POLYOMAVIRUS T ANTIGENS ACTIVATE AN ANTIVIRAL STATE

by

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ABSTRACT

The large T antigen (TAg) of the polyomavirus Simian virus 40 (SV40) is known to play an important role in several events during the viral life cycle, including DNA replication, transcription, and virion assembly. Recent analysis revealed that primary mouse embryonic fibroblasts (MEFs) expressing exogenous TAg showed increased transcription of interferon stimulated genes (ISGs). Interestingly, this activation was shown to be stimulated in the absence of interferon production. The mechanisms by which TAg upregulates the pathway(s) and the consequences of that upregulation remain unknown. Thus, investigation into this mechanism may allow the elucidation of novel host factors moderating the interferon response. I tested several mutants of TAg to understand what functions are necessary to induce the interferon response. My investigation has revealed that the early region of SV40 can activate an antiviral phenotype in MEFs, as seen by the attenuation of viral growth during productive infection. Furthermore, the amino terminus of TAg is sufficient to stimulate this response, and alternative splice forms from the early region (e.g. small T Antigen) do not seem to be critical to the upregulation of ISGs. Other experiments indicate that the STAT1 transcription factor is necessary for establishment of the antiviral state. I have also shown that two human polyomaviruses, Human Polyomavirus BK (BKV) and human Polyomavirus JC (JCV), follow suit with our observations of SV40 TAg. I hypothesize that the TAg-mediated activation of ISGs contributes to an environment inhibitory for viral growth and that this phenomenon is extended to multiple polyomaviruses. This research

offers new insight into the interactions of polyomaviruses with its host. This new knowledge could be used in combating human disease, and therefore serves as a valuable contribution to public health.

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PREFACE

I cannot express my gratitude enough for the help of my friends in the Pipas Lab, and everyone who has supported me through my time there. Since my lucky inception into the lab during the junior year of my undergraduate education, I have been blessed to work alongside intelligent, understanding, and caring individuals. I could not have produced this work without them. Through my experience in the lab I have learned more than I had ever hoped. My duty now is to go on with the tools and experience I've been granted, and continue in the pursuit of science for the betterment of our world.

1.0 INTRODUCTION

Viruses are an important part of our world and affect our lives every day. Viruses are nanoscopic agents that grow within the cells of organisms. They have evolved a multitude of ways to exploit or alter cellular processes in order to survive and spread. Some viruses persist undetected, hiding in cells for years without any deleterious consequence. While other more injurious viruses can cause a great deal of damage to their host, and even, although mostly unintentionally, lead to their death. Studying the lifestyle and activities of viruses can serve as an opportunity for the elucidation of viral and cellular biology, and can also contribute to advances in human health. For example, studies involving the Simian Vacuolating virus 40 (SV40) lead to the discovery of the tumor protein 53 (p53), a cellular transcription factor whose activity is abrogated in as many as 50% of all human cancers (**Damania & Pipas 2009**). This example, among many others that could be stated, lends itself to the notion that continued study of viruses and their activities may offer further insight into viral/cellular biology, and possibly human disease.

1.1 POLYOMAVIRUS TUMOR ANTIGENS

Members from the family Polyomaviridae are known to cause cancer or induce tumors in laboratory animals. Polyomaviridae is comprised of non-enveloped, small, dsDNA viruses.

Within the family are some notable kindred of the genus polyomavirus: SV40, Human Polyomavirus BK (BKV), and Human polyomavirus JC (JCV). SV40 has been shown to be capable of immortalizing and transforming primary cells in culture, and to induce tumorigenesis during *in vivo* studies of hamsters (**Eddy 1962, Girardi 1962**, **Black 1964, Pipas 2009**). Additionally, a few reports have shown a link between these viruses and human cancers, but other studies have not found any association (**Bulut 2013, Shah 2007**). This link remains controversial. Hence, further studies of these viruses are necessary to fully understand their transformative and oncogenic potential as well as the mechanisms behind those phenotypes.

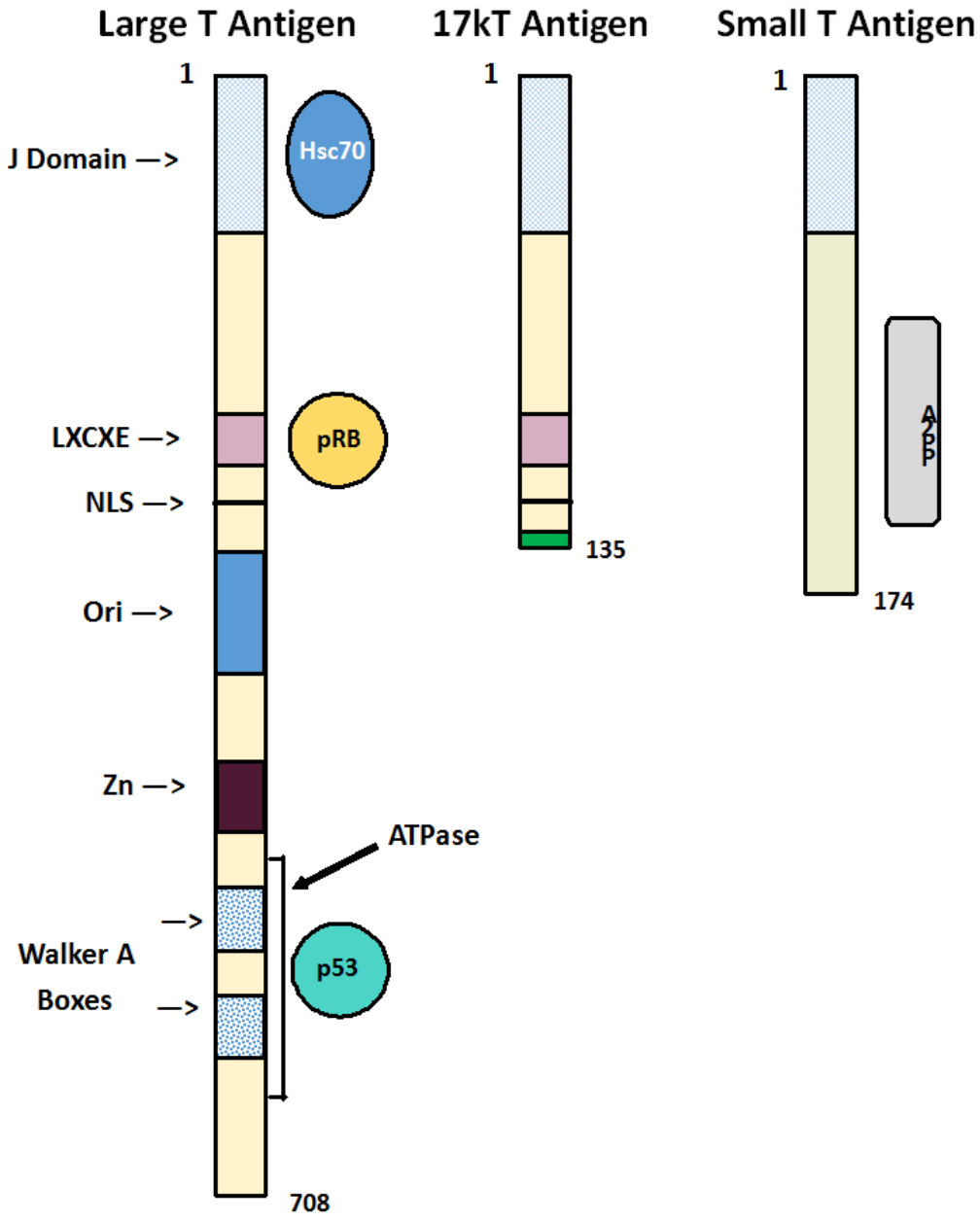
Polyomaviruses encode oncogenes that are important during viral replication, but can also induce the transformation of certain cell types. That is, a productive polyomavirus infection in a permissive host normally proceeds with viral genome replication, and production of progeny virions. However, occasionally in non-permissive host cells, viral replication can fail and produce an abortive infection (**Damania & Pipas 2009**). The consequence of the latter may result in cellular transformation via prolonged expression of the viral oncoproteins, the tumor antigens (T antigens). The T antigens are among the first proteins expressed from the viral genome during its replicative cycle. Specifically, they are needed to drive the host cell into S phase so as to aid in amplification of the viral genome and progeny assembly (**Pipas 2009, Damania & Pipas 2009**). The polyomavirus genome itself, approximately 5000bp in length, is comprised of early and late regions, along with a non-coding regulatory region. SV40, BKV, and JCV, share a relatively high degree of genome sequence homology (approximately 70%) and all transcribe T Antigens from within their early regions (**McKees 2005**). The early regions have the potential of transcribing multiple differentially spliced T Antigen products. Specifically, all three encode the large T antigen (LTAg), and a small t antigen (sTAg). Additionally, the 17kT antigen (17kT) from SV40, a

truncated T antigen from BKV, and three T prime T antigen, T'135, T'136, T'165, from JCV, and are also known to be made respectively **(Trowbridge and Frisque 1995, Gjoerup 2010, Abend 2009)**.

