying secondary tumor. This is not prominent in sections from wild type MRV-infected rabbits. H&E, original magnification, x 400.

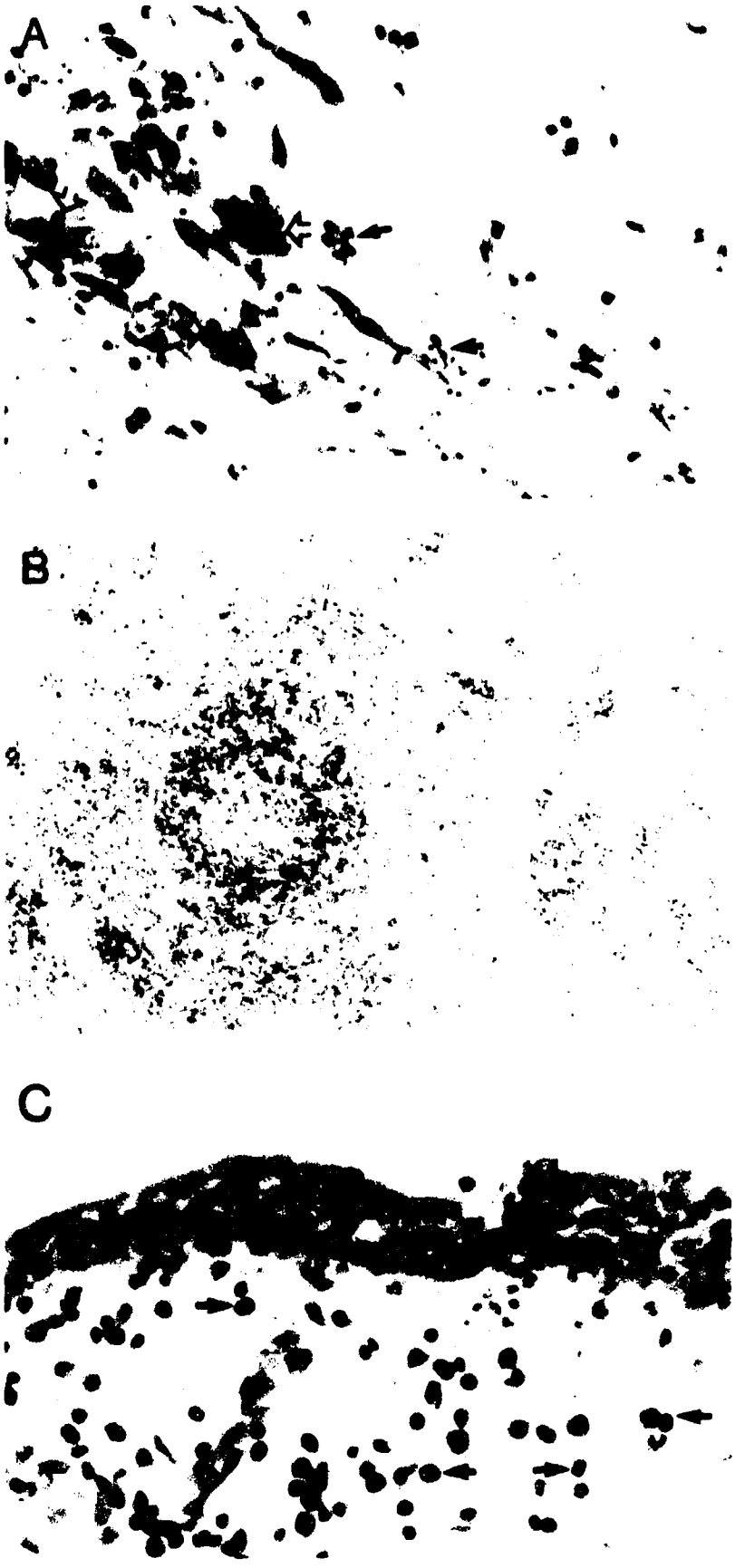
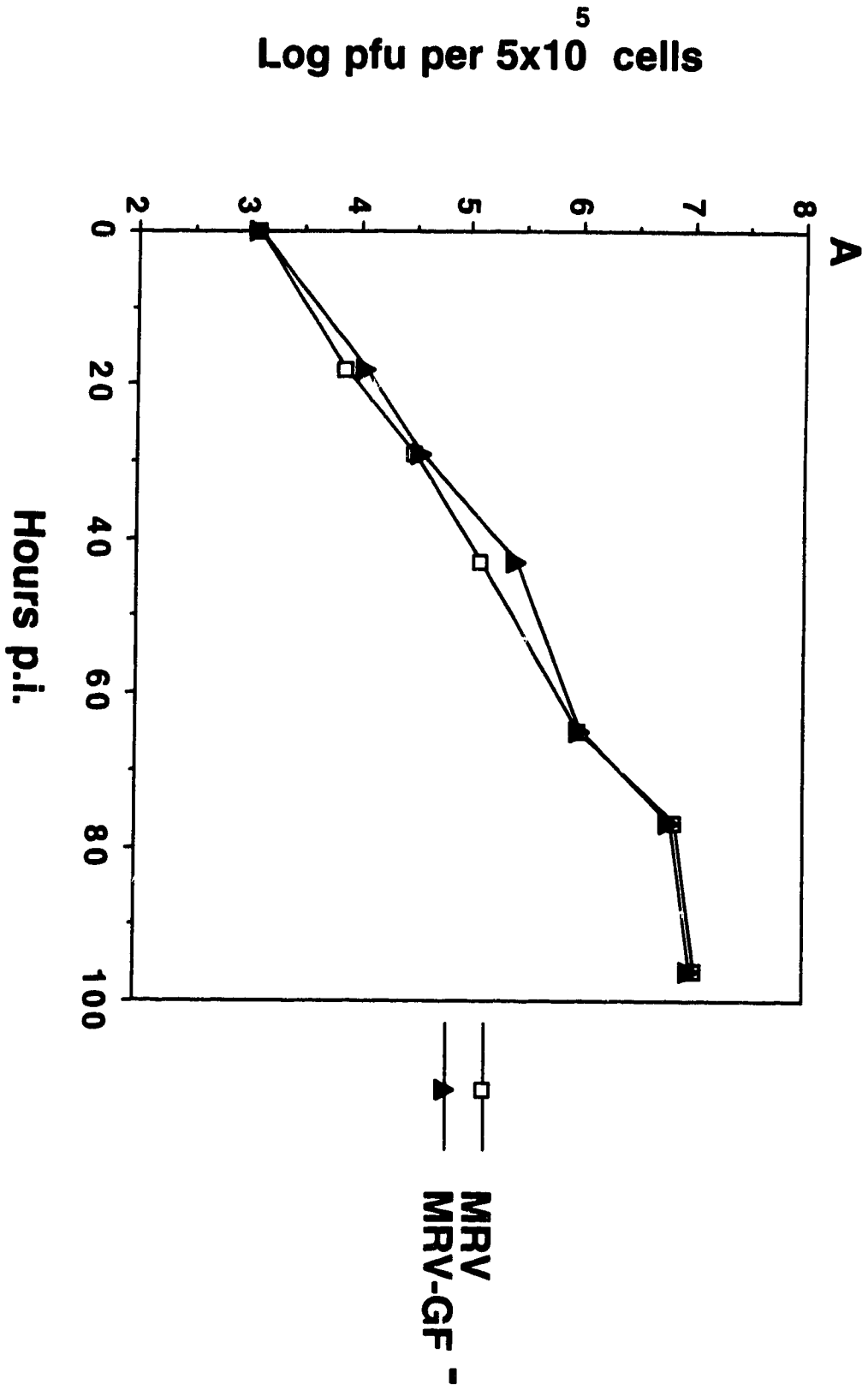
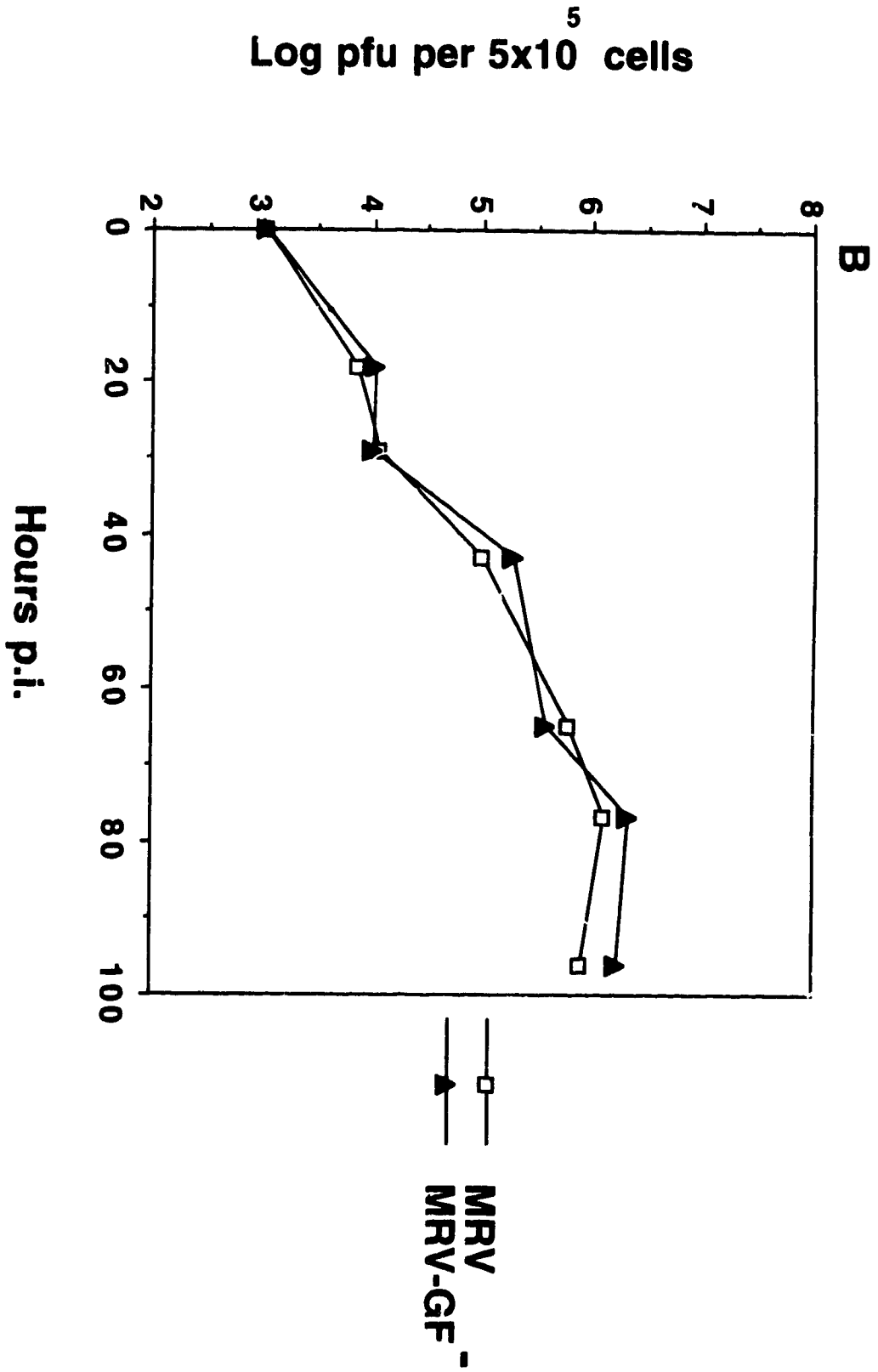

A
B
C

Fig. II-6. Growth of MRV and MRV-GF$^-$ in tissue culture. Monolayers of rabbit cells (RK-13 or SIRC) were infected at a m.o.i. of 0.002, harvested at various times post-infection and titered on RK-13 cells. (A) Growth in RK-13 cells. (B) Growth in serum-depleted RK-13 cells. (C) Growth in serum-depleted SIRC cells.


Log pfu per 5x10^5 cells
2
3
4
5
6
7
8
A
0
20
40
60
80
100
Hours p.i.
MRV
MRV-GF -


Log pfu per 5x10^5 cells
2
3
4
5
6
7
8
B
0
20
40
60
80
100
Hours p.i.
MRV
MRV-GF-

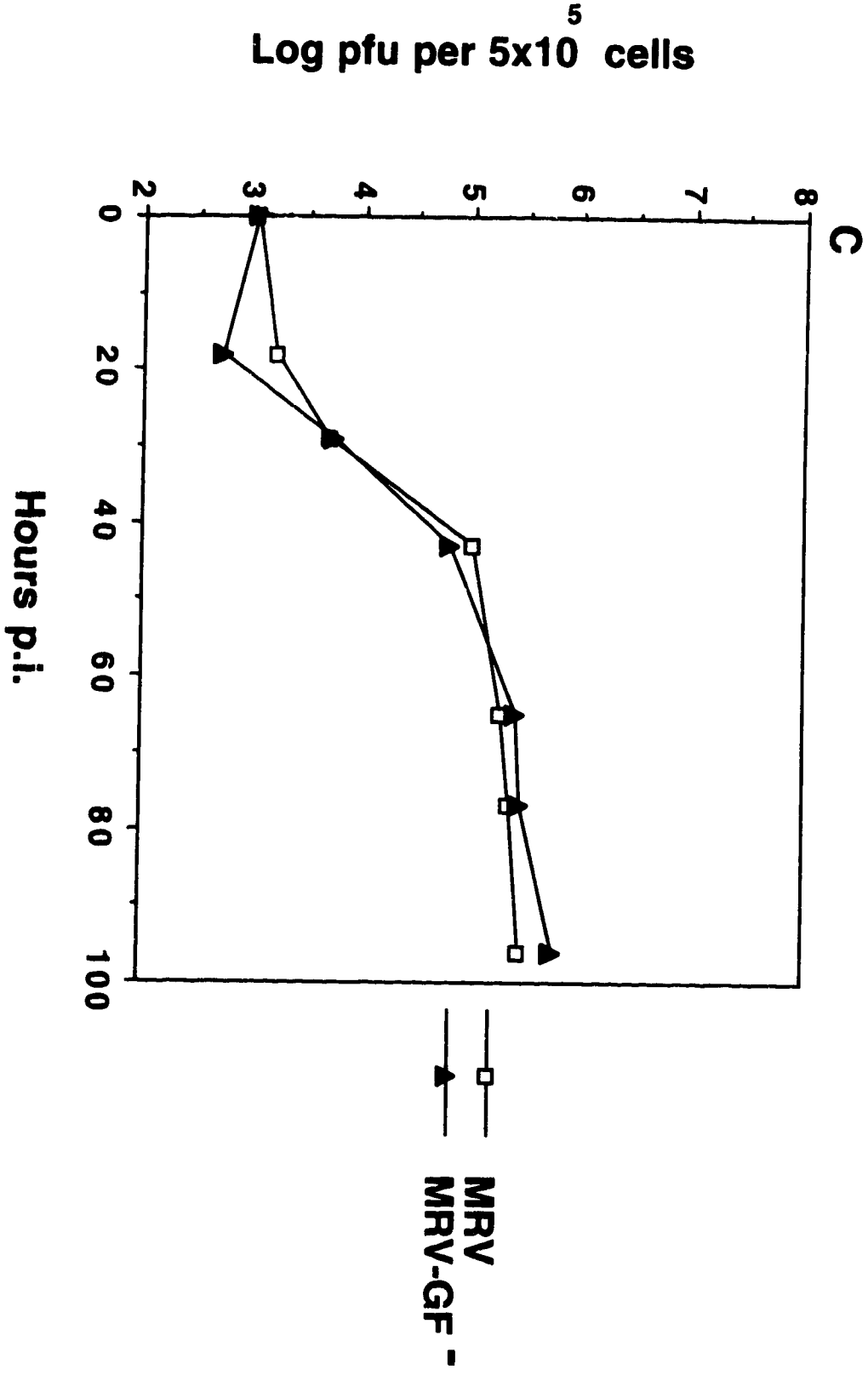

Log pfu per 5x10^5 cells
2
3
4
5
6
7
8
C
0
20
40
60
80
100
Hours p.i.
MRV
MRV-GF -

Fig. II-7. Growth of MRV and MRV-GF$^-$ in primary spleen cell cultures. Cultures of spleen cells isolated from normal rabbits were infected with MRV or MRV-GF$^-$ at a m.o.i. of 0.001 in the presence or absence of the non-specific T cell mitogen Con A. Cultures were harvested at various times p.i. and titered on RK-13 cells.