1.2 POLYOMAVIRUS T ANTIGEN FUNCTIONAL DOMAINS AND CELLULAR TRANSFORMATION

SV40 LTAg has multiple structural domains that induce oncogenic transformation **(DeCaprio 2009, Pipas 2009, Levine 2009)**. SV40 LTAg mediates transformation via the disruption of cellular p53 and retinoblastoma protein (pRB) family members (including pRB, p107, and p130) through activities in both its amino and carboxyl termini. In the amino terminus, the J-domain (with the Hsc 70 binding motif) and the pRB binding domain, the LXCXE motif, work in concert to inactivate pRBs and release cellular E2F transcription factors driving S phase and DNA replication (**Sullivan 2002**). Sequences within the carboxyl-terminal half of T antigen bind and stabilize p53 within its ATPase domain (i.e., Walker A box motifs) (**An 2012**). This inhibits p53 mediated regulation of the G2 to M and G1 to S phase transitions, and the ability to induce apoptosis (**Damania & Pipas 2009**). **Figure 1** illustrates the three T antigens of SV40. The T antigens from BKV, and JCV have common features with SV40 that result in similar functionality (**An 2012**). The LTAg of all three viruses possess a J domain, LXCXE motif, and Walker A boxes necessary for the establishment of cellular transformation (**An 2012**). (**Table one** summarizes the few differences between domains of SV40, BKV, and JCV LTAgs.) Furthermore, each virus produces smaller alternative splice products which share some of the same features. For example, the three T prime transcripts produced by JCV are identical in the first 132 amino

acids in their LTAg. Thus, they contain sequences for the J domain and LXCXE motif (**Frisque 2001**). Similarly, SV40 17kT contains both the J domain and LXCXE motif. BKV also makes a truncated T antigen product that is analogous to the T' 136 from JCV, and therefore contains a J domain an LXCXE motif (**Gjoerup 2010**). An interesting difference between SV40 small T antigen and those of BKV and JCV, is that those of the latter two both exhibit a J domain and LXCXE motifs (**Gjoerup 2010**).



Diagrams of Simian Virus 40 tumor antigen(s): Large T antigen (708aa), 17k T antigen (135aa), and small T antigen (174aa). Domains within T antigen(s) are indicated by arrows. The "Ori" and "Zn" domains indicated and the origin binding domain and the zinc finger, respectively. The respective binding partners are indicated alongside binding sites

Figure 1. Diagrams of Simian Virus 40 T Antigens

Table 1. Comparison of large T Antigen

Viral LTAg	J Domain (Hsc70 Binding)	LXCXE (Rb Binding)	ATPase (Walker A) (P53 Binding)
SV40	HPDKGG	LFCSE	G/AXXXXGKT/S
BKV	HPDKGG	LFCHE	G/AXXXXGKT/S
JCV	HPDKGG	LFCHE	G/AXXXXGKT/S

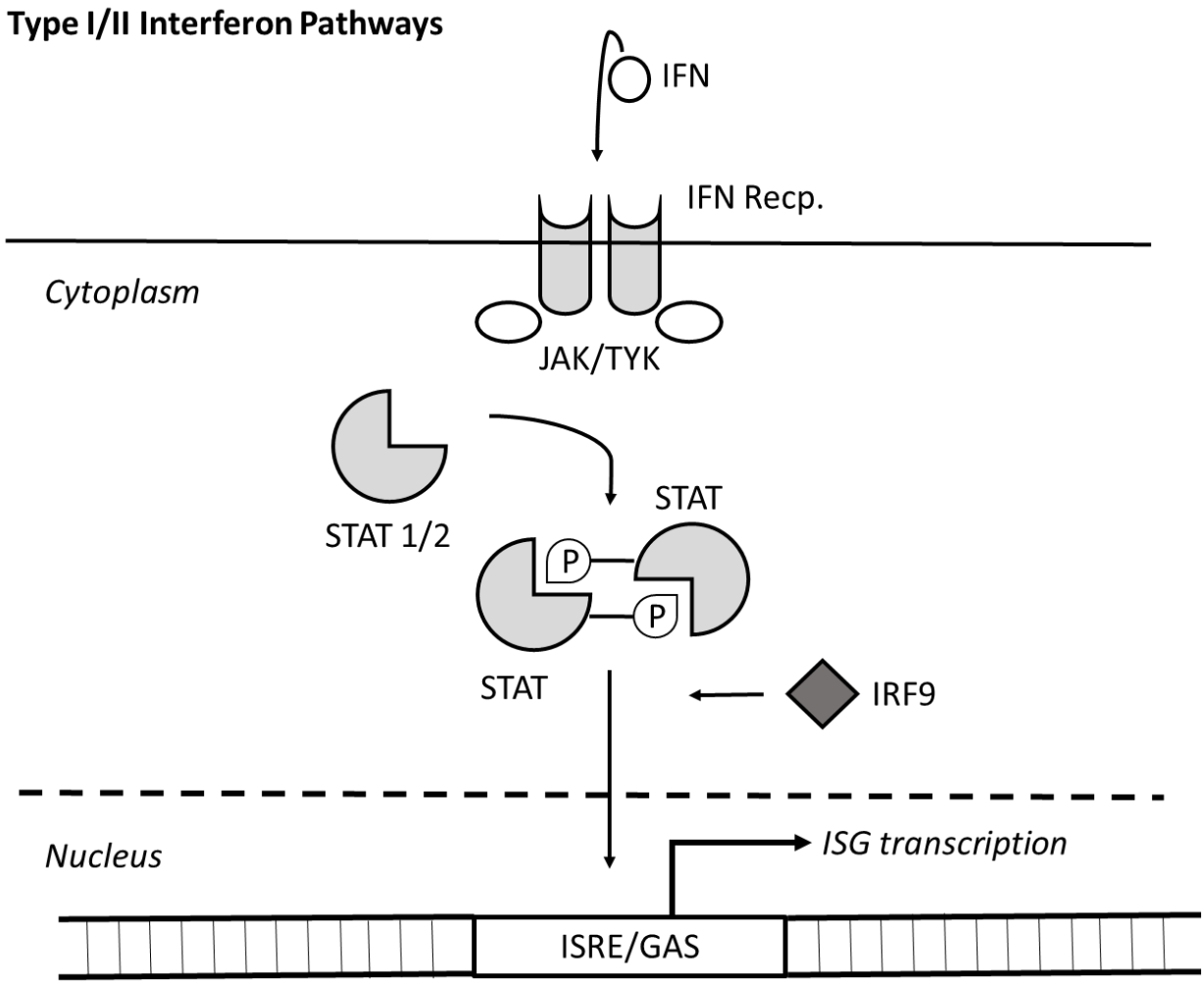
Comparison of Large T antigen domain amino acid sequences important for transformation.

1.3 T ANTIGEN ACTIVATION OF THE INTERFERON RESPONSE

It was recently observed that the expression of the large T Antigen of SV40 in primary mouse embryonic fibroblasts results in the upregulation of many interferon stimulated genes (**Rathi 2010**). Other constituents of type I and type II interferon signaling pathways were shown to be activated as well. Interestingly, this phenotype was activated in the absence of interferon (IFN) signal production. Under normal circumstances of viral infection, the viral nucleic acid may be recognized by pathogen recognition receptors such as RIG-I, or toll-like receptors (**Kawai 2006**). Binding of pathogen molecular patterns to the intracellular receptor induces a signaling cascade leading to the translocation of transcription factors NFκB and IRF3 to the nucleus (**Takeuchi 2010**). In the nucleus, those factors promote the transcription of IFN-α and IFN-β production. The IFN signal is translated and secreted by the infected cell so as to stimulate neighboring cells. IFN receptor expressed on the surface of nearby cells bind the IFN signal and

stimulate type I or II IFN mediated signaling. After binding the IFN signal, membrane associated kinase proteins Janus Kinase 1/2 (Jak1, Jak2) and Tyrosine Kinase (Tyk2) autophosphorylate and activate Signal Transducers and Activator of Transcription (STAT1/2) (**Ivashkiv 2014**). At this point, STAT1 can heterodimerize with STAT2, then associate with IRF-9. This results in the complex Interferon-stimulated gene factor 3 (ISGF3) to form. ISGF3 then translocates to the nucleus and binds to Interferon stimulated response elements (ISRE) in promoters, which then promotes the transcription of ISGs. Alternatively, STAT1 can homodimerize, translocate directly to the nucleus, and bind the gamma activated system (GAS) also promoting transcription of ISGs (**Ivashkiv 2014**). Interferon stimulated genes are known to have various roles, but are often associated with antiviral activities (**Schoggins 2011**). **Figure 2** illustrates the type I and II IFN signaling pathways.

Studies by Verma and others in 2006 revealed elevated levels of ISG transcripts following transfection of primary human fetal glial cells with infectious JCV DNA. This was observed by microarray and real-time RT-PCR. Additionally, a subset of ISGs examined following JC virus infection were also shown to be upregulated (**Verma 2006**). With the observations made by Rathi and Verma as a foundation, this investigation sought to understand the underlying mechanism behind SV40 LTAg's upregulation of the interferon response, to uncover possible consequences of the upregulated response, and to determine if the phenomenon could be extended to other polyomaviruses as well.



The diagram illustrates normal activation of interferon signaling. The signaling cascade results in the promotion of interferon stimulated genes.

Figure 2. Interferon Signaling Diagram

2.0 STATEMENT OF THE PROJECT

Previous studies have revealed that the cellular interferon response is upregulated upon expression of SV40 large T Antigen in several mammalian cell lines (**Rathi 2010**). **Thus, I hypothesized that other members of the polyomaviridae family, encoding similar T Antigen proteins, are able to induce the interferon response; an antiviral state may result from activation of the response.** I have investigated these hypotheses, and have attempted to uncover the mechanism by which the phenotype is established.

The examination of the ability of T antigen to upregulate the interferon response was done so on the presumption that it may reveal previously unknown cellular factors, cellular processes, or viral interactions with the host. More specifically, the possibility that the upregulation of the interferon response by T antigen may be important to T antigen's ability to induce cellular transformation of mouse primary cells.

3.0 SPECIFIC AIMS

Specific Aims:

1. Determine if the interferon response and ISG transcription is altered in the presence of T Antigens from multiple polyomavirus in primary mouse embryonic fibroblast (MEFs) cells.

I have expressed the genomic early region of polyomaviruses SV40, BKV, and JCV in primary mouse embryonic fibroblasts and monitor the expression of modulators of type I and II interferon signaling, as well as the transcription levels of their downstream products, the interferon stimulated genes (ISGs). Pooled clones of MEFs expressing the virus early regions will be assessed. We have chosen MEFs based on their ability to transform with T antigen expression, and the opportunity to perform genetic manipulation on the mice the MEFs are harvested from.

2. Identify if an antiviral state is generated by the induction of ISGs in T Antigen expressing MEFs. VSV is a virus sensitive to the interferon response. Thus, I will perform one step viral growth curves with the Vesicular stomatitis virus (VSV) on MEF pools expressing viral early regions. I will then determine if viral titer is attenuated when compared to the productive infection of normal MEFs.

3. Evaluate the role of STAT1 in ISGs upregulation induced by T antigen expression. Using mutant mice lacking the STAT1 transcription factor, MEF pools will be produced expressing viral early regions. The interferon response will be examined in these pools.

4. Identify the activity of T Antigen necessary for the upregulation of ISGs. For complete understanding of the viral mechanism, elucidation of the specific activity of T antigen is paramount. Using mutants of the viral early region, MEF pools will be generated. These pools will be assayed for their ability to upregulate the interferon response.

4.0 MATERIALS AND METHODS

Isolation of primary mouse embryonic fibroblasts, conditions of cell culture, and establishment of pools.

Mouse embryonic fibroblasts (MEFs) and STAT1(-/-) MEFs were harvested from FVB embryos after 13.5 days using the method previously described (**Markovics 2005**). MEFs were grown in DMEM (Corning #10-013-CV) supplemented with 10% fetal bovine serum (HYCLONE#: SH30070.03) and additionally 1% penicillin/ streptomycin (Invitrogen #15140163). MEFs were grown in a 37C, 5% CO2 incubator. MEF pools were generated via lentiviral transduction (Invitrogen ViraPower #K4975-00). Transductions were carried out with the aid of polybrene reagent (Sigma #H9268). Lentivirus transduction conveys the pL6.3 lenti/V5 TOPO plasmid (Invitrogen #K5315-20) expressing a genomic version of the mutant large T antigens (N136 equivalents, C257, or N136-3213(E107K, E108K)) under the control of the CMV promoter. Plasmids were transfected with lipofectamine 2000 reagent (Invitrogen 11668-027) into the producer cell line, 293FT (Life technologies #R700-07) for production of lentivirus in accordance with manufacturer's instructions. Following lentiviral infection of MEFs, cells were grown to confluency and selected for 7-10 days in 2ug/mL blasticidin (R210-01). Surviving colonies were "pooled" and passaged until passage four, when pellets were collected for further analysis. MEF pools of viral early regions were generated by a similar transduction using retrovirus. Genomic viral early regions were cloned into the pBABE puro plasmid (AddGene Plasmid 1764) and transfected into phoenix eco-cells (ATCC #CRL-3214). Retrovirus made from producer cell lines were used to infect naïve MEFs. The Large T antigen cDNA expressing pools were generated via introduction of pEF1/V5-HisA plasmids expressing the cDNA of the various

LTAg's. Plasmids were introduced via FUgene 6 (Promega) transfection in accordance with manufactuer's instructions. MEF pools were generated via selection for 7-10 days. Full length 3213 clones were a donation by Dr. M.T. Sáenz Robles, prepared by Dr. A. Rathi. These clones were MEFs expressing the full length 3213 mutation. Clones were isolated, grown, and whole cell protein extract samples were prepared as described below.

One Step Viral Growth Curves and Plaque Assay.

Wild-type MEFs or MEF pools expressing T antigen were plated in 6cm dishes and infected with VSV the following day at a multiplicity of infection (MOI) of 0.1. At the time of infection, cells were counted to establish the correct MOI. Based on the different growth rates of cells, the number of cells infected ranged from 2.5e5cells/plate to 2.5e6 cells/ plate. The average of cells infected was ~9e5cells/plate, with a median of 7.5e5cells/plate. Statistical calculations for MEF pools and viral titer were made using R statistical software. A complete list of the number of cells infected for each pool is listed in **table 3**. Infection lasted for 1 hour in a 37C, 5% CO2 incubator, rocking the plates every 15min. Inoculum was removed and 2.5mL of growth medium was supplied. Plates were collected at respective time points and frozen at -80C. Viral supernatant was collected via centrifugation at 3000rpm for 15min.

Infections with EMCV were performed with wt MEFs or MEF pools expressing T antigen. The cells were plated and allowed to grow overnight so as to reach a density of 2e6 cells/plate. The following day cells were counted for accuracy. Cells were infected with EMCV at an MOI of 0.005. Infection lasted for one hour at 37C in a 5% CO2 incubator, rocking the plates every 15min. Afterward, the viral inoculum was removed; plates were washed three times in 1xPBS; 2.5mL of was supplied. Plates were collected at 0, 3, and 6 hours post infection, and frozen at -80C. Viral supernatant was collected via centrifugation at 3000rpm for 15min.