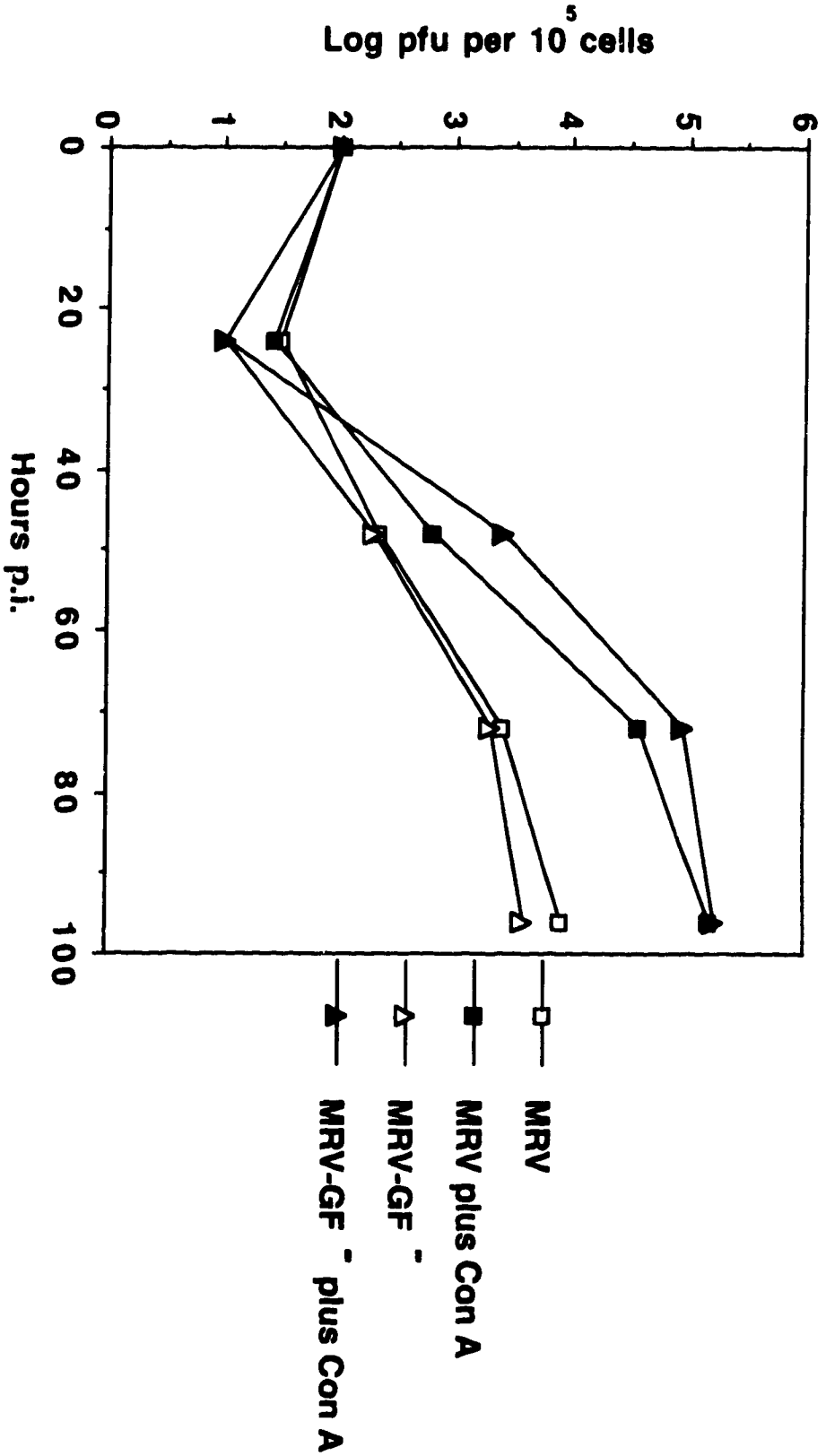
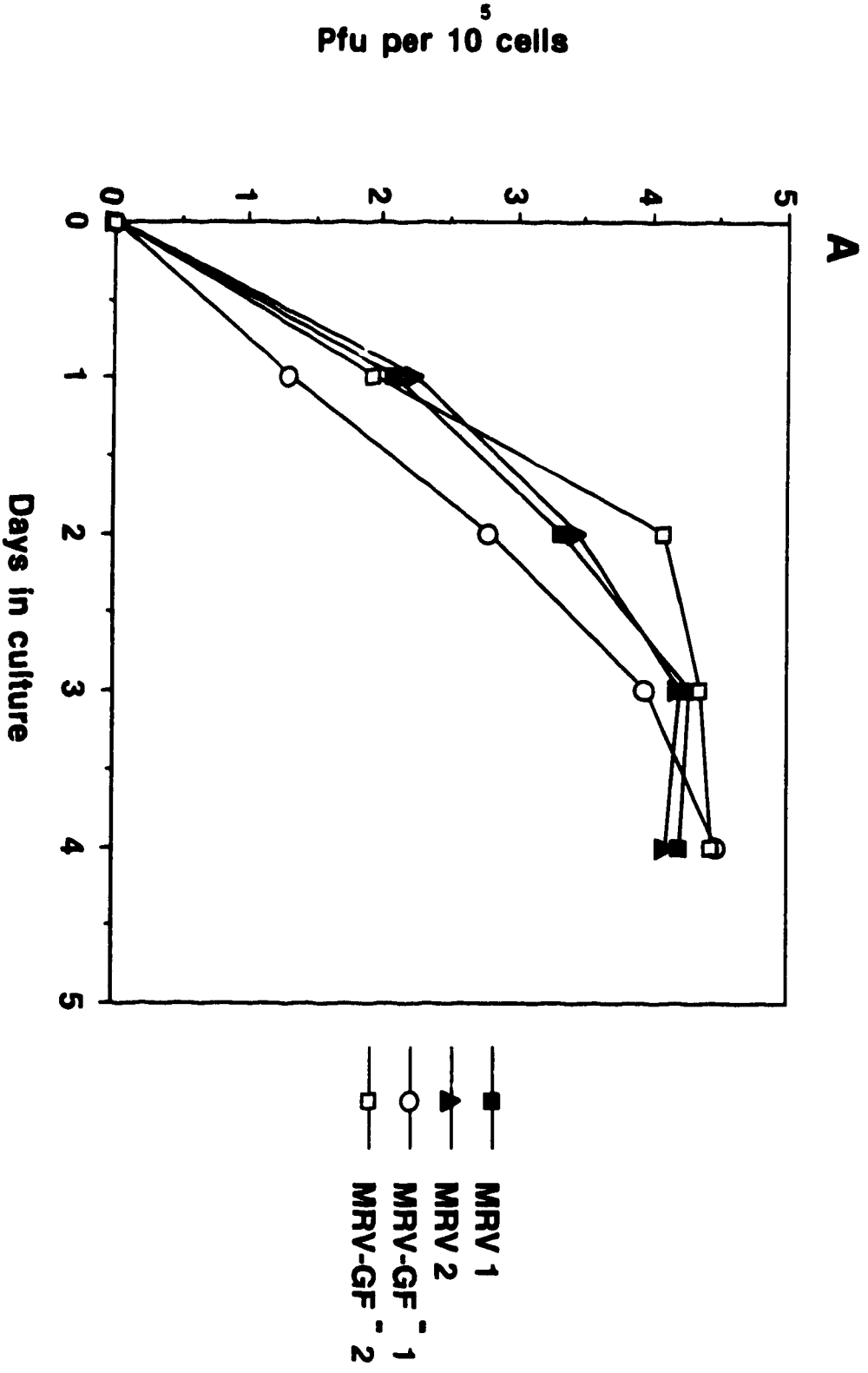

Log pfu per 10^5 cells
0
1
2
3
4
5
6
Hours p.i.
0
20
40
60
80
100
MRV
MRV plus Con A
MRV-GF -
MRV-GF - plus Con A

Fig. II-8. Recovery of virus from spleens of rabbits infected with MRV or MRV-GF$^-$. Spleens from animals that had received MRV or MRV-GF$^-$ 7 days previously were cultured in triplicate in the absence (A) or presence (B) of Con A. At various times after stimulation, cultures were harvested and virus was titered on RK-13 cells.


Pfu per 10^5 cells
A
Days in culture
0
1
2
3
4
5
MRV 1
MRV 2
MRV-GF - 1
MRV-GF - 2

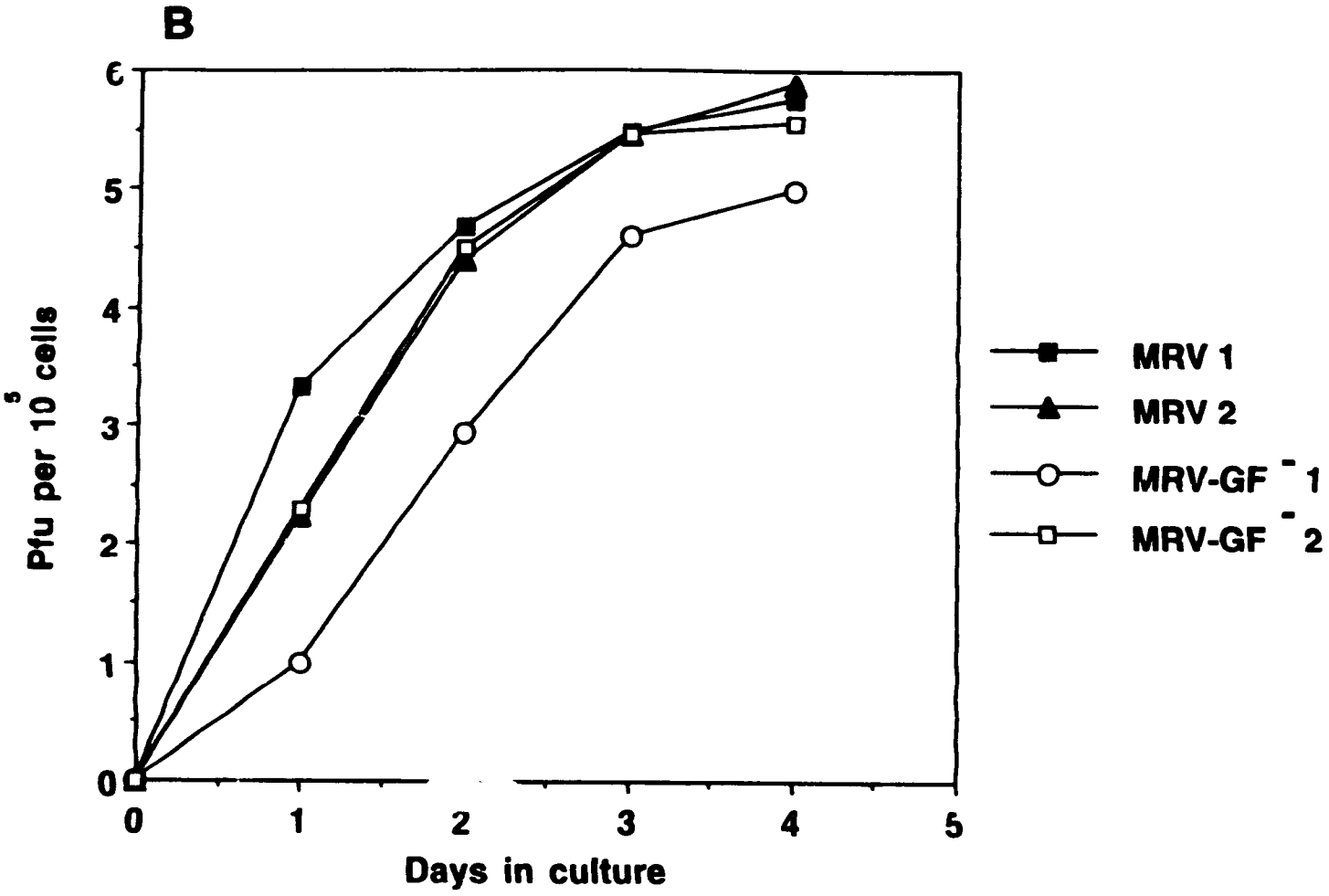

B
6
5
4
3
2
1
0
Pfu per 10 cells
5
0
1
2
3
4
5
Days in culture
MRV 1
MRV 2
MRV-GF⁻1
MRV-GF⁻2

Table II-1. Pathogenicity of MRV and MRV-GF$^-$ in Rabbits

Virus	Inoculum	Symptoms		Fate
MRV	10^3 pfu	day 7	localized tumor	0 % survival
		day 10-11	Gram negative infection disseminated tumor severe purulent conjunctivitis, rhinitis and dyspnea death	
MRV-GF$^-$	10^3 or 10^4 pfu	day 7	localized tumor	75 % survival
		day 10-11	Gram negative infection disseminated tumor moderate to severe purulent conjunctivitis and rhinitis crisis stage (death {25%} or survival 75%})	
		day 17	tumors regress bacterial infection wanes	
		day 30	complete recovery animals immune to rechallenge	

Table II-2. Summary of Major Histological Differences Between MRV and MRV-GF⁻ Infection in Rabbits

	MRV Day 7	MRV-GF⁻ Day 7
Primary Tumor	minimal inflammatory response necrosis of tumor cells not observed	prominent infiltrate of heterophile leukocytes substantial necrosis of tumor cells
Spleen	perifollicular depletion	perifollicular proliferation
	MRV Day 11	**MRV-GF⁻ Day 11**
Primary Tumor	moderate lymphocytic infiltration of skin appendages and acantholysis of epithelium	no lymphoid infiltrate around cutaneous appendages; minor architectural disruption of epithelium
	moderate infiltration with heterophile leukocytes	copious infiltration with heterophile leukocytes and necrosis of individual tumor cells
Spleen	moderate increase in germinal center activity; moderate perifollicular and perivascular hyperplasia	profuse B and T lymphocyte zone proliferative activity
Conjunctiva	extensive proliferation of epithelium moderate inflammatory reaction little necrosis in underlying tumor	mild proliferation of epithelium severe inflammatory reaction extensive necrosis in underlying tumor

Table II-3. Proliferation of Mitogenically Stimulated Spleen Cells Infected by MRV or MRV-GF$^-$ *in vitro* [a]

	Unstimulated		Con A-Stimulated	
	MRV	**MRV-GF$^-$**	**MRV**	**MRV-GF$^-$**
m.o.i.=0.001	3.61+/- 0.05	3.52+/- 0.08	4.30+/- 0.16	5.28+/- 0.19
m.o.i.=0.01	3.32+/- 0.11	3.25+/- 0.02	3.34+/- 0.04	3.83+/- 0.14
m.o.i.=0.1	3.13+/- 0.06	3.13+/- 0.07	3.28 +/- 0.09	3.38+/- 0.05
uninfected	3.09+/- 0.17		5.12+/- 0.04	

[a]Numbers are log (counts per minute) of incorporated 3H-thymidine over 24 hours +/- standard deviation (average of three experiments).

Table II-4. Proliferation of Mitogenically Stimulated Spleen Cells From Infected Rabbits[a]

	Unstimulated	Con A-stimulated
MRV 1	3.07 +/- 0.18	3.67 +/- 0.19
MRV 2	2.66 +/- 0.19	3.78 +/- 0.03
MRV 3	2.47 +/- 0.08	4.81 +/- 0.03
MRV-GF$^-$ 1	2.77 +/- 0.10	4.24 +/- 0.08
MRV-GF$^-$ 2	3.20 +/- 0.06	3.52 +/- 0.04
MRV-GF$^-$ 3	3.10 +/- 0.04	4.82 +/- 0.03
control cells	2.75 +/- 0.03	5.10 +/- 0.08

[a]Numbers are log (counts per minute) of incorporated 3H-thymidine over 24 hours +/- standard deviation (average of three experiments) into spleen cells of rabbits that had been infected with MRV or MRV-GF$^-$ 7 days previously. The 3H-labelling was performed on the third day after Con A stimulation.

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CHAPTER III- DELETION ANALYSIS OF TWO TANDEMLY ARRANGED GENES IN MYXOMA VIRUS, M11L AND MYXOMA GROWTH FACTOR[1]

INTRODUCTION

The Poxviridae comprise a very large family of DNA viruses whose members have been isolated from most vertebrate species and some insects (Moss, 1990; Fenner, 1990b). They produce a remarkably diverse spectrum of disease pathologies in their infected hosts and encode many products which are non-essential for viral replication but directly or indirectly affect virulence, host range, and tissue tropism (Buller and Palumbo, 1991; Traktman, 1990; Turner and Moyer, 1990). Examples of such poxviral products include epidermal growth factor homologues (Brown *et al.*,1985; Chang *et al.*,1987; Porter *et al.*,1988; Porter and Archard, 1987; Twardzik *et al.*,1985; Upton *et al.*, 1987 and 1988) serine protease inhibitors (SERPINS) (Boursnell *et al.*,1988; Kotwal and Moss, 1989; Smith *et al.*,1989; Pickup *et al.*,1986; Upton *et al.*,1990a), soluble forms of tumor necrosis factor-alpha receptor (Upton *et al.*,1991; Smith *et al.*,1991), and an inhibitor of the classical complement pathway (Kotwal *et al.*,1990; Kotwal and Moss, 1988).

Despite the cytoplasmic site of poxviral replication and assembly, a number of poxviruses are considered to be "tumorigenic" because they cause extensive cellular proliferation at or near sites of viral replication (Fenner, 1990a and b; McFadden, 1988). Some of these tumorigenic poxviruses are also able to induce a lethal, invasive disease syndrome due to concomitant immune dysfunction brought about in part by the ability to

[1] A version of this chapter has been published. A. Opgenorth, K. Graham, N. Nation, D. Strayer, and G. McFadden. 1992. Journal of Virology.66: 4720-4731.

replicate in, and interfere with the normal function of, host lymphocytes (McFadden, 1988; Strayer, 1988). One example is myxoma virus (MYX), the causative agent of myxomatosis which, because of its extreme virulence and narrow host range, was deliberately released in Australia in 1950 to facilitate control of the European rabbit (*Oryctolagus cuniculus*) population. The epidemiology and pathological effects of MYX on feral rabbit populations have been studied in detail (Fenner and Marshall, 1957; Fenner and Ratcliffe, 1965; McFadden, 1988).

Characterization of the 160 kb MYX genome (Block *et al.*, 1985; Russell and Robbins, 1989) has shown that it is organized in a fashion similar to that of other poxviruses, containing crosslinked termini, terminal inverted repeats (TIR), and an estimated coding capacity for over two hundred gene products. Despite the variation in degree of virulence between different strains of myxoma virus, their restriction enzyme cleavage maps are relatively well conserved (Russell and Robbins, 1989). MYX is very closely related to other members of the *Leporipoxvirus* genus, such as Shope fibroma virus (SFV). Analysis of several open reading frames (ORFs) encoded within the TIR of MYX reveals a high degree of conservation with the SFV ORF counterparts, both at the nucleotide (>75 %) and amino acid (>80%) levels (Upton *et al.*, 1987, 1988, and 1991). Malignant rabbit fibroma virus (MRV) is an SFV/MYX recombinant virus in which approximately 8 kb of MYX sequences near the left terminus were deleted and replaced with homologous SFV sequences, and a 4 kb subset of this was substituted for MYX sequences at the right terminus (Block *et al.*, 1985; Strayer *et al.*, 1983a and b; Upton *et al.*, 1988). Thus, MRV is 95% identical to MYX, except for the acquisition of 5 genes from SFV plus the generation of two MYX/SFV fusion genes. The syndrome induced by MRV has been examined in detail, and because of its similarity to MYX infection, it is believed that results of studies on MRV are generally applicable to MYX as well (Buller and Palumbo, 1991; McFadden, 1988; Strayer, 1988). Upon intradermal (i.d.) infection with even very low doses of MRV or MYX, recipients develop a primary tumor-like lesion

which disseminates throughout the dermis and subcutis to multiple external and internal tissues. Dissemination of the virus to secondary sites by productively infected lymphocytes is accompanied by profound generalized immunosuppression and concomitant secondary bacterial infections which cause purulent conjunctivitis and rhinitis, leading to dyspnea and death within 10-14 days. The pathological effects of MYX and MRV have been well characterized (Mare , 1974; Strayer and Sell, 1983; Strayer *et al.*,1983; Fenner and Marshall, 1957; Fenner and Ratcliffe, 1965), but the molecular mechanisms by which these viruses induce tumor formation and immunosuppression remain to be established.(McFadden, 1988; Strayer, 1988).

Most, if not all, poxviruses encode products that are members of the epidermal growth factor (EGF) family of growth factors (Brown *et al.*,1985; Chang *et al.*,1987; Porter and Archard, 1987; Porter *et al.*,1988; Twardzik *et al.*,1985; Upton *et al.*,1987 and 1988). The prototype of this family, vaccinia growth factor (VGF), is modified by proteolytic cleavage and glycosylation and is secreted from vaccinia virus-infected cells. The fully mature VGF ligand has been shown to compete with EGF for binding to the EGF receptor at the cell surface, to be mitogenic for responsive cell types bearing the EGF receptor, and to play a role in vaccinia virus pathogenicity (Buller *et al.*,1988a and b; Buller and Palumbo, 1991; King *et al.*,1986; Stroobant *et al.*,1985; Twardzik *et al.*,1985). The EGF analogue encoded by MRV is one of the 5 gene products derived from SFV sequences and is designated Shope fibroma growth factor (SFGF) (Chang *et al.*,1987; Upton *et al.*,1988). Whereas wild type MRV infection in the European rabbit is 100% fatal, even with very small inocula, 75% of rabbits infected with MRV containing an SFGF deletion (MRV-GF$^-$) undergo a less severe disease course and completely recover from infection. In addition, the primary and secondary tumors induced by MRV-GF$^-$ display a reduced degree of cellular hyperplasia compared with those of wild type MRV recipients, particularly in the nasal mucosa and conjunctiva (Opgenorth *et al.*,1992). MYX contains a related but distinct EGF-like gene, called myxoma growth factor (MGF), which is 80%

conserved with SFGF (Upton *et al.*, 1987). During the course of our studies to ascertain whether the biological roles of MGF in MYX infection are comparable to those of SFGF in MRV infection, we constructed a deletion mutant of MYX (vMYX-GF$^-$ ΔM11L) in which the entire 460 bp MGF coding sequence was deleted from the genome. This resulted in a drastic attenuation of MYX that was far more profound than that predicted by our studies on the SFGF deletion in MRV and, in fact, the ability to induce virtually all the symptoms of myxomatosis were abrogated. Fine analysis of the deletion construct used to produce this MYX mutant revealed that the manipulation also resulted in the removal of the carboxy terminus of the partially overlapping upstream ORF, designated M11L. M11L encodes a potential 166 a.a. polypeptide whose carboxy terminus overlaps the amino terminus of MGF by 6 residues (Upton *et al.*, 1987). Here we show that M11L and MGF are two independent virulence factors in MYX, and that mutations in both genes simultaneously results in complete loss of viral pathogenicity in rabbits.