Infectious virus production was quantified by way of plaque assay. Supernatant (inoculum) produced from one step growth curves was serially diluted in growth media and plated on BKH21 cells (ATCC: #CCL-10). BHK21 cells were infected for 1 hour in a 37C, 5%CO2, incubator with rocking every 15min. Inoculum was removed and 1.5% Agarose overlay (LONZA: #50100) was added with 2X MEM (LifeTechonologies: #61100) supplemented with 10%FBS. Twenty-four to 36hrs post infection viral plaques were visualized with the addition of 1% neutral red solution (Sigma). The same procedure was used to quantify EMCV production with two exceptions: VERO cells (ATCC # CCL-81) were used for viral infection, and plates were stained at 36-48 hours post infection to visualize plaques.

Table 2. MEF Pools

BKT	9.00E+05	5.70E+0
BKT	8.00E+05	8.40E+0
JCT	4.00E+05	2.30E+0
JCT	4.50E+05	2.30E+0
JCT	4.50E+05	1.00E+0
JCT	4.00E+05	5.00E+0
JCT	2.00E+06	1.60E+0
MEF	7.00E+05	2.40E+0
MEF	5.00E+05	1.80E+0
MEF	1.50E+06	2.30E+0
MEF	1.50E+06	2.70E+0
MEF	5.50E+05	3.60E+0
N136	2.50E+05	3.30E+0
N136	2.50E+05	1.00E+0
N136	1.00E+06	5.90E+0
N136	1.00E+06	6.00E+0
N136	1.75E+06	4.60E+0
N136	6.00E+05	1.60E+0
N136	8.50E+05	1.60E+0
N137	6.00E+05	5.00E+0
N137	6.00E+05	3.70E+0
N137	9.00E+05	2.50E+0
N137	9.00E+05	2.80E+0
N137	1.20E+06	1.90E+0
N137	5.00E+05	4.60E+0
N138	1.50E+06	3.30E+0
N138	1.50E+06	2.50E+0
N138	4.00E+05	2.00E+0
N138	1.00E+06	8.00E+0
N138	7.00E+05	2.00E+0
N138	5.00E+05	2.60E+0
N138	6.00E+05	1.20E+0
SVT	1.75E+06	1.00E+0
SVT	1.50E+06	1.00E+0
SVT	2.60E+06	1.30E+0
SVT	1.60E+06	2.00E+0

This table indicates the pools, number of cells at the start of infection, and the peak viral titer at 24hrs post infection during the VSV one-step growth curves.

Immunoblot Analysis

Western blots were conducted as previously described (**Markovics 2005**). Dilutions of antibodies tested: Total STAT1 (Cell Signal #9172), Phospho-STAT1 Ser727 (Cell Signal #9177), Phospho-STAT1 Tyr701 (Cell Signal #9171), and Total STAT2 (Cell Signal #4597), were prepared in accordance with the manufacturer's instructions. T-Antigen mouse monoclonal antibodies Ab416, Ab419, and Ab901 were used as described previously (Harlow et al., 1981). Additional mouse monoclonal antibodies against JCV T antigens Ab962 and AB2003 were used as previously described (**Bollag 2000, Munoz-Marmol 2004**). GAPDH loading mouse monoclonal was used as the loading control (US Biologicals #G8140-11). Goat anti-mouse (A2554), and goat anti-rabbit (A0545) from Sigma-Aldrich were used as secondary antibodies.

Whole Cell Extract, RNA Extraction, and Reverse Transcription PCR

MEFs and MEF pools were grown to two days post confluency, collected, and pelleted. Cell pellets were washed in 1x PBS and frozen at -80C. Total RNA extraction was conducted using Qiagen RNeasy kit (#74104) in accordance with manufacturer's instructions. Samples were quantified using a NanoDrop2000.

Reverse transcription PCR for the production of cDNA from 2ug total RNA was conducted with SuperScript III First-Strand Synthesis System for RT-PCR (#18080-051) in accordance with manufacturer's instructions. PCR analysis with gene specific primers was performed using 1uL of cDNA and GOTaq polymerase (Promega) for 25-30cycles. RPL5 served as the normalizing loading control. The reactions were visualized with 1% agarose gels and were stained with ethidium bromide (Sigma-Aldrich). Primers are listed in **table 3**.

Whole cell extract was prepared from 2-day post confluent MEF pools. Cells were pelleted at 3000rpm for 5 min and frozen at -80C. Pellets were lysed in Branton's lysis buffer (Hepes pH7.9 50mM, KCl 0.4M, EDTA 0.5mM, NP40 0.1%, Glycerol 10%). Lysed pellets were centrifuged for 15min at 4C. Supernatant was quantified and stored at -80C. Quantification of extract was performed by Bradford protein assay (BioRad) in accordance with manufacturer's instructions. Bradford samples were quantified with a BioRad iMArk microplate reader.

Table 3: Primers Used During Study

Gene(s)	Direction	Primer	Product Length (bp)	Annealing °C
Ifi44	F	GAGAGAACAGGGAATGAAGAAGGC	134	52.4
	R	CCAACAGAATTGCGATTGGTCC		
Ifi27	F	CCATAGCAGCCAAGATGATGTCTG	121	55
	R	GCATTTGTTGATGTGGAGAGTCC		
Oas2	F	AAAACCAACCGCTCCCAGTTCGTC	488	56
	R	GCAATGTCAAAGTCATCTGTGCC		
Oasl2	F	TTACAGAACAGCCAGAGCTATACGG	548	56
	R	CAAGGGAGATAGATTTACGTCCACG		
GTPase2	F	CTTCCACCTGCTTGTTCTTTGG	266	55.4
	R	TCACAGTTTCCTCAGTGCTGGG		
Rsad2	F	CAATCACACCCAGCAGCAGTTAG	209	54.4
	R	AGCGATGCCTCAGAACACAGTG		
RPL5	F	CCAAACGATTCCCTGGTTATGAC	260	56
	R	GACGATTCCACCTCTTCTTCTTCAC		
IL-6	F	CTTCCTACCCCAATTTCCAA	191	55
	R	ACCACAGTGAGGAATGTCCA		
SV LTAg/stAg	F	TGCAAGGAGTTTCATCCTGA	stAg = 469, LTAg = 188	56
	R	TCATTAAAGGCATTCCACCA		
BK LTAg / stAg	F	GGCGACGAGGATAAAATGAA	stAg = 492, LTAg = 215	56
	R	GCTTCTTCATCACTGGCAAA		
JC LTAg / stAg	F	GATAAAGGTGGGGACGAAGA	stAg = 479, LTAg = 202	56
	R	TTTCTTCATGGCAAAACAGG		

5.0 RESULTS

5.1 MULTIPLE POLYOMAVIRUS T ANTIGENS UPREGUALTE THE INTERFERON RESPONSE

In order to confirm the monoclonal T antigen antibody mixture used for the detection of SV40 T antigen (416 & 419) can successfully cross react with BKV and JCV large T antigens, western blots were conducted with purified LTAg protein (purified samples were donated by Dr. Ping An at the University of Pittsburgh Deptartment of Biological Sciences) from SV40, BKV, and JCV. **Figure 3** shows a western blot of the purified proteins. The blot shows both JCV LTAg and BKV LTAg proteins detected along with SV40 LTAg. These results display that the antibodies used for detection of large T antigen from SV40, BKV, and JCV are indeed functional in their ability to detect the different polyomavirus large T antigens.