MATERIALS AND METHODS

Cells and Virus

Rabbit SIRC cells were obtained from the American Type Culture collection (ATCC) and maintained in Dulbecco modified Eagle's medium (DME) supplemented with 10% fetal bovine serum (FBS). RK-13 cells (provided by S. Sell) and BGMK cells (provided by S. Dales) were grown in DME supplemented with 10% newborn calf serum (NCS). The Lausanne strain of myxoma virus used in this study (Bouvier, 1954; Fenner and Burnet, 1957; Fenner and Marshall, 1957) was obtained from the ATCC.

Enzymes and Chemicals

Restriction enzymes were supplied by Bethesda Research Laboratories, Boehringer Mannheim, and New England Biolabs. T4 DNA polymerase, T4 DNA ligase, calf intestinal phosphatase (CIP), and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) were purchased from Boehringer Mannheim. The Kpn I and Bgl II linkers were obtained from the Regional DNA Synthesis Laboratory, University of Calgary. Lipofectin™ was obtained from Bethesda Research Laboratories.

Plasmid Constructs

A 4.5 kb Sma I fragment containing the MGF gene was isolated from the MYX 8.6 kb Bam HI fragment E, which maps at the left end of the viral genome and encompasses the unique sequence/TIR border (Block *et al.*, 1985; Russell and Robbins, 1989), and ligated into the Sma I site of pUC 13. The resulting plasmid, pMyS2a, was unstable in standard *rec A* bacterial strains (e.g. HB101), but could be propagated in DB1256 which contains *recA*, *recBC*, and *sbcB* loci (DeLange *et al.*, 1986). pMyS2a was linearized by digestion with Kpn I, whose unique enzyme recognition site maps within the first 5 nucleotides (nt) of MGF coding sequences (Upton *et al.*, 1987) . Partial digestion of this DNA with Nru I, for which there are 2 sites in pMyS2a, yielded a product in which 460 nt, including the entire MGF coding region, were deleted. The Kpn I-Nru I deletion therefore spanned from the third codon of MGF to a point 37 nucleotides 5' to the start of the downstream M9L gene. The DNA was blunt-ended, Kpn I linkers were added, the deletion construct was circularized with T4 DNA ligase, and used to transform DB1256. The product, designated pMyK-1, was cleaved with Kpn I and blunt-end ligated to a 3.15 kb Bgl II fragment containing the *E. coli* β-galactosidase gene under the control of the vaccinia p11 promoter (β-gal cassette) (Buller *et al.*, 1988a). The DNA was used to transform DB1256 and a plasmid (pMyPL-5) was isolated in which sequences encompassing the entire MGF gene and the carboxy (C) terminal five amino acids of the

M11L ORF were deleted and replaced with the β-gal cassette. To create an insertion of the β-gal cassette at a position that did not interrupt any known ORFs, plasmid pMyLac-25 was constructed by blunt-ending the Bgl II β-gal cassette into the Sst I site of pMTL-25 (Chambers *et al.*, 1988), then excising the β-gal cassette with Kpn I. This Kpn I-ended β gal cassette was then blunt-end ligated into the Nru I site of pMyS2a downstream of the MGF gene, a site which does not interrupt either MGF or M9L (Upton *et al.*, 1987). To disrupt the MGF gene alone, plasmid pΔMGF-1 was constructed as follows. The 1.5 kb Hinc II fragment spanning the MGF gene (Upton *et al.*, 1987) was blunt-end ligated into the Sma I site of pMTL23p (Chambers *et al.*, 1988), and the DNA was used to transform *E. coli* JM83 (Sambrook *et al.*, 1989). The product, designated pMGF-3, was treated with Bgl II, blunt-ended, recircularized in order to destroy the unique Bgl II site in the vector sequences, and used to transform JM83. The product (pMGFB-4) was isolated and cleaved with Bsa BI, whose unique recognition site (GATNNNNATC) occurs near the middle of the MGF coding sequence (Upton *et al.*, 1987), Bgl II linkers were added, the product was used to transform DB1256, and a plasmid was isolated in which the Bsa BI site was destroyed and replaced with a Bgl II enzyme recognition site (pBsa-7). This plasmid was cleaved with Bgl II, ligated to the β-gal cassette, and used to transform DB1256, yielding pΔMGF-1. Plasmid pΔM11-2 was constructed in the following manner. pMGF-3 was cleaved with Bgl II and ligated to the 3.15 kb Bgl II β-galactosidase cassette from pSC20. This DNA was used to transform DB1256 and the resulting plasmid was designated pMGF-3lac-7. A 0.9 kb Eco RV fragment spanning the 5' end of the M11L gene was isolated from the MYX 8.6 kb Bam fragment E and ligated into the Stu I site of pMGF-3lac-7. Upon transformation of DB1256, the plasmid pΔM11-2 with the proper orientation of the M11L 5' sequences was isolated. This plasmid thus contains an M11L coding sequence that has been disrupted by a 52 bp deletion between the Eco RV and Hinc II sites (see Fig. III-1) and contains a β-gal marker. G. McFadden performed some of these procedures.

Construction of recombinant viruses

The recombinant viruses were constructed by modified standard procedures used to generate poxvirus mutants (Chakrabarti *et al.*, 1985). 35 mm dishes of 70% confluent BGMK cells were infected with MYXV at a multiplicity of infection (m.o.i.) of 0.05 at t=0 hrs; after 2 hrs, a calcium phosphate/DNA precipitate containing 500 ng Hind III-linearized pMyLac-25, pMyPL-5, or pΔMGF-1 was added. At t=6 hrs, the medium was replaced. In the case of vMYX-M11L$^-$, at t=2 hrs the medium was replaced with serum-free DME and a Lipofectin^{TM}/DNA mixture (1:1) containing 1 μg Hind III-cleaved pΔM11-2 was added to the medium. At t=24 hrs. the medium was replaced with DME+10% NCS. For all recombinants, at t=48 hrs, the virus was harvested and replated on BGMK cells at low m.o.i.. After 4 days, recombinant viruses (blue foci or plaques) were identified by overlaying the monolayers with 1% low melting point agarose in DME plus 5% NCS containing 500 μg/ml X-gal. For simplicity, both foci and plaques will be generally referred to as "plaques". After 3 cycles of plaque purification under 1% LMP agarose, stocks were grown to high titer in RK-13 cells. The genomic structures of candidate recombinant viruses were confirmed by polymerase chain reaction and by Southern blot analysis (not shown) as described previously (Innis *et al.*, 1990; Wills *et al.*, 1983). Recombinant MYX viruses containing the β-gal cassette at the Bsa B1 site (vMYX-GF$^-$), the β-gal cassette at the Nru I site immediately downstream of the MGF gene (vMYXlac), or deleted Kpn I - Nru I sequences replaced with the β-gal cassette (vMYX-GF$^-$ ΔM11L), were identified and the stocks were expanded in RK-13 cells (see Fig.III-2).

Virus Replication Analysis in Cell Culture

Cultures of RK-13 or SIRC cells were infected in 35 mm dishes in triplicate at a m.o.i. of 5 for single step growth analysis, or 0.002 to generate low multiplicity growth curves, for 1 hr at 37^{o}C in 0.3 ml DME. The inoculum was removed and replaced with

DME containing the appropriate amount of serum and the cultures were harvested at various times post infection (p.i.). When viral growth was studied in growth arrested cells, the cells were rendered quiescent by reducing serum content to 0% (RK-13) or 0.2% (SIRC) for 4 days prior to infection. Virus titers were determined by plaque assay on RK-13 cells followed by X-gal staining of fixed monolayers. Monolayers of RK-13 cells were fixed in PBS containing 0.02% glutaraldehyde, 1% formaldehyde, and 0.02% NP40 at room temperature for 5 minutes, washed 2x with PBS, then stained with 300 µg/ml X-gal in PBS containing 5mM potassium ferricyanide, 5mM potassium ferrocyanide, and 1mM $MgCl_2$, for 30 min at 37 ^{0}C (Sanes *et al.*, 1986).

Primary Rabbit Spleen Cell Cultures

Spleen cell suspensions from normal and virus-infected rabbits were prepared as described previously (Strayer and Leibowitz, 1987). Briefly, cells were suspended by passage through a steel mesh and cultured in 96-well flat-bottomed microtiter plates (Costar) at a concentration of 10^5 per well in 200 µl of RPMI 1640 (Gibco) supplemented with 10% bovine calf serum, L-glutamine (2mM), 50 µM 2-mercaptoethanol, and antibiotics. Where present, concanavalin A (Con A) and virus were added to the cultures at the time of culture initiation. The data represent averages of experiments performed in triplicate.

Infection of Rabbits With MYX, vMYXlac, vMYX-GF$^-$, vMYX-GF$^-$ ΔM11L, and vMYX-M11L$^-$

Adult female New Zealand White rabbits (2.5 - 3 kg) were purchased from local suppliers. They were housed and maintained according to standard procedures and sacrificed with euthanyl administered intravenously after anaesthesia. Rabbits were inoculated intradermally (i.d.) in the thigh with doses ranging from 10^2 to 10^6 plaque forming units (pfu) in 1 ml normal saline and monitored daily for external signs of

developing myxomatosis, including appearance of tumors, febrility, conjunctivitis, and rhinitis. Tissues from infected rabbits were isolated after necropsy at 7 or 10 days after infection, fixed in neutral buffered 10% formalin, paraffin embedded, and sectioned at 5 μm. Sections were stained with hematoxylin and eosin and viewed by light microscopy. Interpretation of histological slides was performed by D. Strayer and N. Nation. K. Graham assisted with infection, observation, and care of the animals, as well as the processing of tissue samples.

RESULTS

Construction of vMYX-GF$^-$ ΔM11L and vMYXlac

Figure III-1 shows the arrangement of viral open reading frames (ORFs), including MGF and M11L, which map near the left terminus. Note that the orientation of the MYX genome has been reversed from previous publications (Block *et al.*, 1985; Chang *et al.*, 1987; McFadden, 1988; Russell and Robbins, 1989; Upton *et al.*, 1987 and 1988; Wills *et al.*, 1983) to standardize the arrangement of conserved *Leporipoxvirus* genes with those of other poxviruses (Upton *et al.*, 1990b). We have also revised our nomenclature of open reading frames such that MYX ORFs mapping within the TIR will be designated M-T1 to M-T8, whereas those mapping outside the TIR will be prefaced with only M. In addition, unique ORFs will be designated left (L) or right (R) to indicate from which end of the genome the sequences are derived. As described in Materials and Methods, we constructed a recombinant virus (vMYX-GF$^-$ ΔM11L) in which 460 nt, including the entire MGF coding sequence, were deleted and replaced with a β-gal cassette under the control of the vaccinia p11 promoter (see Fig. III-2). Sequencing analysis upstream of MGF (Upton *et al.*, 1987) has indicated that an open reading frame homologous to an SFV ORF (originally called T11-R) (Upton *et al.*, 1988), designated M11L, is present immediately upstream and partially overlaps with the MGF gene (see Fig.III-1). These two genes are encoded within

different reading frames such that the C terminal 6 amino acids of M11L overlap the first 6 amino acids of MGF. Thus, deletion of nucleotide sequences from the Kpn I site to the Nru I site in pMyS2a resulted not only in deletion of the entire MGF gene but also the truncation of M11L at the 3' end and fusion of the M11L coding sequences to the antisense orientation of the C-terminus of the β-gal cassette. Since we wished to confirm that expression of the β-gal cassette itself would have no effect on the virulence or growth properties of MYX, we also constructed vMYXlac as a virus by inserting the β-gal cassette at the Nru I site downstream of the MGF gene, a region that does not interrupt any open reading frames or perturb the function of the M9L promoter. In addition, by converting the Bgl II termini of β-gal cassette into Kpn I sequences before insertion into the Nru I site of pMyS2a, the 3' junctions between the β-gal cassette and the Nru I site in vMYXlac and vMYX-GF$^-$ ΔM11L were made to contain identical nucleotide sequences (see Materials and Methods). Thus, any possible effect of the β-gal insertion on downstream M9L gene expression would be identical in both viruses. Furthermore, to check for any effect of β-gal cassette orientation on the phenotype of the knockout recombinant, we constructed a second MGF$^-$, ΔM11L virus in which the β-gal cassette was inserted in the opposite orientation to that shown in Fig.III-2 (not shown). The orientation of the β-gal cassette had no detectable effect on any properties of the MGF$^-$, ΔM11L viruses *in vivo* or *in vitro* (not shown), indicating that the nature of the inserted sequences fused to the M11L ORF did not affect the observed attenuated phenotype of the deletion.

Effect of the Kpn I-Nru I deletion on MYX virulence

A total of 40 rabbits were infected intradermally with 10^3 pfu of either MYX, vMYXlac, or vMYX-GF$^-$ΔM11L in order to examine the effects of the deletion on the pathogenic properties of MYX during the progression of myxomatosis. Our observations are summarized in Table III-1. As expected, MYX and vMYXlac recipients were indistinguishable and always developed a lethal disease characterized by large (4-5 cm),

raised, hemorrhagic primary tumors, diffuse, swollen secondary tumors, severe purulent conjunctivitis and rhinitis. In contrast to this, vMYX-GF$^-$ΔM11L recipients remained uniformly healthy throughout the course of the infection and developed a smaller (2-4 cm) non-hemorrhagic primary tumor which completely disappeared by 14 days after infection. To determine if increased doses of vMYX-GF$^-$ΔM11L could recapitulate the wild type MYX syndrome, we infected rabbits either intradermally or intravenously with 10^6 pfu vMYX-GF$^-$ ΔM11L. The only additional symptoms seen in these recipients were slight reddening of the conjunctiva and the occasional development of a small scratch-like lesion on the eyelid or ear. We observed no signs of the purulent secondary bacterial infections, secondary tumors, conjunctivitis, or rhinitis that invariably occur in the wild type MYX syndrome. vMYX-GF$^-$ΔM11L recipients were immune to challenge with wild type MYX at 21 days after infection. We conclude that a deletion of 460 nt encompassing the MGF gene virtually abolishes the disease symptoms associated with myxomatosis and that this dramatic attenuation is neither a simple dosage effect nor dependent on the route of infection.

Comparative histological analysis of tissues from wild-type MYX and vMYX-GF$^-$ΔM-11 recipients

We examined tissue sections of primary and secondary tumors, spleen, liver, kidney, lung, conjunctiva, and nasal mucosa from MYX and vMYX-GF$^-$ ΔM11L recipients. The histopathological characteristics of wild type MYX infection have been described previously (Fenner, 1990a; Fenner and Ratcliffe, 1965; Strayer *et al.*, 1983a, Strayer and Sell, 1983). A summary of the major histological differences between MYX and vMYX-GF$^-$ΔM11L is presented in Table III-2.

MYX recipients, day 7.

The tumors seen in MYX (strain Lausanne) recipients were highly myxoid, with abundant connective tissue matrix proteins and mucopolysaccharides surrounding scattered atypical tumor cells (see Fig.III-3. panel A). Compared with their counterparts in MRV-induced tumors (Strayer and Sell, 1983), these cells were less atypical and less abundant. A slight heterophilic infiltrate within the tumor was observed. The conjunctiva in these animals show extensive squamous metaplasia and hyperplasia (see Fig.III-3 panel B). In the spleen, perifollicular cell proliferation and sinusoidal congestion were observed, but the expansion of the red pulp characteristic of MRV infection was not prominent. Examination of the lungs in these animals showed no edema, though intraalveolar hemorrhage was detected.

MYX-GF$^-$ΔM11L recipients, day 7.

Several aspects of the primary tumors in these animals differed greatly from observations made with wt MYX recipients at this time point. An extensive inflammatory infiltrate was noted in myxomas from vMYX-GF$^-$ΔM11L rabbits (see Fig.III-3 panel C). It was composed of a mixture of lymphocytes and heterophils, resembling much more the inflammation seen within and around Shope fibromas than that observed within malignant fibromas or myxomas (Strayer *et al.*, 1984). In addition, subepidermal edema was prominent. Spleen sections from these animals showed somewhat less perifollicular hyperplasia than that seen in wild type MYX recipients. Follicular proliferation, however, was prominent, indicative of a vigorous cell-mediated immune response to the viral infection. The red pulp was expanded in these animals, and sinusoids were moderately congested. Other organs examined were normal.

MYX recipients, day 11.

The primary tumors in these animals showed classic myxoma-like tumor cells, extensive hemorrhage and infiltration with heterophils (Fenner and Ratcliffe, 1965). Copious amounts of extracellular matrix were deposited in areas surrounding skin appendage structures, especially hair follicles. The conjunctiva showed increased squamous metaplasia and hyperplasia, with many bizarre squamous cells in the epithelium, multiple large, atypical tumor cells in the underlying stroma and a large subepithelial tumor. In the spleen, the pattern of perifollicular hyperplasia was prominent, but follicular zones were unremarkable.

vMYX-GF$^-$ ΔM11L recipients, day 11.

Much loss of primary tumor mass was observed, with considerable subepidermal edema and extensive infiltration of the tumor area by lymphocytes and heterophils (see Fig.III-3 panel D). Though myxoma-like tumor cells were identifiable, they were sparse and were often surrounded by a ring of heterophils and/or lymphocytes. In these respects, vMYX-GF$^-$ΔM11L myxomas closely resemble regressing Shope fibromas (Strayer *et al.*, 1984). Other organs examined from these animals were normal.

Growth of vMYX-GF$^-$ΔM-11 in cultured cells *in vitro*

The growth properties of vMYX-GF$^-$ΔM11L, vMYXlac, and MYX were examined in a variety of susceptible rabbit and primate cells *in vitro*. In all experiments, vMYXlac and MYX behaved identically. We observed no morphological differences in the foci induced by wild type MYX, vMYXlac, or vMYX-GF$^-$ΔM11L on BGMK, SIRC, or BSC-1 cells (not shown). On RK-13 cells, however, MYX and vMYXlac produced characteristic foci, whereas vMYX-GF$^-$ΔM11L lesions contained few cells in the center of the infected area and thus had a more plaque-like morphology (not shown). The growth curves of high multiplicity (m.o.i.=5) single step productive infections of RK-13 cells by

MYX and vMYX-GF$^-$ΔM11L, however, were very similar (not shown). We examined the ability of MYX, vMYXlac, and vMYX-GF$^-$ ΔM11L to propagate through cultured cells by performing low multiplicity (m.o.i.=0.002) infections in RK-13 (not shown) and SIRC cells (Fig. III-4) and observed only minor differences in titers, whether or not the infected cells had been actively growing or quiescent at the time of infection (not shown).

The ability to grow in resting and mitogenically stimulated lymphocytes is crucial to the pathogenesis of MYX infection because it provides a means for impairing immune function as well as expediting dissemination in the host via the lymphatic channels (Strayer *et al.*, 1985 and 1987). The ability of the *Leporipoxviruses* to propagate in cells of the immune system has been shown to correlate with the extent of pathogenicity upon infection of a susceptible host. For example, the highly virulent MRV and MYX viruses replicate efficiently in cultures of spleen cells of normal rabbits, even without mitogenic stimulation (Strayer, 1988; Strayer and Leibowitz, 1987; Strayer *et al.*, 1985). On the other hand, SFV, a benign virus which induces only a localized primary tumor without concomitant immune suppression, is completely incapable of propagation in primary splenic cultures or in pure lymphocyte populations (Heard *et al.*, 1990; Strayer *et al.*, 1990). Therefore, any impediment to growth of MYX in lymphocytes by the consequence of the 460 bp deletion could in theory explain the observed attenuation. We examined the growth of vMYXlac and vMYX-GF$^-$ ΔM11L in spleen cell cultures in the presence or absence of Con A stimulation at m.o.i.s of 0.1 (not shown), 0.01 (not shown), and 0.001 (Fig.III-5). At all multiplicities tested, in the presence of Con A, vMYX-GF$^-$ΔM11L produces substantially lower amounts of infectious progeny than vMYXlac. Even more dramatically, in the absence of Con A stimulation, vMYX-GF$^-$ΔM11L appears unable to replicate to any significant extent, suggesting that resting T cells are unable to support the replication of this deletion mutant or that replication of the mutant virus is being actively inhibited by other cell types within the splenic cell population. We conclude that vMYX-GF$^-$ΔM11L is markedly restricted in its ability to grow in a mixed lymphocyte population *in vitro* and suggest that

the impaired ability to propagate in lymphocytes *in vivo* would be an important block to the spread of virus to secondary sites.

Construction and analysis of vMYX-GF$^-$ with an intact M11L gene

To evaluate the effect of MGF deletion alone in the presence of an unaltered M11L ORF, we constructed vMYX-GF$^-$ as described in Materials and Methods (see Fig.III-2). vMYX-GF$^-$ contains a β-gal cassette inserted at the Bsa B1 site in the middle of the MGF coding sequences (Upton *et al.*, 1987), thereby interrupting the ORF in a fashion analogous to the SFGF deletion in MRV-GF$^-$ (Opgenorth *et al.*, 1992). A total of 24 rabbits were infected intradermally with 10^3 pfu of either MYX, vMYXlac, or vMYX-GF$^-$ in order to examine the effects of the MGF deletion on the pathogenic properties of MYX during the progression of myxomatosis. Our observations are summarized in Table III-1. The effect of interrupting MGF in MYX was indistinguishable from that resulting from inactivation of SFGF in MRV, both *in vitro* and *in vivo* (Opgenorth *et al.*, 1992). That is, vMYX-GF$^-$ recipients developed a moderated form of myxomatosis that included development of secondary lesions as well as bacterial infections causing purulent conjunctivitis and rhinitis. However, the disease symptoms were milder than those caused by wild type MYX and the majority of rabbits infected with vMYX-GF$^-$ never became seriously ill . As with SFGF-minus MRV (Opgenorth *et al.*, 1992), vMYX-GF- infection induced myxoid primary and secondary tumors that contained fewer proliferating cells than their wild type counterparts. Nasal mucosa and conjunctiva from vMYX-GF$^-$ recipients displayed a marked reduction in the squamous metaplasia and hyperplasia that is characteristic of wild type MRV and MYX infection. Thus, both vMYX-GF$^-$ and vMRV-GF$^-$ viruses are less virulent in rabbits probably because they induce less damage to target epithelia, resulting in a reduced degree of concomitant Gram negative bacterial infections in the respiratory tract. The colony morphology of vMYX-GF$^-$ was identical to those of MYX and vMYXlac on RK-13, SIRC, and BGMK cells (not shown), and growth curves in primary spleen cell cultures were very similar (Fig.III-6). We conclude that MGF contributes to MYX pathogenicity in

a fashion analogous to that of SFGF in MRV, and that the extensively attenuated phenotype of vMYX-GF$^-$ΔM11L is not simply due to the absence of functional MGF.

Construction of a MYX mutant deleted in M11L

We sought to determine the contribution of M11L to the properties of MYX infection by constructing a mutant (vMYX-M11L$^-$) in which 53 bp between the EcoRV and Hinc II sites near the beginning of the M11L coding sequences were deleted and replaced with the β-gal cassette (see Materials and Methods), thereby inactivating the M11L ORF (Fig. III-2). In order to confirm the genomic structure of vMYX-M11L$^-$, Southern blots with wild type and recombinant vMYX-M11$^-$ were performed using the β-gal cassette and the MGF-containing 1.5 kb MYX Hinc II fragment as probes. Digestion with diagnostic restriction enzymes such as Bgl I, Hinc II, and Sma I yielded the predicted products (not shown), indicating that the vMYX-M11$^-$ genome does not contain an intact M11L gene and that the loss of this gene does not affect the viability of the virus in cell culture. Western blot analysis using anti M11L antisera indicates that cells infected with wild type MYX and vMYXlac, but not vMYX-M11L$^-$, contain M11L protein (Graham *et al.*, 1992), confirming that the M11L ORF has indeed been rendered nonfunctional in vMYX-M11L$^-$.

A total of 24 rabbits were infected intradermally with 10^3 pfu of either vMYXlac or vMYX-M11$^-$ in order to examine the effects of the M11L deletion on the pathogenic properties of MYX during the progression of myxomatosis. Our gross pathological observations are summarized in Table III-1. vMYX-M11$^-$ recipients underwent a disease course which differed substantially from vMYXlac or wild type MYX infection. Within 6 days post infection, rabbits that had received vMYX-M11$^-$ developed a local tumor at the site of inoculation that was more clearly demarcated from the surrounding skin than the corresponding lesions induced by wild type MYX or vMYXlac. By 8 days p.i., when vMYXlac and wild type MYX recipients displayed marked signs of purulent bacterial infection in the conjunctiva and nasal mucosa, vMYX-M11$^-$ recipients were asymptomatic

in this respect. However, they developed secondary tumors which were much larger and more protuberant than corresponding wild type lesions, and were especially prominent on the eyelids. The majority (approximately 60%) of vMYX-M11$^-$ recipients were completely free of secondary bacterial infections in the conjunctiva and nasal passages throughout the course of the disease, and the remainder had only mild symptoms. Those vMYX-M11L$^-$ recipients that did develop bacterial infections remained asymptomatic until approximately 15 days post-infection, and then exhibited only very minor signs of conjunctivitis and rhinitis. At no time during the course of the infection did any of the vMYX-M11L$^-$ recipients develop secondary bacterial infections or dyspnea that were comparable in severity to those characteristic of wild type MYX or vMYXlac infection. By 17 days p.i.,the vMYX-M11L$^-$ lesions began to regress and recovery was essentially complete by 40 days p.i. In summary, these results show that vMYX-M11L$^-$ is highly attenuated, indicating that the M11L gene product is important for MYX virulence.

The histopathological profile of vMYX-M11L$^-$ infection was conspicuously different from that of wild type MYX or vMYXlac in several respects (Table III-3). Sections of primary and secondary tumors taken from vMYX-M11L$^-$ recipients 10 days p.i. revealed marked vesiculation occurring within the epidermal layer of skin overlying the tumor, which was not present in tumors of vMYXlac recipients (Fig. III-7). The dermis in vMYX-M11L$^-$ tumors contained a much more prominent heterophilic infiltrate than the corresponding vMYXlac tissue, and the degree of edema was also significantly greater. Splenic macrophages of vMYX-M11L$^-$ recipients contained considerable granular debris, whereas this was not observed in the vMYXlac counterparts. Together, these observations suggest a more intense inflammatory reaction to vMYX-M11L$^-$ infection. The spleen of vMYX-M11L$^-$ recipients also displayed hyperplasia of the periarteriolar lymphoid sheaths, but the proportion of immature lymphocytes was markedly lower than that found in vMYXlac recipients, which indicates a lesser degree of lymphocytic depletion. No

differences between vMYXlac and vMYX-M11L⁻ recipients in the kidney and liver were observed.

Growth of vMYX-M11L⁻ in cultured cells *in vitro*

The growth properties of vMYX-M11L⁻ and vMYXlac were examined in rabbit kidney (RK-13) cells. Wild type MYX and vMYXlac produced characteristic foci, whereas vMYX-M11L⁻ lesions contained very few cells in the center of the infected area and thus had a more plaque-like morphology which was similar to that of vMYX-GF⁻ ΔM11L. We examined the ability of vMYXlac and vMYX-M11L⁻ to propagate in cultured cells by performing low multiplicity (m.o.i.=0.002) infections in RK-13 cells and observed no differences in titers between the two viruses, whether or not the infected cells were actively growing or quiescent at the time of infection (not shown).

We examined the growth of vMYXlac and vMYX-M11L⁻ in spleen cell cultures in the presence or absence of Con A at m.o.i.=0.001 (Fig.III-8). In the presence of Con A, vMYX-M11L⁻ grows to less than 10 fold lower titers than vMYXlac. In the absence of Con A stimulation, vMYX-M11L⁻ appears completely unable to propagate in these cultures. These results suggest that the inability of vMYX-GF⁻ΔM11L to propagate in unstimulated primary spleen cell cultures is due to the absence of a functional M11L gene. Furthermore, the M11L gene product may have a role either in virus replication in lymphocytes or in reducing the destruction of infected cells in these cultures by immune effector cells.

The use of myxoma virus in Australia during the 1950's as a biological control agent against feral European rabbits has lead to extensive investigation of the epidemiology and pathogenesis of myxomatosis (Fenner, 1990a; Fenner and Ratcliffe, 1965). This work has resulted in the isolation of many strains of myxoma virus which vary substantially in virulence and in the severity of symptoms induced upon infection of rabbits. To date, however, experiments using naturally occurring attenuated strains have not allowed dissection of the genetic components that contribute to the pathogenicity of MYX infection. In this study we demonstrate that deletion of a small, defined region of the genome of a highly virulent strain of MYX (Lausanne) results in nearly complete attenuation of the viral disease. The two virulence factors whose combined inactivation appears to be responsible for the generation of this non-pathogenic phenotype are MGF, a member of the EGF family of growth factors (Upton *et al.*, 1987), and a novel virulence marker designated M11L.

We chose to examine the role of the MGF locus in pathogenesis because other members of the EGF family, including TGFα, VGF, and SFGF, are known to promote cellular hyperplasia (Burgess, 1989; Carpenter and Cohen, 1990; Laurence and Gusterson, 1990; Lin *et al.*, 1988; Salomon *et al.*, 1990; Ye *et al.*, 1988). A synthetic peptide spanning residues 30-83 of the MGF gene, which includes the six conserved cysteine residues involved in EGF receptor binding, has also been shown to be biologically active, suggesting the *in vivo* role for this protein as an EGF receptor ligand (Lin *et al.*, 1991). Furthermore, abrogation of VGF expression has also been found to decrease the virulence of vaccinia virus in intracranial lethality assays in mice (Buller *et al.*, 1988a) and deletion of the SFGF gene in MRV results in moderated disease symptoms, especially conjunctivitis and rhinitis associated with epithelial metaplasia and hyperplasia at secondary sites of infection (Opgenorth *et al.*, 1992). In order to excise the MGF gene from the MYX genome we made use of a unique Kpn I site in the third codon of the MGF coding sequence and a

downstream Nru I site which maps 5' to the promoter of the adjacent gene M9L. In addition to removing the MGF locus, the 460 nt Kpn I-Nru I deletion also altered the 3' end of M11L because the C terminal 6 amino acids overlap with the N terminus of MGF, although in a different reading frame. The consequence of this M11L alteration has been to truncate M11L and fuse the ORF to vector sequences from the β-gal cassette. Both orientations of the selectable marker gave virus constructs with the identical phenotype, suggesting that the pronounced attenuation of vMYX-GF$^-$ ΔM11L is due to loss of both M11L and MGF functions. The possibility that additional undetected genomic alterations might be responsible for the attenuation are remote for several reasons: 1) multiple independent isolates of vMYX-GF$^-$ΔM11L resulted in the same phenotype for each clone, 2) the orientation of the β-gal cassette in vMYX-GF$^-$ΔM11L had no effect on the phenotype, 3) insertion of the β-gal cassette immediately downstream of MGF at the Nru I site between MGF and M9L to generate vMYXlac had no effect on pathogenicity.

Dramatic differences between MYX and vMYX-GF$^-$ΔM11L both *in vivo* and *in vitro* were observed. Recipients of vMYX-GF$^-$ΔM11L developed a syndrome closely resembling Shope fibroma virus (SFV) infection (McFadden, 1988; Strayer *et al.*, 1985), in which a primary tumor at the site of injection completely regressed by 14 days after infection, without evidence of secondary bacterial infection or any detectable compromise in immune function. Infection with very high inocula of vMYX-GF$^-$ΔM11L either intradermally or intravenously did not alter this SFV-like condition, indicating that the attenuation is indeed profound. Histologically, the tumors induced by MYX and vMYX-GF$^-$ΔM11L differ in the extent and type of inflammation present. Whereas MYX tumors contain a mild, chiefly heterophilic inflammatory infiltrate, those of vMYX-GF$^-$ΔM11L recipients are massively infiltrated with a mixture of lymphocytes and heterophils, indicative of a more effective immune response to the virus infection. In MYX recipients, the conjunctiva displayed considerable squamous metaplasia and hyperplasia, which is thought to increase the susceptibility of such epithelia to bacterial superinfection (Heath,

1979; Jakab *et al.*, 1979; Strayer and Sell, 1983). The lack of such epithelial alterations and, indeed the absence of virtually any secondary site lesions, in vMYX-GF$^-$ΔM11L recipients may partially explain the complete abrogation of the secondary bacterial infections that normally cause purulent conjunctivitis and rhinitis in these animals. *In vitro*, vMYX-GF$^-$ΔM11L behaved very similarly to the wild type parent and vMYXlac in cultured cell lines, although the foci produced in RK-13 cells were rather less proliferative than those produced by the MGF$^+$/M11L$^+$ controls. Much more dramatically, in mitogenically stimulated spleen cell cultures vMYX-GF$^-$ΔM11L replicated to 10 fold lower titers than wild type MYX. In the absence of mitogenic stimulation, vMYX-GF$^-$ΔM11L appeared completely incapable of replication in spleen cells, whereas MYX grows well in these cultures. These results suggest that vMYX-GF$^-$ΔM11L may be impaired in its ability to propagate in lymphocytes *in vivo*, precluding effective dissemination of virus via infected lymphocytes to secondary sites. Our inability to culture virus from spleens of vMYX-GF$^-$ΔM11L recipients at 7 days p.i. supports this notion (not shown). Thus, it appears that the absence of induced epithelial hyperplasia and metaplasia at secondary sites of viral replication combined with decreased capacity to disseminate through infected lymphocytes contribute greatly to the loss of virulence by vMYX-GF$^-$ΔM11L.

The attenuation displayed by vMYX-GF$^-$ΔM11L is much more severe than that expected by inactivation of MGF alone. Deletion of the growth factor gene in MRV, a virus which is closely related to MYX but instead of possessing MGF encodes the related SFGF gene, resulted in an MRV-like syndrome of reduced severity but still qualitatively similar to that of the parent virus, except that target epithelia were less affected by the virus than those of wild type MRV recipients, showing less severe bacterial infections and decreased squamous hyperplasia and metaplasia (Opgenorth *et al.*, 1992). Although 75% of MRV-GF$^-$ recipients survived infection, MRV-GF$^-$ was indistinguishable from wild type MRV in *in vitro* assays, including colony morphology and growth in spleen cell cultures. In order to ascertain if the more dramatic attenuation resulting from the 460 bp Kpn I-Nru I deletion

in MYX could be explained by differing biological roles of MGF in MYX as compared to SFGF in MRV, we inserted the β-gal cassette into the single Bsa B1 site in the middle of the MGF open reading frame and obtained a recombinant virus (vMYX-GF$^-$) whose pathological profile was now almost identical to that observed for MRV-GF$^-$. Therefore, vMYX-GF$^-$ΔM11L is more profoundly attenuated than either MRV-GF$^-$ or vMYX-GF$^-$, and this attenuation cannot be attributed solely to deletion of the MGF gene alone.

These results strongly indicated that the partially overlapping M11L ORF immediately upstream of MGF is probably a virulence determinant in addition to MGF. Although the Kpn I-Nru I excision was constructed to remove only five amino acids from the M11L C terminus, this alteration directly affected M11L function. In fact, localization experiments with anti-M11L antibody suggest that M11L is normally inserted at the infected cell surface but cannot be detected at the surface of cells infected with vMYX-GF$^-$ΔM11L (Graham *et al.*, 1992). In order to confirm the possibility that alteration of the M11L open reading frame affected the virulence properties of vMYX-GF$^-$ΔM11L, we also constructed a unique M11L deletion mutant of MYX (vMYX-M11L$^-$) in which a small portion of the M11L coding sequence was excised and replaced with a β-gal cassette, nevertheless leaving MGF intact and functional. The properties of this virus *in vivo* and *in vitro* demonstrate that M11L is indeed an independent virulence factor in MYX. Deletion of M11L had a profound effect on the ability of MYX to propagate in primary spleen cell cultures (Fig.III-8), and the *in vitro* growth properties in spleen cell cultures of vMYX-M11L$^-$ were similar to those of vMYX-GF$^-$ΔM11L. In either the presence or absence of the non-specific T cell mitogen Con A, the productive replication of vMYX-M11L$^-$ was severely impaired in these cultures compared with that of vMYXlac or wild type MYX. These results suggest that the M11L product is necessary for efficient growth in lymphocytes, or, alternatively, that the absence of M11L may facilitate destruction of virus-infected cells by phagocytic or cytotoxic cells present in the spleen cell cultures. .

The disease course upon infection of rabbits with vMYX-M11L$^-$ is clearly attenuated compared with wild type MYX or vMYXlac infection since 100% of vMYX-M11L$^-$ recipients survived infection whereas MYX and vMYXlac infections are invariably fatal (Table III-1). vMYX-M11L$^-$ recipients developed only very minor secondary bacterial infections in contrast with the severe purulent conjunctivitis and rhinitis that always accompany wild type MYX and vMYXlac infection. Despite the mildness of vMYX-M11L$^-$ infection, the protuberant tumors induced by this virus were much larger and more demarcated compared with those of vMYXlac recipients. Histological examination of primary and secondary tumors indicated that the large size of the tumors was due primarily to greater edema and massive heterophilic infiltration at sites of viral replication, rather than a more pronounced fibroblastic proliferative response. These observations, together with the much more severe dermatitis in vMYX-M11L$^-$ tumors, are indicative of a strong acute inflammatory response which is not present in control vMYXlac or MYX lesions. Sections of spleens from vMYX-M11L$^-$ recipients showed much less depletion of mature lymphoid cells in the periarteriolar lymphoid sheaths compared with their vMYXlac counterparts, reflecting a state of greater T-cell immune activation. The large amounts of cellular debris observed within splenic macrophages are also indicative of a more vigorous acute inflammatory activity. Together, these observations indicate a role for M11L in inhibiting the generation of a cellular immune response to viral infection at some early step, for example,the chemotactic recruitment and/or activation of infiltrating inflammatory cells. Experiments with anti-M11L antisera (Graham *et al.*,1992) indicate that M11L is expressed and transported to the surface of MYX-infected cells. Importantly, M11L function is lost if the protein does not localize at the cell surface, suggesting that the M11L protein may function by dampening an effective early inflammatory reaction. We conclude that M11L is an important virulence factor in MYX, possibly by acting as a membrane-bound "viroceptor" (Upton *et al.*,1991) involved in inhibition of an early step in the development of an acute inflammatory response to viral infection. It appears clear that M11L is

pathological marker whose effects, when combined with those of other genes such as MGF, contribute to the development of full blown myxomatosis. The vMYX-GF$^-$ΔM11L mutant described here is a dramatically attenuated form of MYX and provides the first example of a defined deletion in myxoma virus that renders it essentially avirulent in susceptible rabbits. In addition, vMYX-GF$^-$ΔM11L should provide a useful genetic background for evaluating the effects of other putative virulence factors on poxvirus pathogenesis.