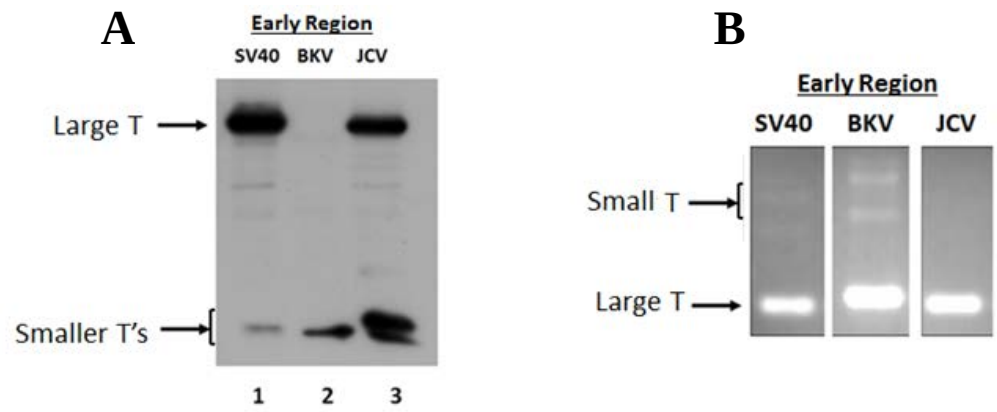
Comparison of purified LTAg protein from SV40 (Control), BKV, and JCV. The 416 monoclonal antibody was used for detection of T Antigen.
Figure 3: Detection of purified T antigen

To assess the level of large T antigen expression in MEFs as well as the possible alternative splice forms, western blots using T antigen specific antibodies were performed. Three separate MEF pools from different embryos were generated by lentiviral transduction to convey the different polyomavirus genomic early regions into the primary MEF cells. The early regions allow for the potential transcription of the various alternative splice forms of the large T antigen. Because of the transformative ability of the early regions (**An 2012**), the cell pools were immortalized. The pools are initially a heterogeneous mixture of cells exhibiting different morphologies. The different pools grew at approximately the same rate, faster than primary wild-type MEFs, however this was not rigorously examined. The levels of T antigen expressed by the pools are shown in **figure 4**. **Figure 4** reveals T antigen expression with a mixture of monoclonal antibodies (416 & 419) against sites in the amino terminus of SV40 large T antigen. Panel A of the figure depicts the protein expression profile of the genomic early region (**Figure 4A**). The

Large T antigen product dominates for SV40 while its small T and possibly 17kT are in much lower proportion (Figure 4A Lane1). BKV early region appears to show no large T antigen expression, but does exhibit expression of either small T, or 17kT product (Lane 2). Lane 3 shows expression of JCV genomic early region, and indicates a clear presence of large T product as well as other T prime or small T products.

The relative abundance of large T antigen and small T antigen mRNAs was determined by a differential PCR that was performed with primers capable of amplifying both large and small T antigen transcripts simultaneously. The primers are located within the genomic sequence flanking the large T antigen and small T antigen introns. Primer design locations for SV40 are depicted in **Figure 4C**. Primers for BKV and JCV were made in similar fashion flanking the introns of large and small T antigens. Table 2 provides the sequences for all primers used. **Figure 4B** depicts the SV40, BKV, or JCV transcript levels of their large T antigens compared small T antigen. The large T antigen products are clearly shown in greater proportion to the small T antigen for both SV40 and JCV. Surprisingly, the large T antigen mRNA levels for BKV also appear to be high relative to its small T antigen product.

Figure 4 indicates that expression of the polyomavirus genomic early region from SV40, BKV, and JCV result in the production of multiple T antigen splice forms, and that the large T antigen dominates transcription and expression for SV40 and JCV. Surprisingly, for BKV the large T antigen product seems to dominate transcription, but there does not appear to be any large T antigen is expressed above detectable levels. Instead smaller products which may or may not be BKV small T antigen dominate expression. Despite the differences in T antigen protein expression, the SV40, BKV, and JCV T antigen expressing MEFs all upregulate total STAT1/2 protein levels, phosphorylated STAT1 protein levels, and ISG transcription.



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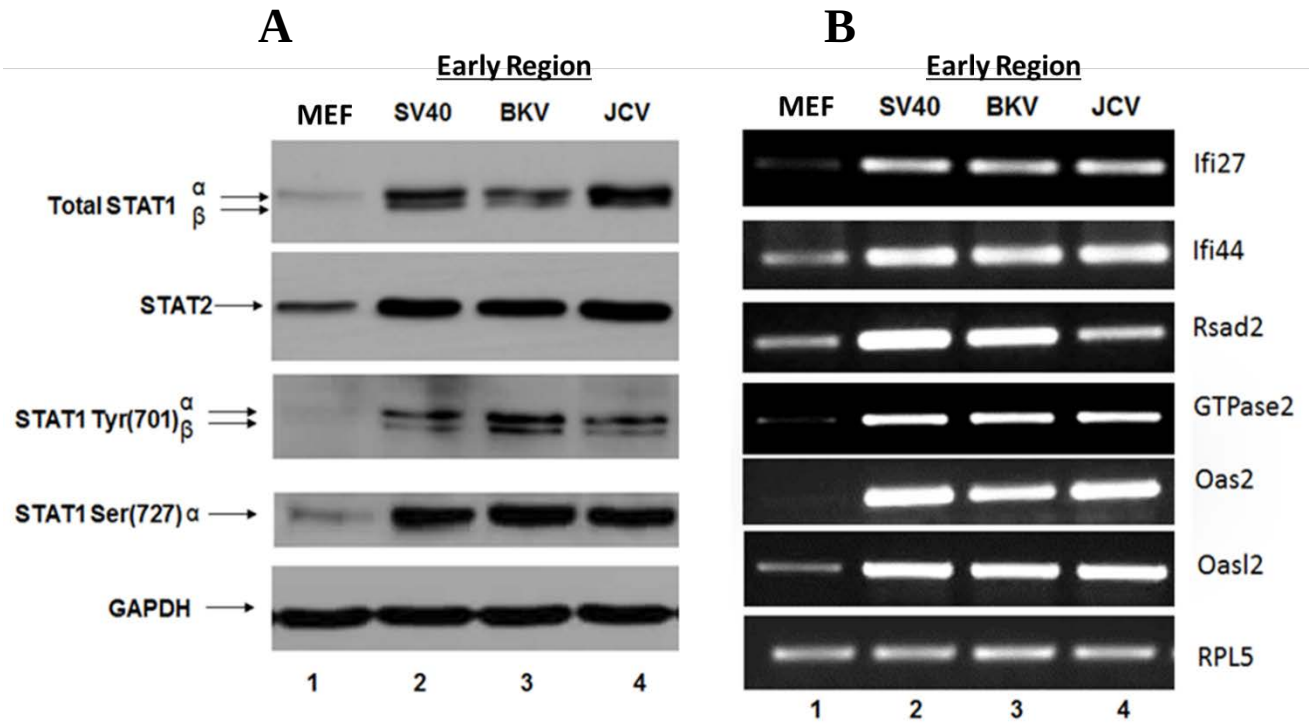
ATTTAAAGCTCTAAGGTAAATATAAAATTTTTAAGTGTATAATGTGTTAAACTACTGATTCTAATTGTTTGTGTATTTTAGATTCCAACCTATGGAACTGATGAA
TGGGAGCAGTGGTGGAATGCCTTTAATGAGGAAAA

Left Panel (A): Whole cell extract of 2 days post confluent MEF pools expressing SV40, BKV, JCV early region, or wt MEF cells without T Antigen (MEF). Right Panel: (B) Reverse transcription PCR of total RNA transcript levels of ISGs from the pools of MEFs with T antigen or wt MEFs (MEF). GAPDH and RPL5 serve as loading controls, respectively. Bottom Panel: (C) The first 651 nucleotides of the genomic T antigen region of SV40. The blue text indicates the first intronic region that is spliced to produce the large T antigen. The underlined portion indicates the intron for small T antigen. Primer sequences are indicated in red.

Figure 4: Genomic Early Region Expression

To determine if the T antigens of BKV and JCV upregulate the interferon response, whole cell extracts were examined and total RNAs were collected from at least three separate pools of MEFs. The pools expressed the genomic early region from different polyomaviruses (SV40, BKV, or JCV) (**Figure 4A**). Western blot analysis confirmed the previous results that SV40 T antigen upregulates expression of total STAT1 and its activated form at the tyrosine-701 site (**Rathi 2010**).

Additionally, STAT1 activation at the serine-727 site as well as total STAT2 expression also increased in the presence the SV40 T-Antigen (**Figure 5A**). Furthermore, a subset of the downstream targets of interferon induction, the ISGs, were examined by semiquantitative reverse transcription PCR analysis of total cellular RNA (**Figure 5B)**. This analysis confirmed that there was an increase in the transcription of the ISGs examined. The same analysis was used to examine pools of MEFs expressing BKV and JCV genomic early regions. Both BKV and JCV pools upregulated total STAT1, activated STAT1, and ISG transcripts (**Figure 5**). The results reflected in **Figure 5** hold true for all three pools examined with some exceptions. The BKV genomic early region from one of three pools, did not show an upregulation of total STAT1, however that same pool did still show upregulation for activated STAT1 S727 (data not shown). Tyrosine 701 (P-STAT1) was only examined in one pool from SV40, BKV, or JCV, as the supply of antibody was depleted and the manufacturer discontinued production. Therefore it cannot be determined if activated STAT1 Y701 is upregulated in all MEF pools generated. In conclusion, the expression of T antigens from BKV and JCV upregulate the interferon response in MEFs similar to SV40 T antigen.

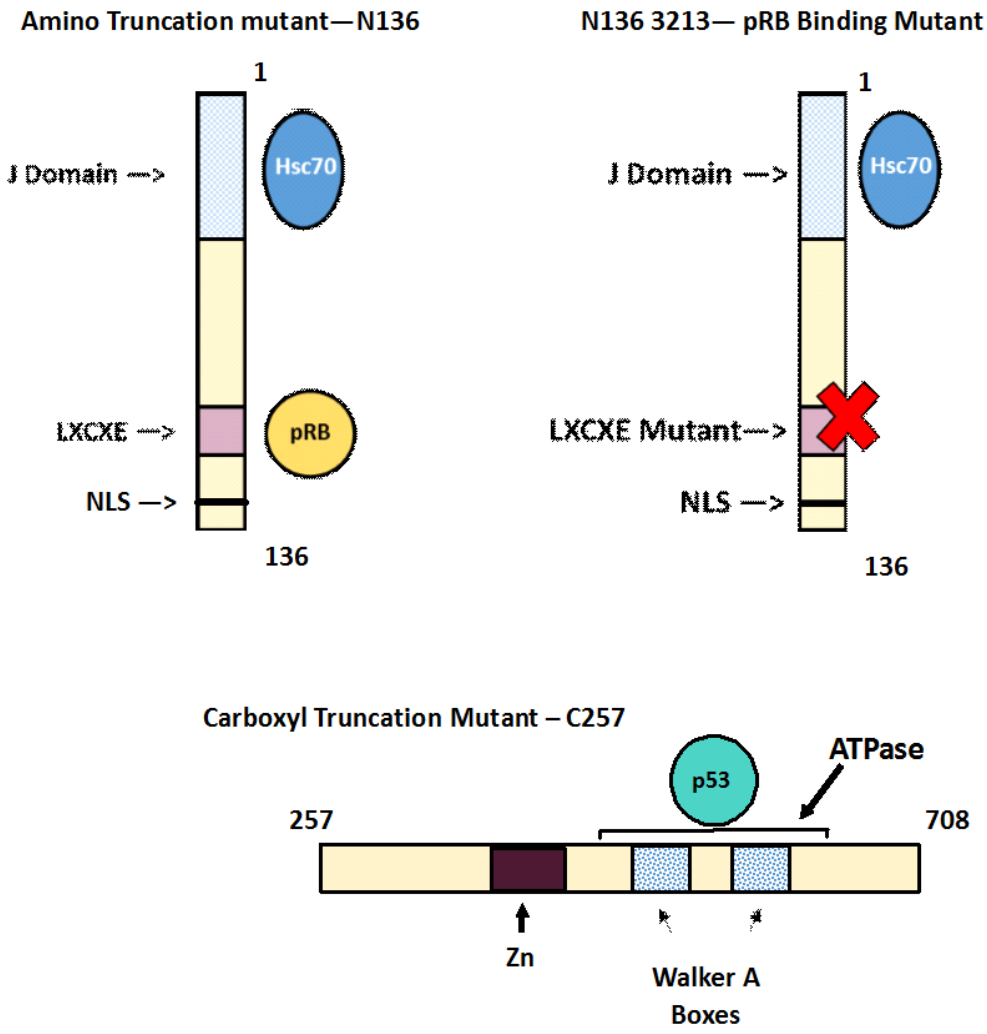


Left lane (A): Western blot of MEF pools expressing the genomic early region of SV40, BKV, JCV, or wt MEF cells without T Antigen (MEF). Right lane: (B) Reverse transcription PCR of ISG subset from the pools of MEFs with T antigen or wt MEFs (MEF). GAPDH and RPL5 serve as loading controls, respectively.

Figure 5: Early region upregulation of interferon response

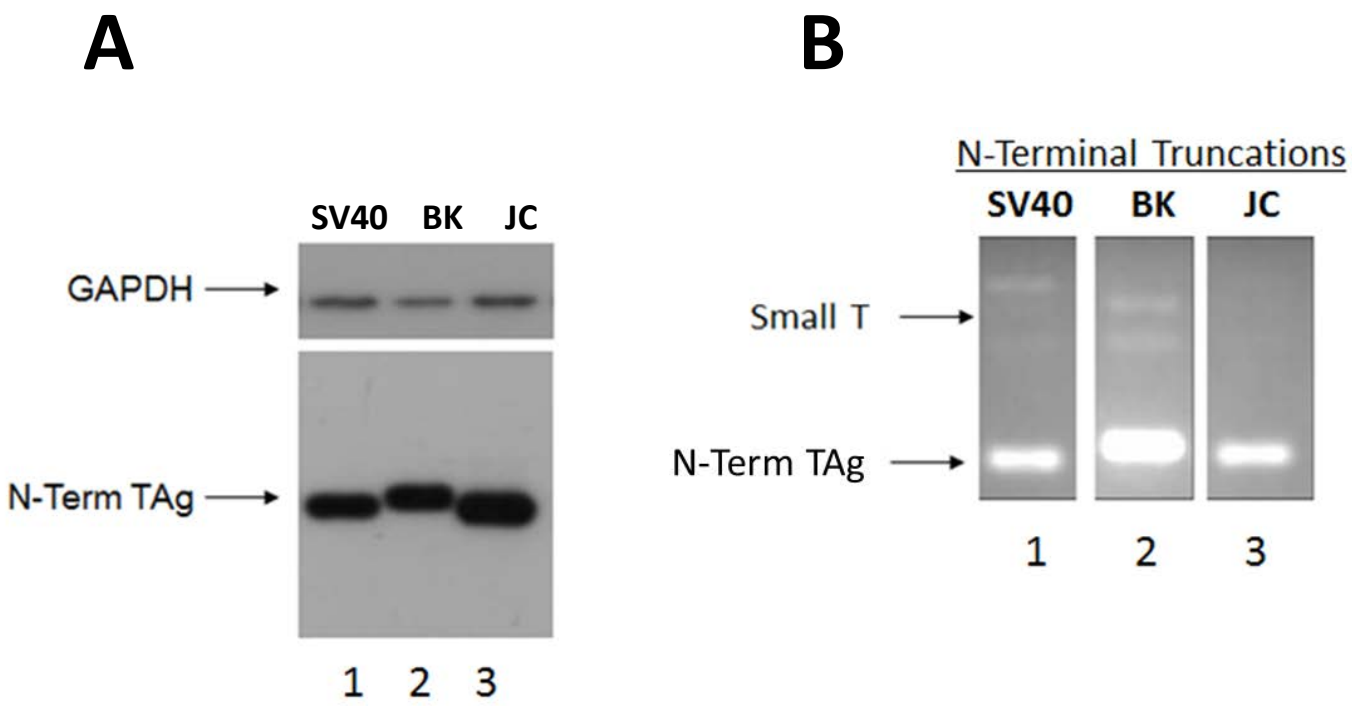
5.2 THE N-TERMINUS OF THE LARGE T ANTIGEN IS SUFFICIENT FOR THE INTERFERON RESPONSE

To map the activity or activities of large T antigen necessary for ISG upregulation, a series of SV40, BKV, or JCV amino-terminal truncation mutants was examined. The amino truncated large T antigen forms carry a J-domain along with the retinoblastoma (Rb) protein binding motif (LXCXE) which together have been shown to bind and release E2F transcription factors leading to stimulation of cellular division (**Sullivan 2002**). **Figure 6A** illustrates SV40's N-terminal truncation mutants. As described above, three MEF pools each were produced expressing the different polyomavirus N-terminal T antigen mutants. As observed in the genomic early region expressing pools, these new pools grew as a heterogeneous mixture of cells with different morphologies. Again, the N-Terminal mutant pools grew faster than wt MEFs. To examine the level N-Terminal LTAg truncation mutants proteins are expressed in the MEF pools, western blots were performed to assess the level protein production, and PCR was performed to estimate the level of transcript mRNA. Additionally, because the amino truncation LTAgs are expressed from genomic sequences truncated at the 136th amino acid, it was necessary to test for the presence of the stAg splice variant. **Figure 7A** shows the expression level of each truncation mutant protein. In each case a single band was observed of the expected size of the N136 mutant equivalents. **Figure 7B** depicts the differential PCR for mRNA levels of N-Term LTAg or sTAg transcript. **Figure 7B** reveals the most robust signals correspond to the expected sizes of the N-Terminal truncation mutants. Thus, the dominate transcripts produced are the N-terminal mutants for all three polyomaviruses tested.