Figure III-1. Diagram of left end of myxoma virus genome showing location of the MGF and M11L ORFs. The Bam HI fragments AA, S, CC, K, DD, and E are as in (37) except the orientation has been reversed (see text). Upper arrow denotes the viral TIR and lower arrows denote open reading frames. The site of the Kpn I-Nru I 460 bp deletion is indicated. The solid vertical arrow indicates site of β-gal cassette insertion at the Nru I site in the control virus vMYXlac. The dashed vertical arrow indicates the site of β-gal cassette insertion at the Bsa B1 site in vMYX-GF$^-$. The site of the Eco RV-Hinc II deletion in vMYX-M11L$^-$ is shown. Abbreviations are Bm, Bam HI; B, Bsa BI; E, Eco RV; H, Hinc II, K, Kpn I; N, Nru I.

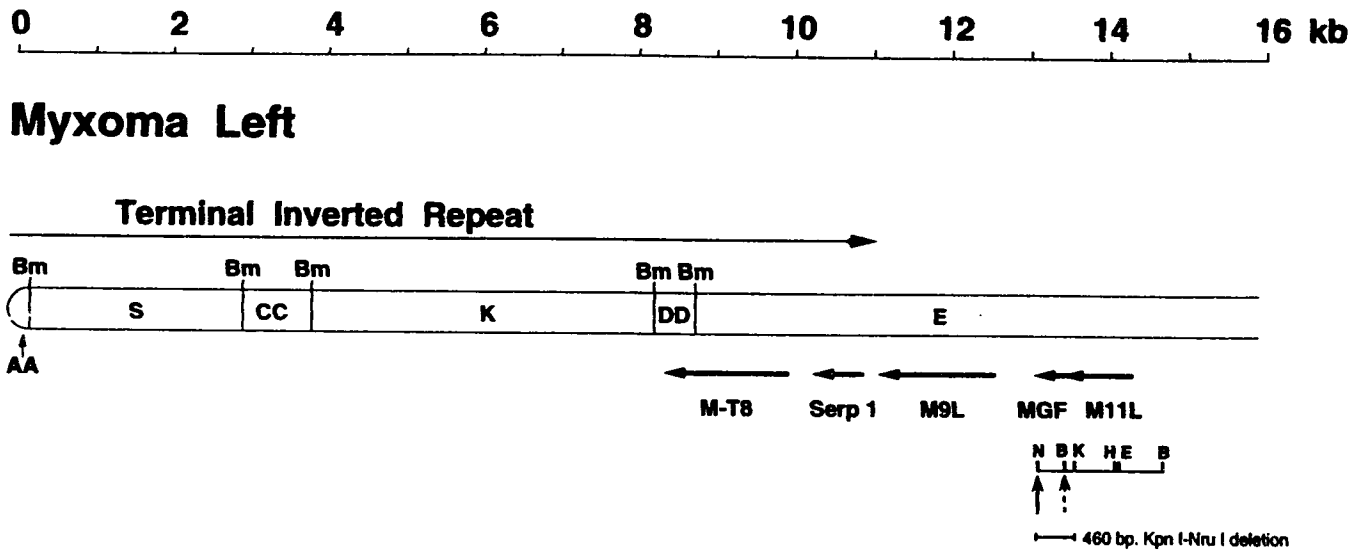
0
2
4
6
8
10
12
14
16 kb
Myxoma Left
Terminal Inverted Repeat
Bm
Bm
Bm
Bm Bm
S
CC
K
DD
E
AA
M-T8
Serp 1
M9L
MGF
M11L
N B K H E B
460 bp. Kpn I-Nru I deletion

Figure III-2. Structures of MYX, vMYXlac, vMYX-GF$^-$ ΔM11L, vMYX-GF$^-$, and vMYX-M11L$^-$ genomes near the MGF locus. The orientations are reversed from Figure 1. Abbreviations are B1, Bgl I; B, Bsa BI; E, Eco RV; H, Hinc II; K, Kpn I; N, Nru I.

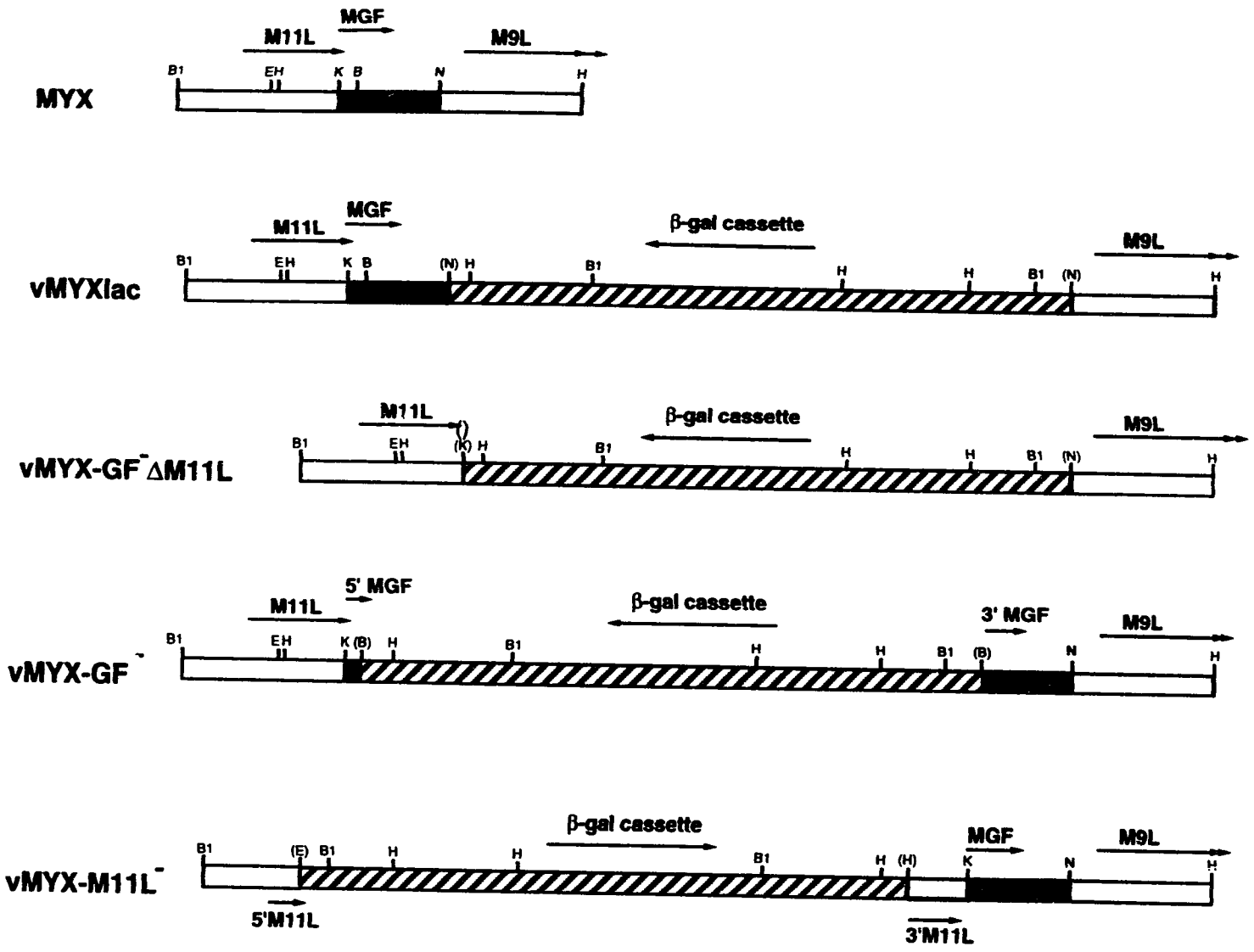
MYX
M11L
MGF
M9L
B1
EH
K B
N
H
vMYXlac
β-gal cassette
(N) H
vMYX-GF⁻ΔM11L
(K) H
vMYX-GF⁻
5' MGF
3' MGF
K (B) H
(B)
vMYX-M11L⁻
(E) B1
(H)
5'M11L
3'M11L

Figure III-3. (Panel A) MYX primary tumor, day 7. The tumor is composed of proliferations of fibroblasts and extensive elaboration of connective tissue matrix filaments, with an infiltrate of heterophil leukocytes.

(Panel B) MYX conjunctiva, day 7. Normally 2-3 cell layers thick, the epithelial layer of the conjunctiva in these rabbits has proliferated to several times its normal thickness. Underlying tumor is seen at the bottom of the frame. Conjunctiva from vMYX-GF$^-$ΔM11L recipients is unaffected at both 7 and 11 days p.i.

(Panel C) vMYX-GF$^-$ΔM11L primary tumor, day 7. The cells comprising these tumors are similar to those seen in wild type tumors except that a prominent inflammatory infiltrate is noted (arrows). This type of infiltrate is absent in MYX or vMYXlac tumors.

(Panel D) vMYX-GF$^-$ΔM11L primary tumor, day 11. These tumors differ from their day 7 counterparts in showing a much more prominent lymphoid infiltrate. Abundant clusters of lymphocytes (arrows) are shown.

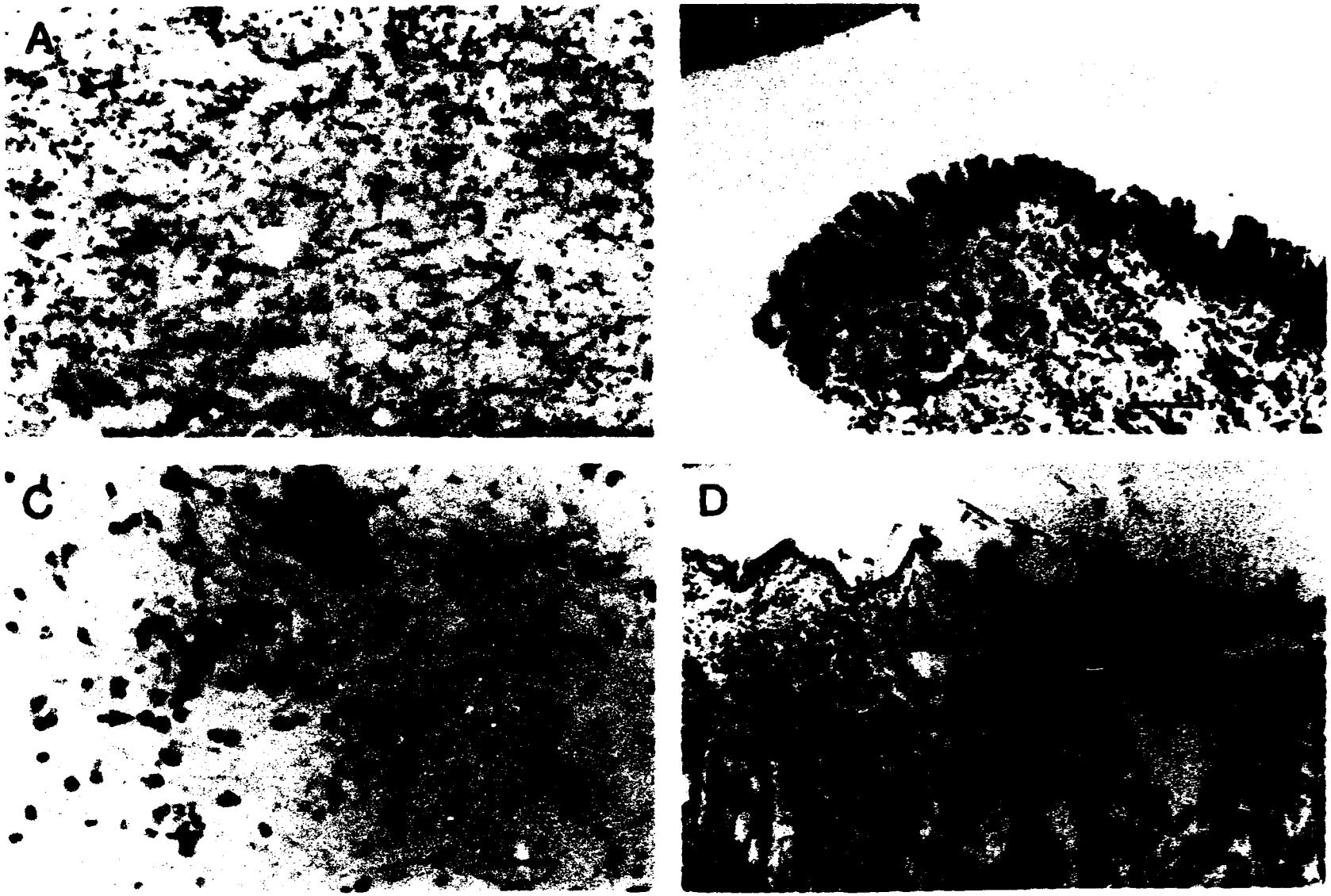
A
C
D

Figure III-4. Growth of vMYXlac and vMYX-GF$^-$ΔM11L in tissue culture. SIRC cells were infected at a m.o.i. of 0.004, harvested at various times p.i., and infectious virus titered on RK-13 cells.

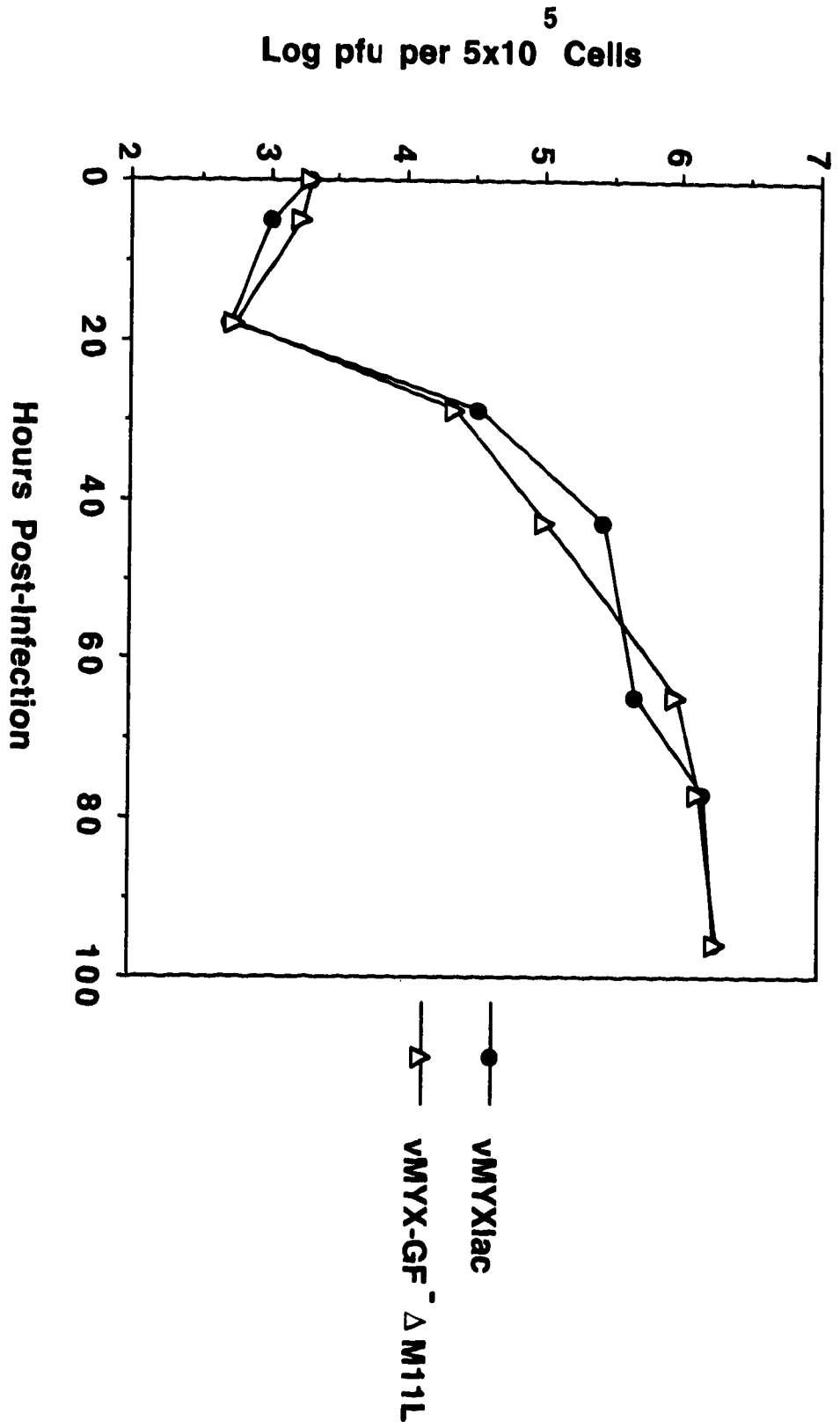
Log pfu per 5x10^5 Cells
2
3
4
5
6
7
0
20
40
60
80
100
Hours Post-Infection
vMYXlac
vMYX-GF⁻ ΔM11L

Figure III-5. Growth of vMYXlac and vMYX-GF$^-$ΔM11L in primary rabbit spleen cell cultures. Cultures of spleen cells isolated from normal rabbits were infected with vMYXlac or vMYX-GF$^-$ΔM11L at a m.o.i. of 0.001 in the presence or absence of the non-specific T cell mitogen Con A. Cultures were harvested at various times p.i. and titered on RK-13 cells.