Panel A shows the first 136aa of LTAg, along with features and binding partners (N136). **Panel B** shows the N136 –3213 mutant which is identical to N136, but has abrogated pRB binding from mutation in the LXCXE (N136 3213). Panel C depicts the carboxyl truncation mutant of LTAg with features and binding partners. Numbers at the ends of diagrams indicate the amino acid position corresponding to the full length LTAg.

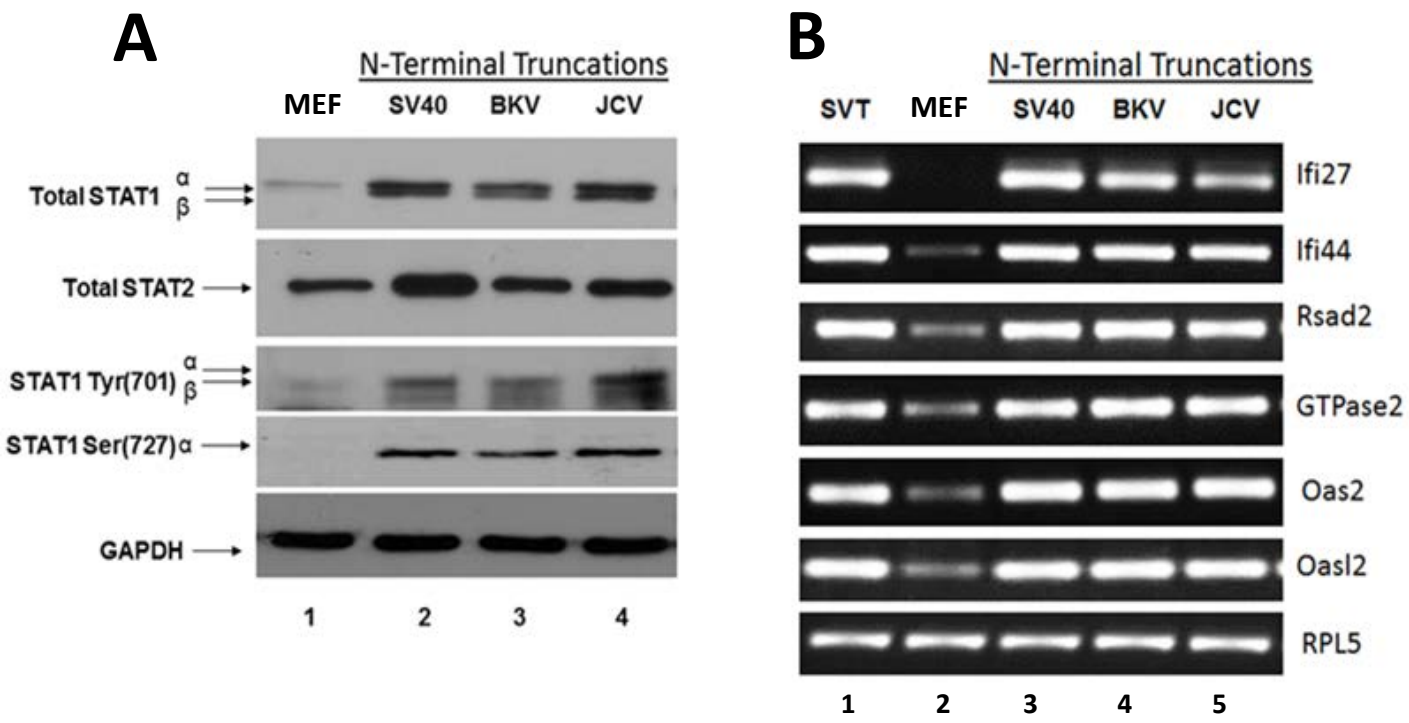
Figure 6: SV40 Large T antigen mutant diagrams



Panel A depicts a western blot using whole cell extract of 2 days post confluent MEF pools expressing the genomic N-terminal truncation mutant from each virus respectively. A cocktail of TAg antibodies (416,419,2003, 701) were used for detection. **Panel B** depicts a reverse transcription PCR of total RNA transcript levels of ISGs from the pools of MEFs with genomic N-Terminal T antigen or naïve wt MEFs (MEF).
Figure 7: N-terminal Truncation Mutant Expression

Examination of three pools from SV40, BKV, or JCV revealed a consistent upregulation of the interferon response signaling modulators and the downstream products, ISGs (**Figure 8B**). These results were comparable with what was observed in the genomic T antigen early regions. However, in each of the three pools tested, total STAT2 failed was not upregulated by BKT-N138 (**Figure 8A**). This was in contrast to the genomic early region expression of BKV. Finally, **Figure 8A** reveals that the amino terminal truncation mutants are capable of upregulating a total levels of

STAT1/2, activated STAT1, and ISG transcripts. I conclude that the amino terminal truncation mutants comprising the first one hundred and thirty-six amino acids in SV40 (or equivalents in the other polyomaviruses) of the large T antigen are sufficient to upregulate the interferon response. The results from figures 7 and 8 indicate that the MEF pools tested preferentially transcribe and express the N-terminal truncation mutant, and that the mutant T antigen expression results in the upregulation of the interferon response.