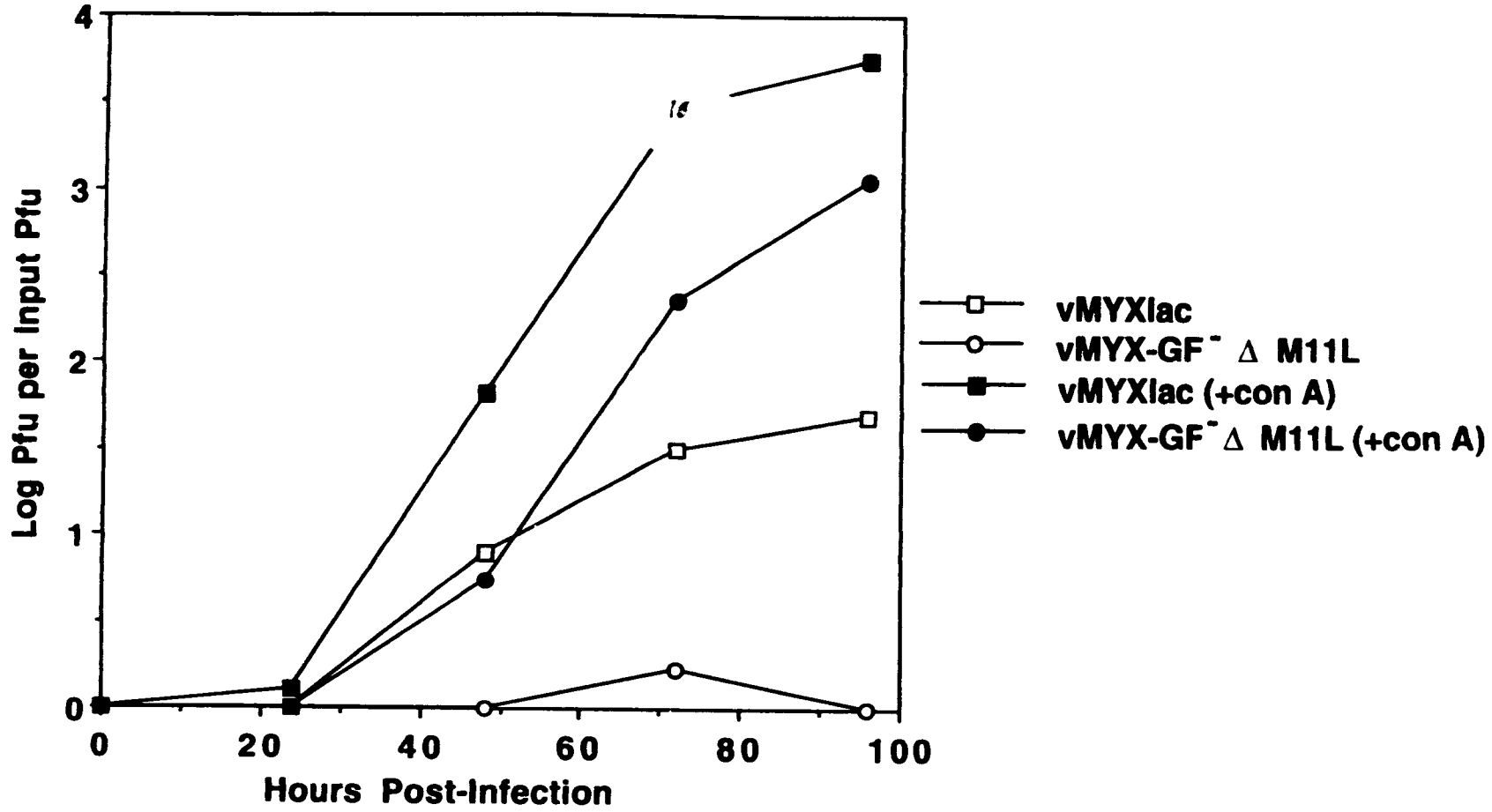
Log Pfu per Input Pfu
0
1
2
3
4
0
20
40
60
80
100
Hours Post-Infection
vMYXlac
vMYX-GF⁻ Δ M11L
vMYXlac (+con A)
vMYX-GF⁻Δ M11L (+con A)

Figure III-6. Growth of vMYXlac and vMYX-GF$^-$ in primary rabbit spleen cell cultures. Cultures of spleen cells isolated from normal rabbits were infected with vMYXlac or vMYX-GF$^-$ at a m.o.i. of 0.001 in the presence or absence of the non-specific T cell mitogen Con A. Cultures were harvested at various times p.i. and titered on RK-13 cells.

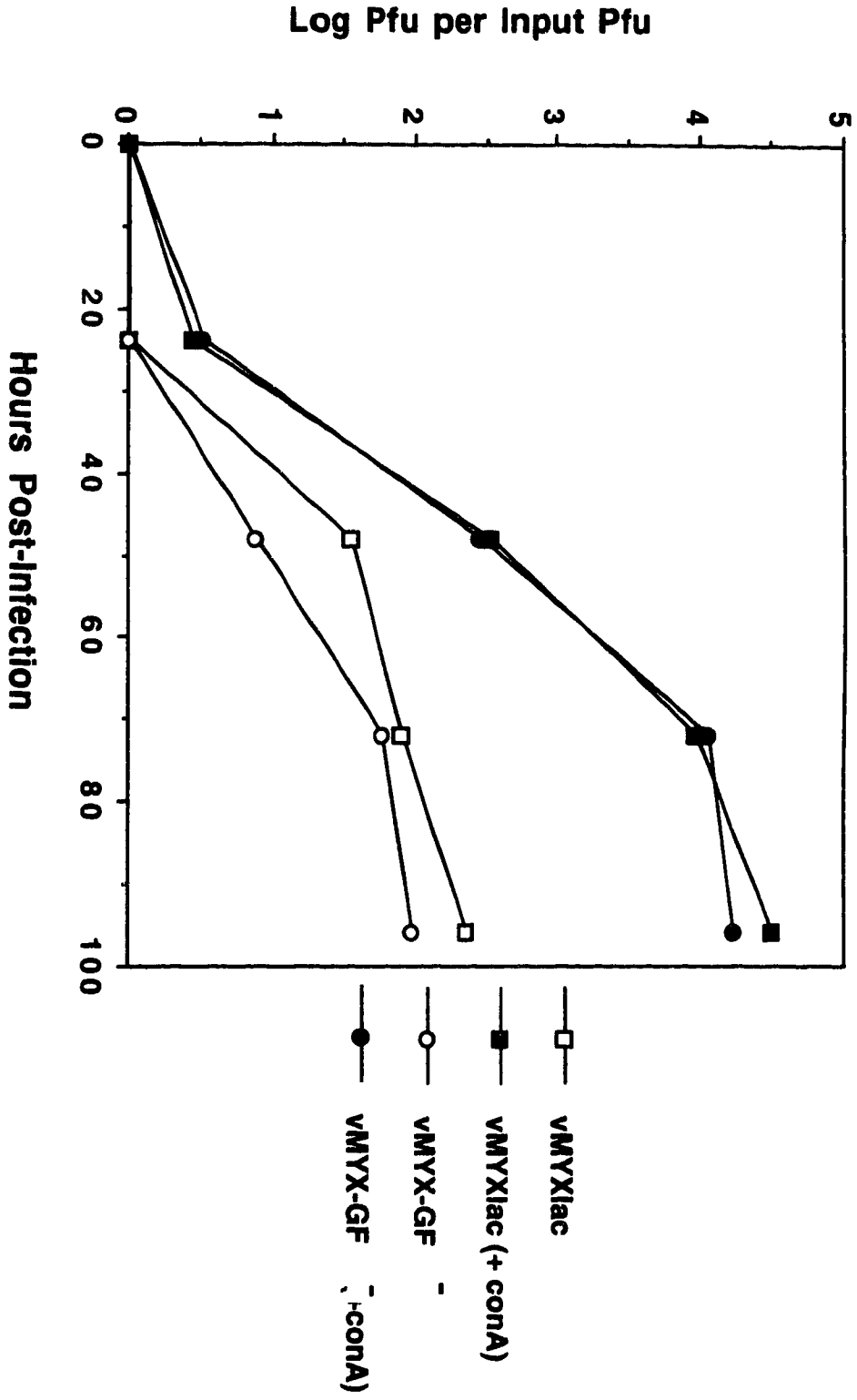
Log Pfu per Input Pfu
0
1
2
3
4
5
Hours Post-Infection
0
20
40
60
80
100
vMYXlac
vMYXlac (+ conA)
vMYX-GF
vMYX-GF (+conA)

Figure III-7. Primary tumor from rabbits that had received vMYXlac (Panel A) or vMYX-M11L$^-$ (Panel B) 9 days previously.

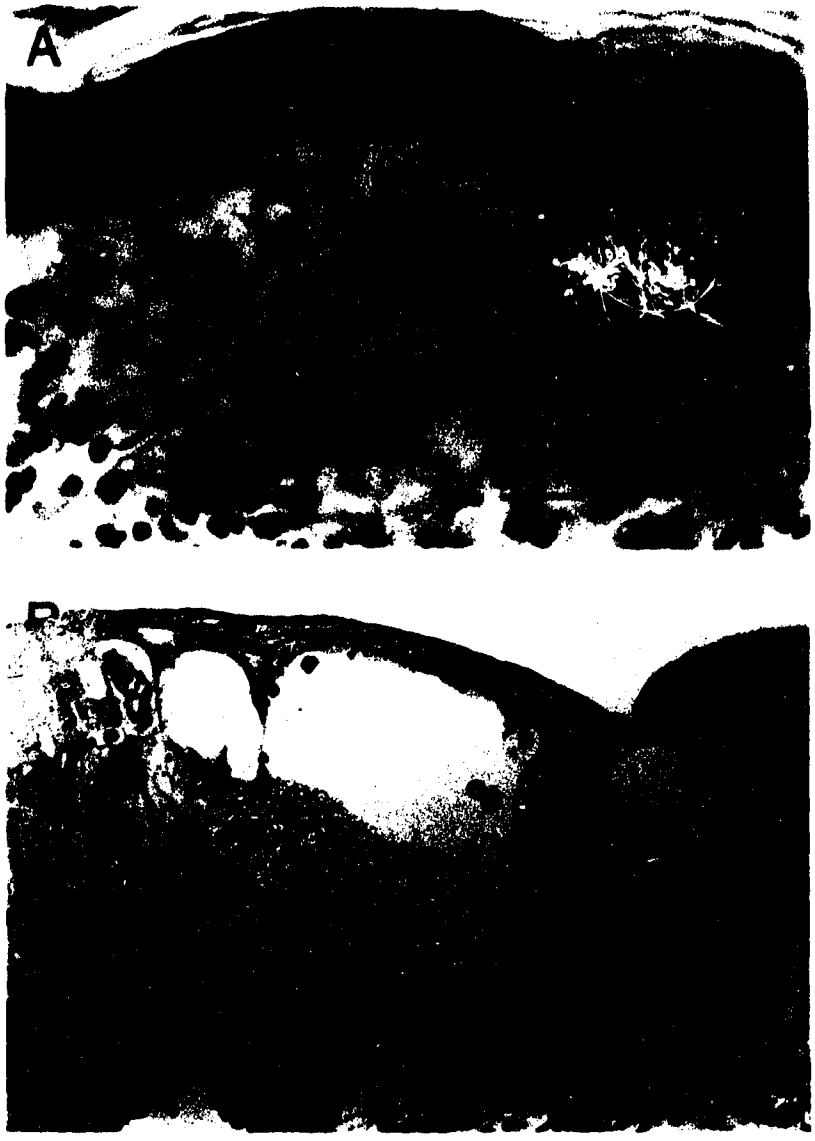
A
B

Figure III-8. Growth of vMYXlac and vMYX-M11L$^-$ in primary rabbit spleen cultures. Cultures of spleen cells isolated from normal rabbits were infected with vMYXlac or vMYX-M11L$^-$ at a m.o.i. of 0.001 in the presence or absence of Con A. Cultures were harvested at various times p.i. and titered on RK-13 cells.

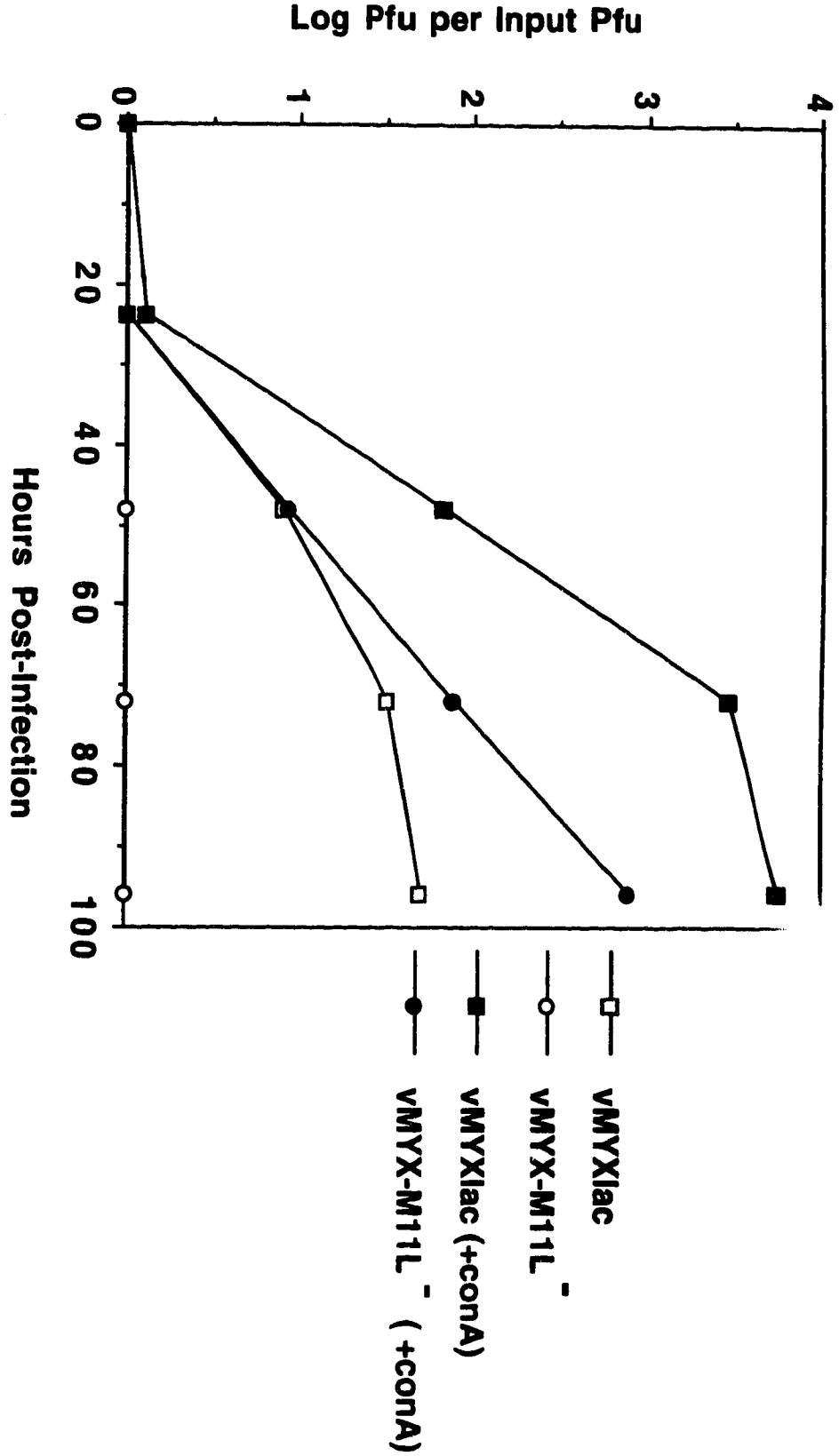
Log Pfu per Input Pfu
0
1
2
3
4
Hours Post-Infection
0
20
40
60
80
100
vMYXlac
vMYX-M11L⁻
vMYXlac (+conA)
vMYX-M11L⁻ (+conA)

Table III-1. Pathogenicity of MYX, vMYXlac, vMYX-GF⁻ΔM11L, vMYX-GF⁻ and vMYX-M11L⁻ in Rabbits

Virus	Inoculum	Symptoms		Fate
MYX or vMYXlac	10^3 pfu	day 5	Localized primary tumor	0 % survival
		day 9	Gram negative infection of nasal mucosa and conjunctiva; Disseminated tumor at multiple secondary sites; Severe purulent conjunctivitis, rhinitis, and death	
vMYX-GF⁻ΔM11L	10^3 pfu	day 5	Localized primary tumor	100% survival
		day 9	Primary tumor begins to regress; No secondary lesions observed; No conjunctivitis or rhinitis	
		day 14	Compete recovery Animals immune to challenge	
vMYX-GF⁻ΔM11L	10^6 pfu	day 5	Localized primary tumor	100% survival
		day 7	Slight redness of conjunctiva; Occasional lesion (1-2 mm) on eyelid or ear; No conjunctivitis or rhinitis	
		day 9	Primary tumor regresses	
		day 14	Complete recovery; Animals immune to challenge by MYX	
vMYX-GF⁻	10^3 pfu	day 5	Localized tumor	75% survival
		day 7	Conjunctiva begin to redden	
		day 8	Bacterial infection in conjunctiva and nares; Secondary lesions appear; Some animals develop labored breathing	
		days 10-15	Primary tumors regress; 25% become moribund	
vMYX-M11L⁻	10^3 pfu	day 5	Localized protuberant tumor at inoculation site	100% survival
		day9	Large demarcated secondary cutaneous tumors	
		day 15	Primary and secondary tumors reach largest size (4-5 cm primary; 1-1.5 cm secondary)	
		days 15-24	Mild conjunctivitis and rhinitis	
		days 17-21	Primary and seconary tumors regress	
		day 40	Nearly complete recovery (remnants of tumor still present); Immune to MYX challenge	

Table III-2. Summary of Major Histological Differences Between Wild Type MYX and vMYX-GF⁻ΔM11L Infection in Rabbits

	MYX, Day 7	vMYX-GF⁻ΔM11L, Day 7
Primary Tumor	Slight heterophilic infiltrate	Extensive lymphocytic and heterophilic infiltrate
Spleen	Prominent perifollicular hyperplasia	Prominent follicular proliferation
Conjunctiva	Squamous metaplasia and hyperplasia	Normal
	MYX, Day 11	**vMYX-GF⁻ΔM11L, Day 11**
Primary Tumor	Extensive hemorrhage; Tumor mass increasing; Heterophilic infiltrate	Considerable reduction of tumor mass; Extensive lymphocytic and heterophilic infiltrate
Spleen	Perifollicular hyperplasia	Normal
Conjunctiva	Squamous metaplasia and hyperplasia	Normal

Table III-3. **Summary of Major Histological Differences Between vMYXlac and vMYX-M11L⁻ Infection in Rabbits**

	vMYXlac	vMYX-M11L⁻
Primary and Secondary Tumors	Mild heterophilic infiltrate present throughout dermis;	Large numbers of infiltrating heterophil leukocytes and macrophages;
	Minimal vesiculation within overlying epidermal layer;	Marked vesiculation within overlying epidermal layer;
	Mild edema of dermis and subcutis	Moderate subepidermal edema
Spleen	Macrophages relatively quiescent and unactivated;	Considerable cellular debris within cytoplasm of splenic macrophages;
	Hyperplastic periarteriolar lymphoid sheaths with predominance of immature lymphocytes	Hyperplastic periarteriolar lymphoid sheaths with moderate increase in % immature lymphocytes

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CHAPTER IV-TRANSFORMING GROWTH FACTOR ALPHA, SHOPE[147] FIBROMA GROWTH FACTOR, AND VACCINIA GROWTH FACTOR CAN REPLACE MYXOMA GROWTH FACTOR IN THE INDUCTION OF MYXOMATOSIS[1]

INTRODUCTION

The poxvirus family comprises a large group of viruses that infects a wide range of vertebrate and invertebrate species (Fenner, 1990a and b). In recent years it has become apparent that the poxviruses encode a multitude of gene products which are non-essential for viral replication but nonetheless contribute to viral virulence, host range and mode of dissemination, which, in aggregate, appear to confer upon each poxvirus species a characteristic biological phenotype in infected host animals (Traktman, 1990; Turner and Moyer, 1990; Buller and Palumbo, 1991). A number of these nonessential virulence genes have been found to exist as families that are distributed across the spectrum of poxvirus genera. For example, it has been shown that most, if not all, poxviruses encode a family of growth factors that are closely related to epidermal growth factor and transforming growth factor alpha (Brown *et al.*, 1985; Chang *et al.*, 1987; Porter and Archard, 1987; Twardzik *et al.*, 1985; Upton *et al.*, 1987). The EGF-like growth factor encoded by vaccinia virus, vaccinia virus growth factor (VGF), contributes to the lethal syndrome following intracranial inoculation of mice and exacerbates the localized lesion produced upon intradermal infection of rabbits (Buller *et al.*, 1988). The biological effect of VGF is achieved by virtue of its ability to function as a *bona fide* ligand for EGF receptors on target

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cells in a paracrine fashion (Twardzik *et al.*, 1985; Stroobant *et al.*, 1985). Myxoma growth factor (MGF) (Upton *et al.*, 1987) and Shope fibroma growth factor (SFGF) (Upton *et al.*, 1988; Chang *et al.*, 1987) are pathogenic markers in myxoma virus and malignant rabbit fibroma virus (MRV), respectively, and are responsible for at least some of the epithelial and fibroblastic proliferation observed at primary and secondary sites of viral infection (Opgenorth *et al.*, 1992a and b). Because the host range, pathological effects, and outcome of the diseases induced by the poxviruses are substantially different from one another (Buller and Palumbo, 1991; Fenner, 1990a; Turner and Moyer, 1990), the extent to which the divergence in sequence between members of any one gene family gives rise to the heterogeneity in phenotype between poxvirus species remains unclear. It has been suggested that the distinctively proliferative nature of the leporipoxviruses, molluscum contagiosum, and Yaba tumor virus may be due to some unique property of their respective growth factors which is absent from growth factors of the cytolytic poxviruses, such as vaccinia virus (McFadden, 1988; Dales, 1990; Pogo *et al.*, 1989). Inspection of the primary amino acid sequences of SFGF and MGF reveals substantial differences from those of VGF, EGF and TGFα (Upton *et al.*, 1987). Whereas the amino acid sequences of SFGF and MGF are 80% identical, they are less than 40% homologous to other members of the EGF family. In addition, SFGF and MGF lack any obvious putative membrane-spanning hydrophobic regions near the C termini that are present in VGF, TGFα, and EGF precursors, suggesting that the mode of expression, and therefore biological activity, of the leporipoxviral growth factors might be unique. We therefore wished to investigate whether SFGF and MGF had unique properties that were necessary for viral pathogenesis in rabbits or whether other members of the EGF growth factor family could substitute for MGF and SFGF as virulence factors.

To address this problem, we constructed variants of myxoma virus (strain Lausanne) (Bouvier, 1954; Fenner and Burnet, 1957) in which the native MGF gene (Upton *et al.*, 1987) was disrupted and replaced with a βgal cassette engineered for expression in poxviruses (Chakrabarti *et al.*, 1985; Buller *et al.*, 1988) in tandem with either SFGF, VGF, or the cDNA of rat TGFα under the control of the vaccinia early/late p7.5 promoter (Venkatesan *et al.*, 1981; Franke *et al.*, 1985). These recombinant viruses are denoted vSΔMGF, vVΔMGF, and vTΔMGF, respectively (Fig.IV-1). In addition, a control MGF-disrupted virus was constructed in which the βgal cassette plus the p7.5 promoter alone were inserted into the MGF locus (vCΔMGF). vMYXlac contains the identical βgal cassette inserted at a site immediately downstream of the MGF gene which does not interrupt any known open reading frames and is indistinguishable from wild type myxoma virus both in vitro and in vivo (Opgenorth *et al.*, 1992a). Plasmids used in the construction of the recombinant viruses were made in the following manner. SFGF, VGF and TGFα coding sequences were placed immediately downstream of the vaccinia p7.5 promoter by ligating appropriate restriction fragments into the Bam H1 site of pVV3.0 (Franke *et al.*, 1985). Rat TGFα cDNA sequences were obtained as a 700 bp Sma I fragment from $prTGF_{0.2}$ (Lee *et al.*, 1985). The intact SFGF gene was isolated as a 600 bp Sau 3a fragment (Chang *et al.*, 1987) and ligated into the Bam HI site of pUC 19. After cleavage with Pst I and Hinc II, unidirectional digestion with exonuclease III was performed and a clone (pSGF-4 Δ1) was selected in which the endogenous SFGF promoter sequences up to 3 nucleotides upstream of the initiating methionine codon were removed (Chang *et al.*, 1987). The DNA fragment containing the SFGF sequence was then isolated as a Hind III-Eco RI fragment and blunt-end ligated into the Bam HI site of pVV3.0. The VGF gene was obtained as a 0.8 kb Hinc II-Xba III fragment from pNK19 and ligated into the Hinc II site of pUC13 (Venkatesan *et al.*, 1982). After cleavage of the product with Pst I and Hinc II, exonuclease III was used to remove DNA sequences

upstream of the VGF gene up to 40 nt upstream of the initiator methionine codon. The VGF gene was then isolated as a Hind III-Eco RI fragment and blunt-end ligated into the Bam HI site of pVV3.0. A Bgl II βgal cassette consisting of the vaccinia late p11 promoter fused to the *E. coli* lacZ gene, derived from pSC20 (Buller *et al.*, 1988), was then ligated into the Bgl II site of pVV3.0 and the pVV3.0 constructs containing the individual growth factor genes. Finally, a Sal I-Hind III fragment containing p7.5-TGFα-p11-lacZ, and Pst I fragments containing p7.5-SFGF-p11-lac Z, p7.5-VGF-p11-lac Z, and p7.5-p11-lac Z, were ligated into the Bgl II site of pBSa-7 (Opgenorth *et al.*, 1992a), which resulted in disruption of the MGF gene. Recombinant viruses were constructed as previously described (Opgenorth *et al.*, 1992a) and genomic structures were confirmed by Southern blot analysis of appropriate restriction enzyme digests (data not shown).

Rabbits were infected intradermally in the flank with 10^3 infectious units of vVΔMGF, vSΔMGF, vTΔMGF (2 rabbits each), vCΔMGF or vMYXlac (3 rabbits each), and monitored daily for external signs of developing myxomatosis, which include appearance of tumors, conjunctivitis, rhinitis, and febrility (Fenner and Marshall, 1957; Fenner and Ratcliffe, 1965). At 8 days after infection, the animals were sacrificed with euthanyl administered after anaesthesia. Tissues isolated from these rabbits at necropsy were fixed in neutral buffered 10% formalin, paraffin embedded, sectioned at 5 μm, and viewed by light microscopy. N. Nation interpreted the histological slides. K. Graham assisted with the inoculation and observation of the animals and the harvesting of tissue samples.

All animals infected with the growth factor-containing recombinants became acutely ill by 8 days after infection and were indistinguishable from those infected with the wild type parental virus with respect to the severity of illness and appearance of the lesions. The differences between clinical symptoms in vMYXlac, vVΔMGF, vSΔMGF and vTΔMGF recipients on one hand, compared with vCΔMGF recipients on the other, were very striking. As expected from previous work with vMYX-GF$^-$, a different growth factor minus myxoma virus construct (Opgenorth *et al.*, 1992a), vCΔMGF recipients developed a tumor at the site of inoculation which was markedly less hemorrhagic at the gross level than those of the other animals. None of the three vCΔMGF recipients became febrile, nor did they develop the purulent conjunctivitis and rhinitis or difficulty in breathing that are characteristic signs of wild type myxomatosis. Secondary tumors were minimal by day 8, and appeared as mild swelling and reddening of the eyelids. In contrast, all of the rabbits infected with vMYXlac, vVΔMGF, vSΔMGF or vTΔMGF became severely ill, with development of all the characteristics signs of myxomatosis including hemorrhagic tumors at the site of inoculation and at secondary sites, fever, and purulent conjunctivitis and rhinitis with dyspnea. These results show that, although MGF plays a critical role in the induction of full-blown myxomatosis, other EGF-like growth factors, including cellular TGFα, can substitute for MGF without loss of any of the hallmark symptoms of fully pathogenic infection. Moreover, SFGF, VGF, and TGFα appear to restore the gross pathogenic profile of the tumors and the extent of bacterial superinfection induced by myxoma virus in an identical fashion.

The histopathological profiles of vMYXlac, vSΔMGF, vVΔMGF, and vTΔMGF infection were also indistinguishable from each other and from that of wild type myxoma virus (Opgenorth *et al.*, 1992a; Fenner and Ratcliffe, 1965) (Figs. IV-2 and IV-3). The primary and secondary tumors induced by all of the growth factor recombinants were highly myxoid, with abundant connective tissue proteins and mucopolysaccharides

surrounding scattered atypical fibroblasts. Extensive hemorrhage and diffuse edema were present in the subcutis immediately underneath the epidermal surface. Sections from secondary tumors revealed epithelial hyperplasia and squamous metaplasia of the conjunctiva and nasal mucosa, in addition to the subepithelial tumor. Tumors from animals infected with vCΔMGF were qualitatively similar to those of wild type myxoma virus, consisting of reactive fibroblasts in a myxoid matrix. However, these lesions revealed a much smaller degree of hemorrhage, edema and necrosis than those infected with the growth factor plus strains. In addition, epithelial hyperplasia of conjunctiva and nasal mucosa were largely absent in animals infected with vCΔMGF. It has been previously suggested that the ability of wild type myxoma virus and MRV to induce hyperplasia in target epithelia may facilitate Gram negative overgrowth in infected animals, causing fatal dyspnea (Strayer, 1988; Strayer and Sell, 1983). These results show that this characteristic feature of myxoma virus infection is fully restored to MGF-minus strains by SFGF, VGF, and TGFα, and suggest that these ligands function in a fashion that is indistinguishable from that of MGF in rabbit tissues.

The EGF family comprises a group of well studied polypeptides with numerous biological activities whose roles in normal physiology and pathogenesis are nevertheless incompletely understood (Guterson and Laurence, 1990; Carpenter and Cohen, 1990; Salomon *et al.*, 1990). Despite many structural and functional similarities between members of the EGF family, certain differences in biological activities have been described. For example, TGFα is substantially more potent than EGF in promoting bone resorption (Ibbotson *et al.*, 1986), neovascularization (Schreiber *et al.*, 1986), and support of neurons (Chalazonitis *et al.*, 1992). EGF, but not VGF or TGFα, is capable of positive regulation of IFN-γ expression in mouse spleen cell cultures (Abdullah *et al.*, 1989). One study showing that a monoclonal antibody to the EGF receptor prevents TGFα but not EGF binding suggests that these two growth factors either bind to different sites on the receptor or stabilize different conformations of the EGF receptor. Recently, it has also been shown

that the ligand for the *neu* receptor is also a member of the EGF family of growth factors (Lupu *et al.*, 1992; Wen *et al.*, 1992; Holmes *et al.*, 1992), and hence the poxviral growth factors could potentially serve to stimulate cellular receptors other than that for EGF. Thus, it is of interest to demonstrate that the target cells for mitogenic stimulation by MGF, such as the epithelial cell layers overlying myxoma tumors in the conjunctiva and respiratory tract, are the same for heterologous poxviral and cellular ligands of the EGF receptor. The results in this communication show that, in the context of myxoma virus pathogenicity, VGF, TGFα, SFGF, and MGF perform qualitatively very similar roles since the lesions and disease course produced by vMYXlac, vSΔMGF, vVΔMGF, and vTΔMGF were indistinguishable at both the gross and histopathological levels. While more detailed experiments examining cellular tropism and the effect of promoter strength may reveal subtle differences between these recombinant viruses, these preliminary experiments suggest that VGF, TGFα, SFGF, and MGF have a similar biological effect on the same target cells, and that the distinctively proliferative nature of myxoma virus and MRV lesions are not due to unique characteristics of the MGF and SFGF ligands themselves. Furthermore, these studies demonstrate that growth factor minus derivatives of myxoma virus can be used to express and examine the targets of nonviral growth factors such as TGFα *in situ*. Potentially, the ectopic expression of other cellular growth factor families from attenuated myxoma virus constructs could be analyzed to assess the distribution of other classes of target cells within native animal tissues as well.

Figure IV-1. Structures of myxoma virus, vMYXlac, vCΔMGF, vVΔMGF, vSΔMGF, and vTΔMGF genomes near the MGF locus. Abbreviations are B1, Bgl I; B, Bsa BI; E, Eco RV; H, Hinc II; K, Kpn I; N, Nru I.

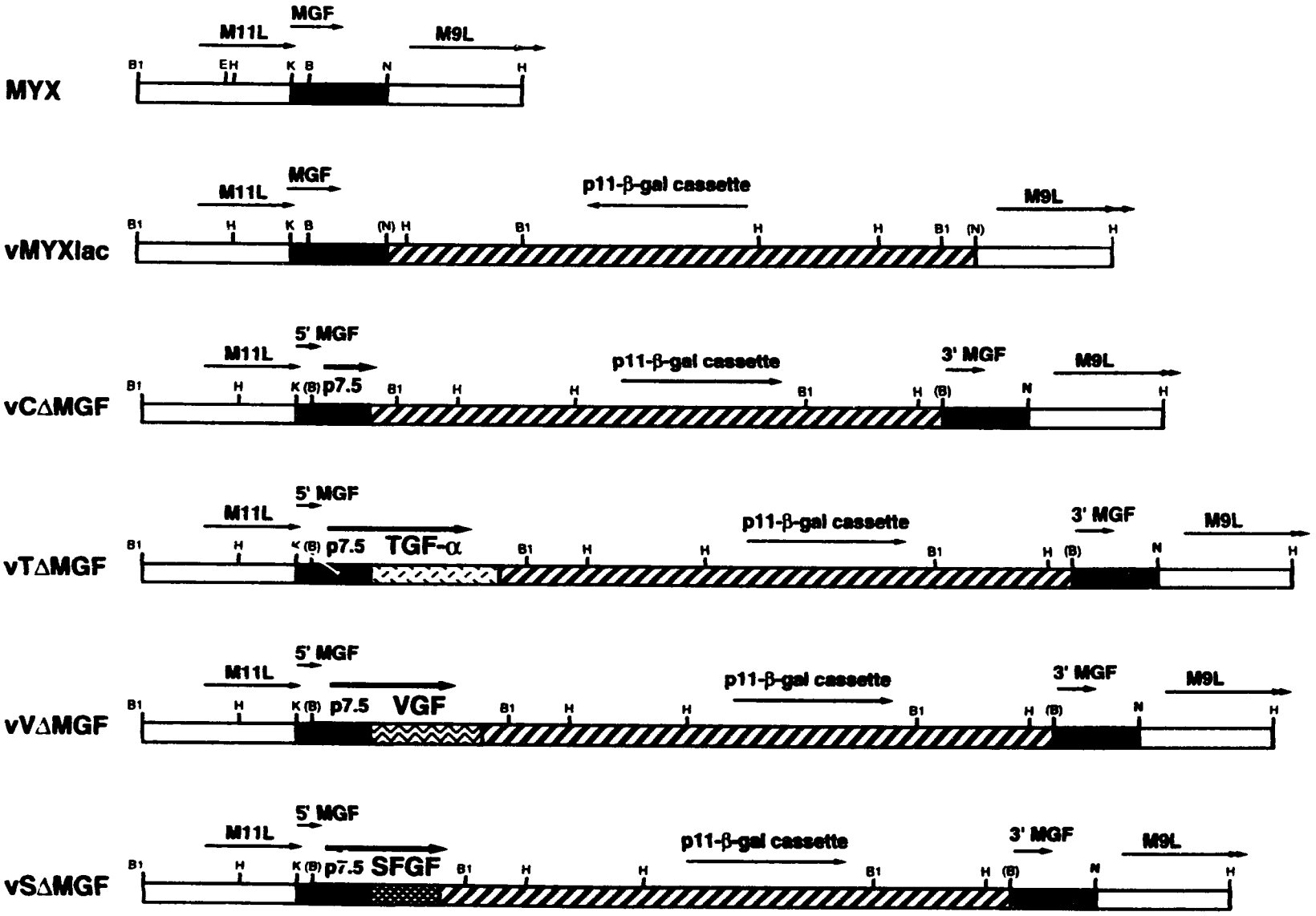
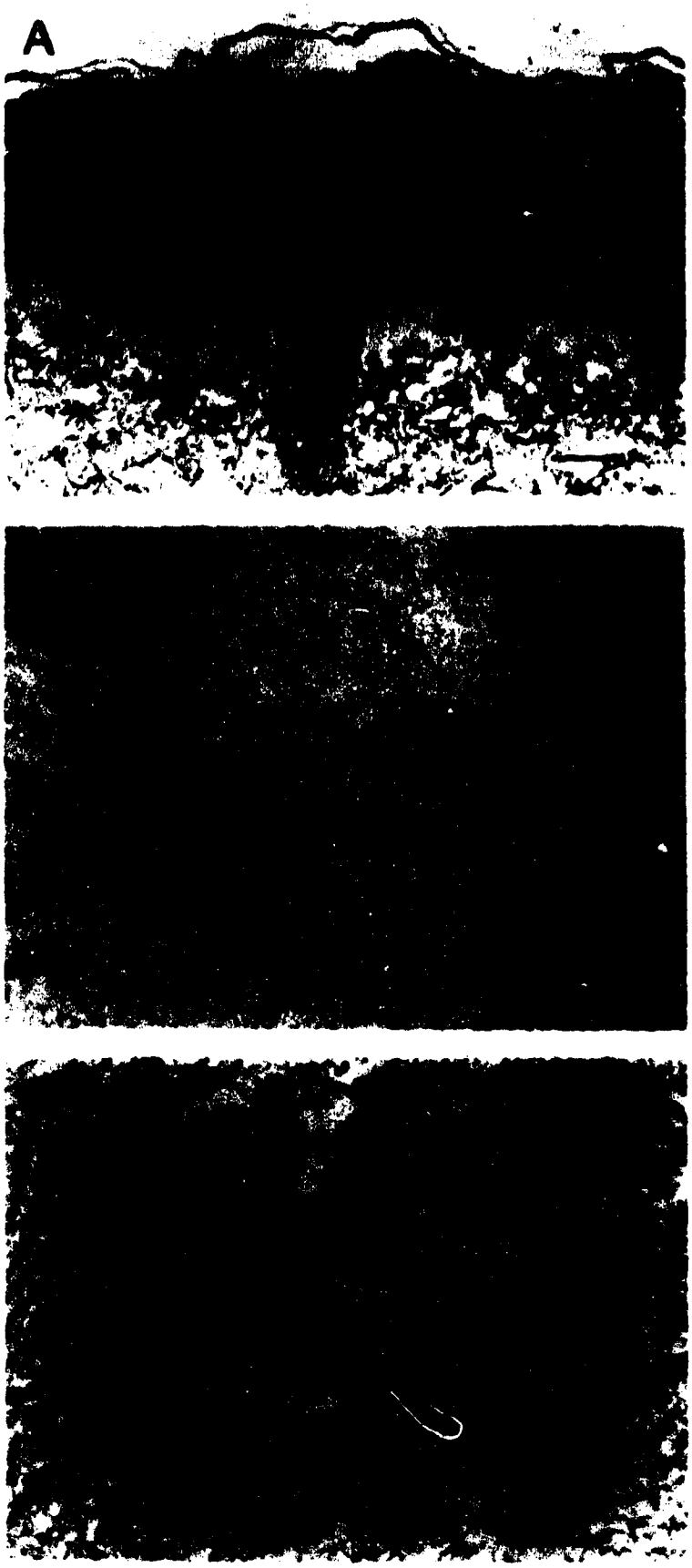

MYX
M11L
MGF
M9L
B1 EH K B N H
vMYXlac
M11L
MGF
p11-β-gal cassette
M9L
B1 H K B (N) H B1 H H B1 (N) H
vCΔMGF
5' MGF
M11L
p11-β-gal cassette
3' MGF
M9L
B1 H K (B) p7.5 B1 H H B1 H (B) N H
vTΔMGF
5' MGF
M11L
p11-β-gal cassette
3' MGF
M9L
B1 H K (B) p7.5 TGF-α B1 H H B1 H (B) N H
vVΔMGF
5' MGF
M11L
p11-β-gal cassette
3' MGF
M9L
B1 H K (B) p7.5 VGF B1 H H B1 H (B) N H
vSΔMGF
5' MGF
M11L
p11-β-gal cassette
3' MGF
M9L
B1 H K (B) p7.5 SFGF B1 H H B1 H (B) N H

Figure IV-2. Sections of primary tumor from rabbits that had received vMYXlac (A), vSΔMGF (B), vVΔMGF (C), vTΔMGF (D), or vCΔMGF (E), 8 days previously. Normal skin from the flank of an uninfected rabbit is shown for comparison (F). Note the severe edema of the dermal connective tissue in panels A-E versus the normal (F). This is characterized by separation of dermal connective tissue fibers and increased distance between adnexal structures. While the full thickness of the skin, from the epidermal surface to the cutaneous muscle, is visible in the normal skin, it is not visible in any of the test animals at the same magnification. The degree of edema is particularly severe in B. Panels A and D demonstrate the extensive epithelial disruption present in the tumors from recipients of growth factor plus viruses compared with normal skin (F). Epithelium in vCΔMGF recipients (E) displays minor epithelial disruption. Bar indicates 250 μm.


A

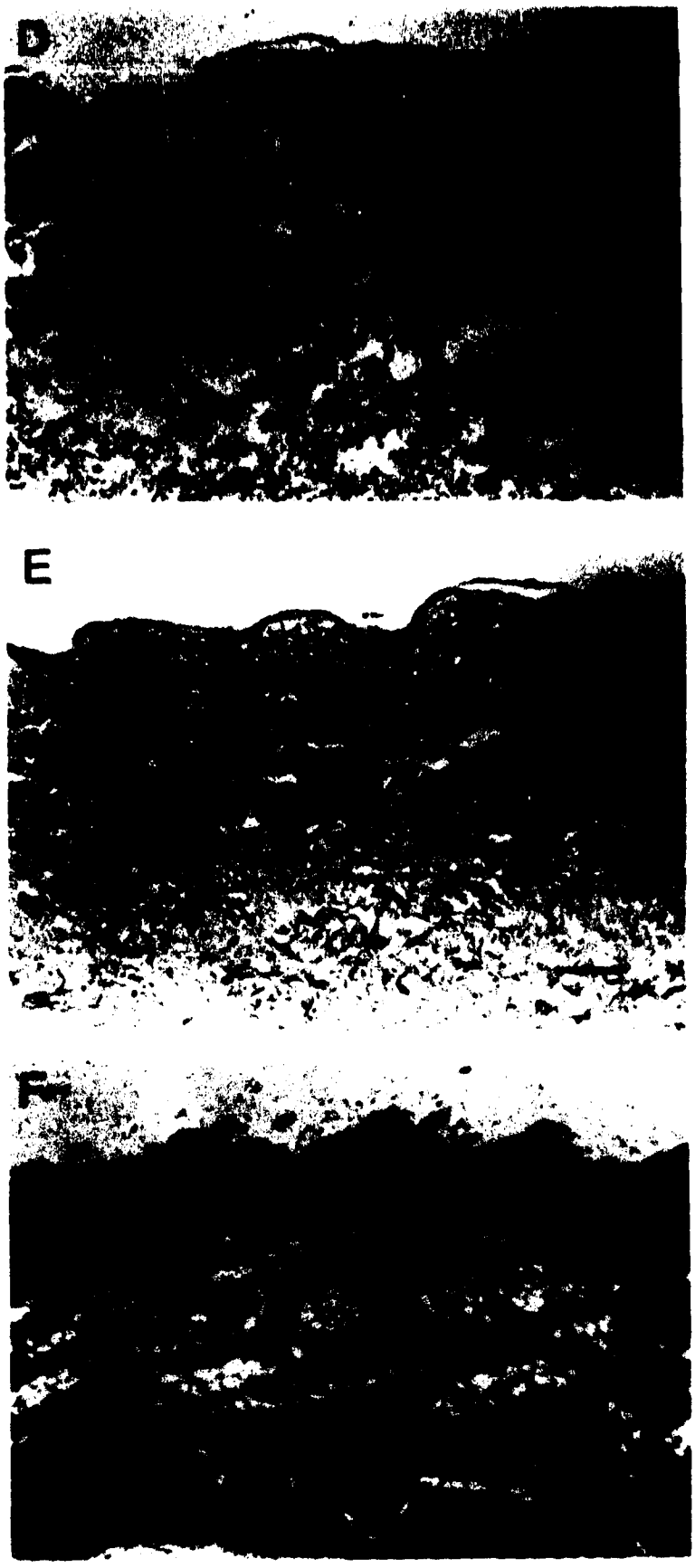
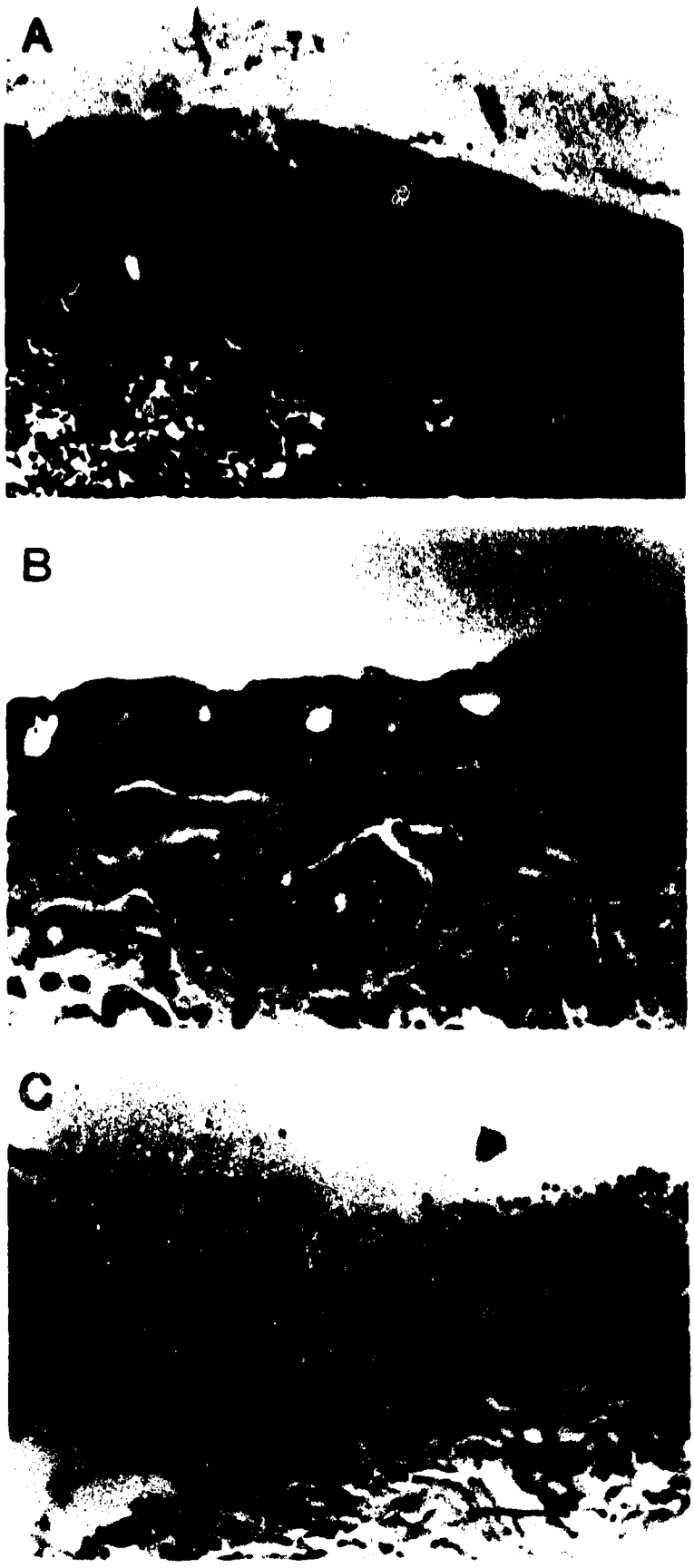

D
E
F

Figure IV-3. Sections of conjunctiva from rabbits that had received vMYXlac (A), vSΔMGF (B), vVΔMGF (C), vTΔMGF (D), or vCΔMGF (E), 8 days previously. Normal conjunctiva from an uninfected rabbit is shown for comparison (F). Note the degree of conjunctival epithelial hyperplasia and disorganization, most evident in D and A, and vascular congestion, most notable in B and C, compared with normal (F). Bar indicates 50 μm.


A
B
C

D

E

F

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Investigation of poxviral pathogenesis in recent years has focussed on the detection and analysis of specific viral gene products that may act as virulence factors by subverting host defense systems or creating conditions optimal for viral growth. This approach has revealed that poxviruses encode an array of non-essential gene products that provide multiple strategies to ensure survival and proliferation within the infected host (Turner and Moyer, 1990; Buller and Palumbo, 1991).

One of the objectives of this work was to determine whether the EGF-like growth factors encoded by the Leporipoxviruses play a role in viral pathogenesis, particularly with respect to the proliferative nature of the lesions induced by MRV and myxoma. The work of this thesis shows that MGF and SFGF are major virulence factors in myxoma virus and MRV, and that SFGF and MGF are biologically active *in vivo* in a natural virus-host system. Although SFGF and MGF are responsible for at least some of the fibroblastic and epithelial cell proliferation at sites of viral replication, much of the hyperplastic response to Leporipoxviral infection is independent of the presence of SFGF or MGF. Reconstitution of full virulence of myxoma virus by replacement of MGF with SFGF, VGF, and rat TGFα has demonstrated that these polypeptides share similar cellular targets in the context of poxviral infection, and that the distinctly proliferative nature of myxoma virus /MRV lesions is not due to unique characteristics of the MGF and SFGF ligands themselves. Combined with other studies (Ye *et al.*, 1988; Lin *et al.*, 1988 and 1991; Chang *et al.*, 1987; Upton *et al.*, 1987), this work supports the notion that SFGF and MGF are *bona fide* members of the EGF family of growth factors.

Deleting the EGF homologues in MRV and myxoma virus profoundly attenuated these viruses since the wild type viruses were invariably lethal whereas approximately 75% of MRV-GF$^-$ and MYX-GF$^-$ recipients recovered from infection. Surprisingly, no significant differences in the *in vitro* growth characteristics were observed between wild

type and growth factor minus strains of MRV/myxoma virus. However, these observations are consistent with other studies in which presence or absence of certain virulence factors has very little effect on viral growth characteristics in cell culture but profound consequences upon infection of the appropriate animal host, and emphasize the possibility that subtle changes in tissue tropism or growth characteristics in a very small subset of specific cell types could dramatically alter the outcome of infection (Buller and Palumbo, 1991; Upton *et al.*, 1990; Twardzik *et al.*, 1985; Rodriguez *et al.*, 1992; Kotwal *et al.*, 1990).

The results of this work indicate several potential roles for EGF-like growth factors in poxviral pathogenesis. The most obvious effect of SFGF and MGF on the clinical course of MRV/myxoma virus infection was an increase in the severity of bacterial conjunctivitis and rhinitis in the later stages of infection. Since bacterial colonization can be promoted by alterations in epithelial structure (Cotran *et al.*, 198[illegible] it is possible that the increased conjunctival and nasal epithelial proliferation due to viral induction of EGF-like growth factors might be directly responsible for the overwhelming severity of the bacterial infections. Whether increased cellular proliferation is a direct or indirect effect of SFGF/MGF remains to be established. For example, elaboration of growth factor from infected cells could directly stimulate surrounding uninfected cells to proliferate. Alternatively, metabolic priming of certain target cells by secreted growth factor could provide a more favorable milieu for vigorous viral replication which could lead to greater cellular damage, and thus a stronger host response involving cellular proliferation. Despite the diverse biological activities attributed to EGF-like polypeptides, this class of growth factors has not yet been shown convincingly to participate in downregulation of inflammatory or immunological responses. In addition, hematopoietic cells do not express the EGF receptor (Carpenter and Wahl, 1991). However, the increase in heterophilic infiltration and cell necrosis observed in tumors from MRV-GF$^-$ infected rabbits suggests that SFGF may directly or indirectly prevent recruitment of phagocytic cells to the site of infection and prevent destruction of infected cells. Finally, the possibility remains that

growth factor expression is required for optimal replication in a small subset of target cells *in vivo* to ensure full lethality of infection.

Several lines of evidence suggest that SFGF and MGF are *bona fide* members of the EGF family of growth factors: a) nucleic acid sequence data indicates primary amino acid homology with EGF-like growth factors (Chang *et al.*,1987; Upton *et al.*,1987); b) synthetic polypeptides corresponding to major portions of SFGF and MGF sequences display specific binding to the EGF receptor on A431 cells and induce mitogenesis in NRK cells (Lin *et al.*,1988 and.1991); c) conditioned medium from cells infected with VGF minus vaccinia virus engineered to overexpress SFGF competes for EGF receptor binding with ^{125}I-EGF (A. Opgenorth, M. O'Connor, and G. McFadden, unpublished data); d) SFGF and MGF have a demonstrable effect on viral pathogenesis; e) MGF,SFGF,VGF and TGFα are functionally interchangeable with respect to virulence of myxoma virus infection. Therefore, this work has contributed *in vivo* data to show that MGF and SFGF are indeed functionally related to EGF and TGFα.

The proliferative nature of the lesions induced by the Leporipoxviruses has intrigued virologists for several decades (McFadden, 1988; Febvre, 1962). When vaccinia virus was found to encode an EGF-like growth factor, numerous authors suggested that a leporipoxvirus specific EGF-like growth factor could be responsible for the tumor-like lesions induced upon Leporipoxvirus infection (McFadden, 1988; Dales, 1990). The results of this work establish that EGF-like growth factors are not the primary cause of the proliferative nature of Leporipoxvirus induced lesions. Rather, it is possible that the fibroblastic and epithelial cell proliferation, secretion of extracellular matrix, and angiogenesis that typify these lesions are an exaggerated form of the normal inflammatory and wound healing responses to tissue injury (Cotran *et al.*,1989).Perhaps the basis for the proliferative character of these lesions could be elucidated by examining how the nature of the host inflammatory and immunological responses to viral infection determines the character of the resulting lesions.

One of the goals of this work in its earlier stages was to determine which of the SFV open reading frames that were transferred to myxoma virus to generate MRV were responsible for its unique phenotype as originally described (Strayer and Sell, 1983; Strayer *et al.*, 1983). However, first hand observation of the clinical courses of MRV and myxoma virus infection revealed that the dramatically increased proliferative nature of MRV compared with myxoma virus was in fact far less remarkable when the Lausanne strain instead of the Moses strain of virus was used for comparison with MRV. This observation further highlights the close relationship between SFV and myxoma virus ORFs and suggests that strain differences in myxoma sequences may be primarily responsible for phenotypic differences between MRV and other myxoma virus strains. This work also shows that the Leporipoxviral growth factors possess no unique features that make them essential for full virulence of MRV/myxoma virus and suggests to a first approximation that SFGF, MGF, VGF, and TGFα affect primarily the same target cells within the context of MRV/myxoma virus infection. These result substantiate the notion that, in addition to the well established conservation of essential genes and regulation of gene expression between poxvirus genera (Macaulay and McFadden, 1989), functional conservation of some non-essential gene products has also occurred.

Traditionally, the study of host responses to poxviral infection has focussed on the interaction of poxviruses with the cellular and humoral arms of the immune system (Buller and Palumbo, 1991). It is becoming increasingly clear, however, that modulation of the inflammatory response is also a critical component of poxviral evasion from host defenses. In addition, it appears that poxviruses have evolved multiple overlapping strategies to downregulate the host inflammatory response to infection. In the case of cowpox virus, it has been shown that the 38-kDa SERPIN gene product is responsible for inhibition of interleukin-1β processing, which prevents the influx of macrophages and neutrophils into the site of viral replication, thereby diminishing the inflammatory response and increasing pathogenicity (Ray *et al.*, 1992). The myxoma/MRV SERP-1 serpin gene product also

downregulates the inflammatory response and contributes to viral virulence in rabbits (Upton *et al.*, 1990; J. Macen, C. Upton, N. Nation, G. McFadden, manuscript in preparation). Other Leporipoxviral products which are likely to play a role in interfering with inflammatory processes are the myxoma virus encoded soluble receptors for TNFα (Upton *et al.*, 1991) and IFN-γ (Upton *et al.*, 1992).

This work has identified a novel type of virulence factor in myxoma virus, M11L. The M11L gene has a counterpart in swinepox virus (R. Massung and R. Moyer, personal communication), but displays no significant homology with sequences in vaccinia virus or in the PIR database. SFV encodes an M11L counterpart, designated S11L, which is 73% identical to M11L and which cross reacts with anti-M11L antibodies (Graham *et al.*, 1992). The M11L ORF encodes a 166 amino acid polypeptide and is expressed as an early gene. The predicted product contains a 142 amino acid extracellular domain containing six cysteine residues and two consensus N-linked glycosylation sites, and a single transmembrane helix near the C terminus (Graham *et al.*, 1992). Cell surface immunofluorescence studies using anti-M11L antibodies indicate that M11L is located at the surface of infected cells. M11L downregulates the inflammatory response at the site of viral replication in the myxoma virus-infected host, and is critical for full viral virulence. In contrast to other anti-inflammatory poxviral gene products mentioned above, M11L is not secreted from the infected cell, but is localized at the infected cell surface (Graham *et al.*, 1992). Moreover, the cell-surface localization of M11L appears to be critical for its activity as a virulence factor, since cells infected with the attenuated vMYX-GF-ΔM11L synthesize an M11L variant which is not transported to the cell surface. These observations suggest that M11L might possibly function as a membrane-bound "viroceptor" that could short circuit the function of an inflammatory mediator by interrupting a critical ligand-receptor interaction.

An intriguing feature of M11L is that it allows viral replication in primary mixed splenocyte culture in the absence of mitogenic stimulation. It is possible that the M11L product is required to overcome a block to viral replication in cells within the splenocyte population that does not exist in fibroblasts. Alternatively, perhaps the presence of M11L prevents destruction of infected cells by phagocytic cells within the splenic cell population, allowing productive replication and spread of virus. In either case, detailed analysis of viral replication within defined leukocyte subsets may help elucidate the role of M11L in poxviral interactions with components of the host immune system.

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