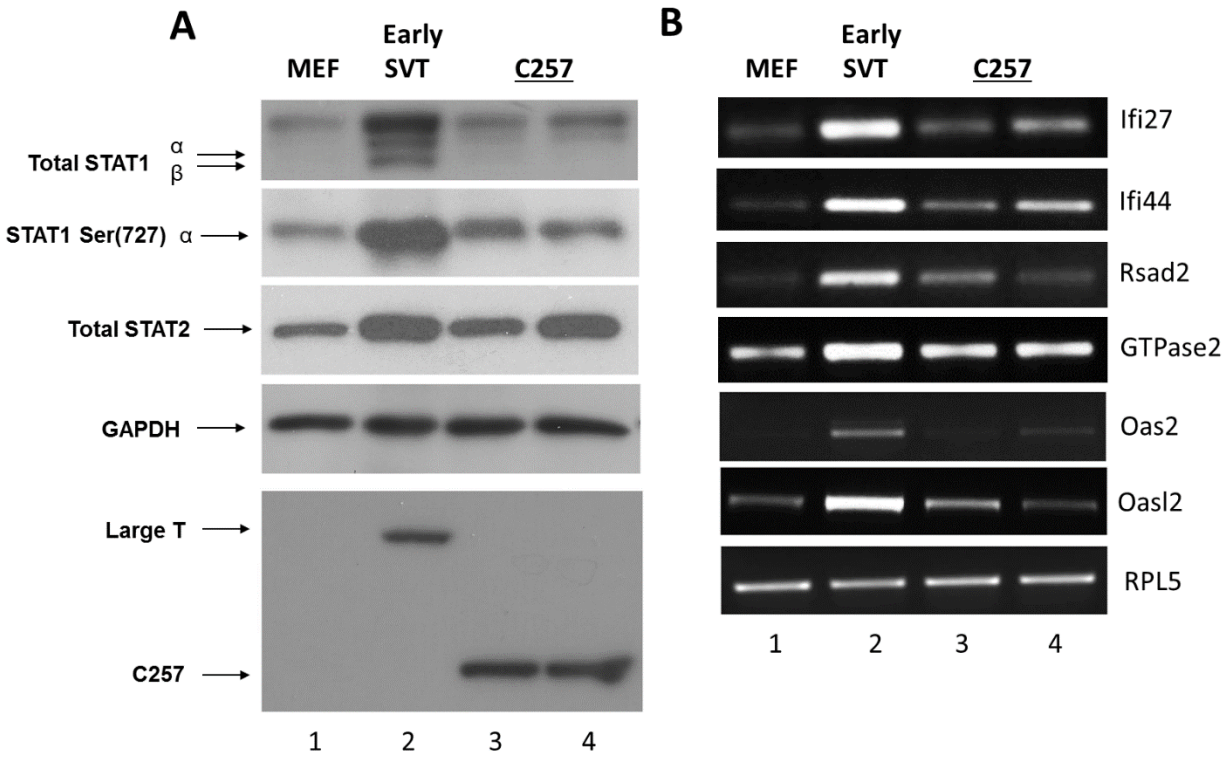


Panel A: Western blot analysis of MEF pools with N-terminal truncation mutants of the viral large T antigen from SV40, BKV, JCV, or naïve MEFs. **Panel B**: ISG transcript levels from MEF pools expressing an N-Terminal truncation mutant of the viral large T antigen or naïve MEFs. Lane 1B indicates upregulated ISG transcript levels from MEF pools expressing the genomic early region of SV40 for comparison.

Figure 8: N-terminal Truncation Mutant Upregulation of Interferon Response

5.3 THE CARBOXYL-TERMINUS OF THE LARGE T ANTIGEN IS NOT SUFFICIENT FOR UPREGULATION OF THE INTERFERON RESPONSE

Next domains within the carboxyl-terminal half of large T antigen were we tested, and unable to upregulate the interferon response in MEFs. I examined two pools of MEFs expressing the carboxy-terminus of the LTAg from the 257-708th amino acids (C257) (**Figure 6C)**. The pools tested were shown to express the C257 truncation mutant (**Figure 9A**). Western blots and PCRs were performed to assess the expression levels of STAT1 and STAT2, and ISG transcription levels (**Figure 9**). Both C257 pools tested were defective for the upregulation of both total and activated STAT1 (**Figure 9A**). Interestingly, total STAT2 varied between the two pools examined, i.e. STAT2 was upregulated over wt in the second MEF pool tested; not the first. Figure 9B shows mRNA transcript levels of selected ISGs, and in nearly every case, the ISGs did not upregulate. These results suggest that the in the carboxyl-terminal domains of large T antigen, cannot upregulate ISGs and is likely not important to the upregulation of the interferon response.



Panel A depicts western blot using whole cell extract of 2 days post confluent MEF pools expressing the carboxyl truncation mutant (C257) from SV40 compared to naïve MEFs and MEF pools expressing the genomic early region of SVT. C257 expression was tested with mouse monoclonal 901. **Panel B** depicts ISG transcription levels of C257 mutant MEF pools compared to naïve MEFs and MEF pools expressing the genomic early region of SVT.

Figure 9: C-terminal truncation mutant expression

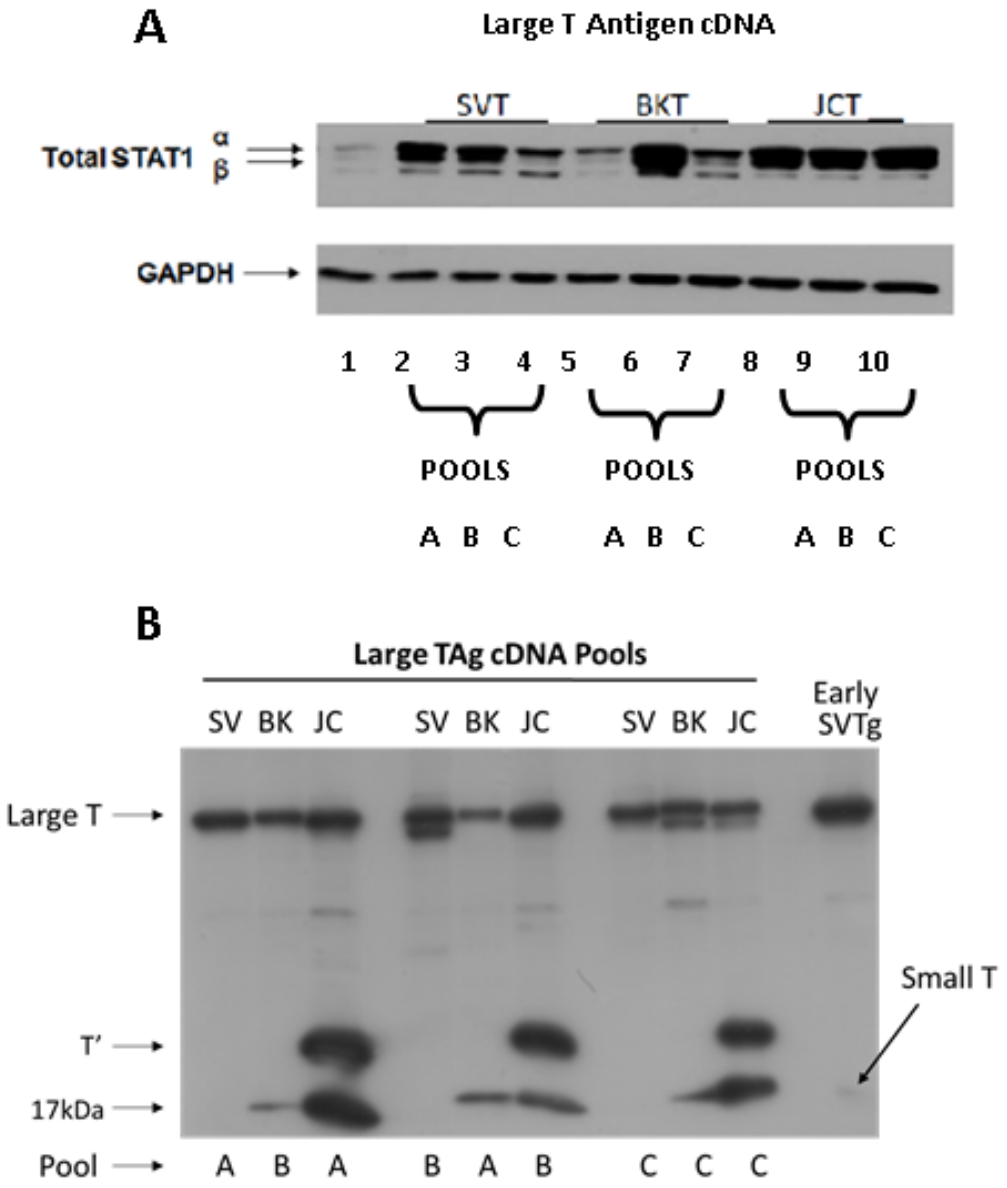
5.4 SMALL T ANTIGEN IT NOT NECESSARY FOR THE UPREGULATION OF THE INTERFERON RESPONSE

Since the SV40 genomic early region can express at least three transcripts (large T, small T, and 17k T), it was tested whether the small T antigen contributes to the induction of the interferon response. Although the previous evidence in this study indicates a relatively low level of stAg transcription/ expression within the pools tested (**Figures 4 & 7**), it is possible that a small amount of the protein can upregulate the interferon response. Thus, constructs encoding the cDNA of the viral LTAg that are incapable of expressing small t antigen were tested. These constructs were expressed in MEFs and cell pools were tested for T antigen expression and upregulation of the interferon response.

To reveal the level of T antigen expression in the MEF pools possessing constructs encoding the cDNA of the viral LTAg, a mixture of monoclonal antibodies against SV40 TAg (416 and 419) and JC TAg (962 and 2003) were used in detection of T antigen (**Figure 10B**). The figure reveals expression of LTAg cDNA in the all of three pools tested (**Figure 10B**). However, some pools may express degraded forms of the LTAg, i.e. slightly smaller appeared bands near the expected signal (**Figure 10B**). Additionally, numerous alternative, non-small T antigen, T antigen splice forms are observed in the different pools for BKV and JCV (**Figure 10B**).

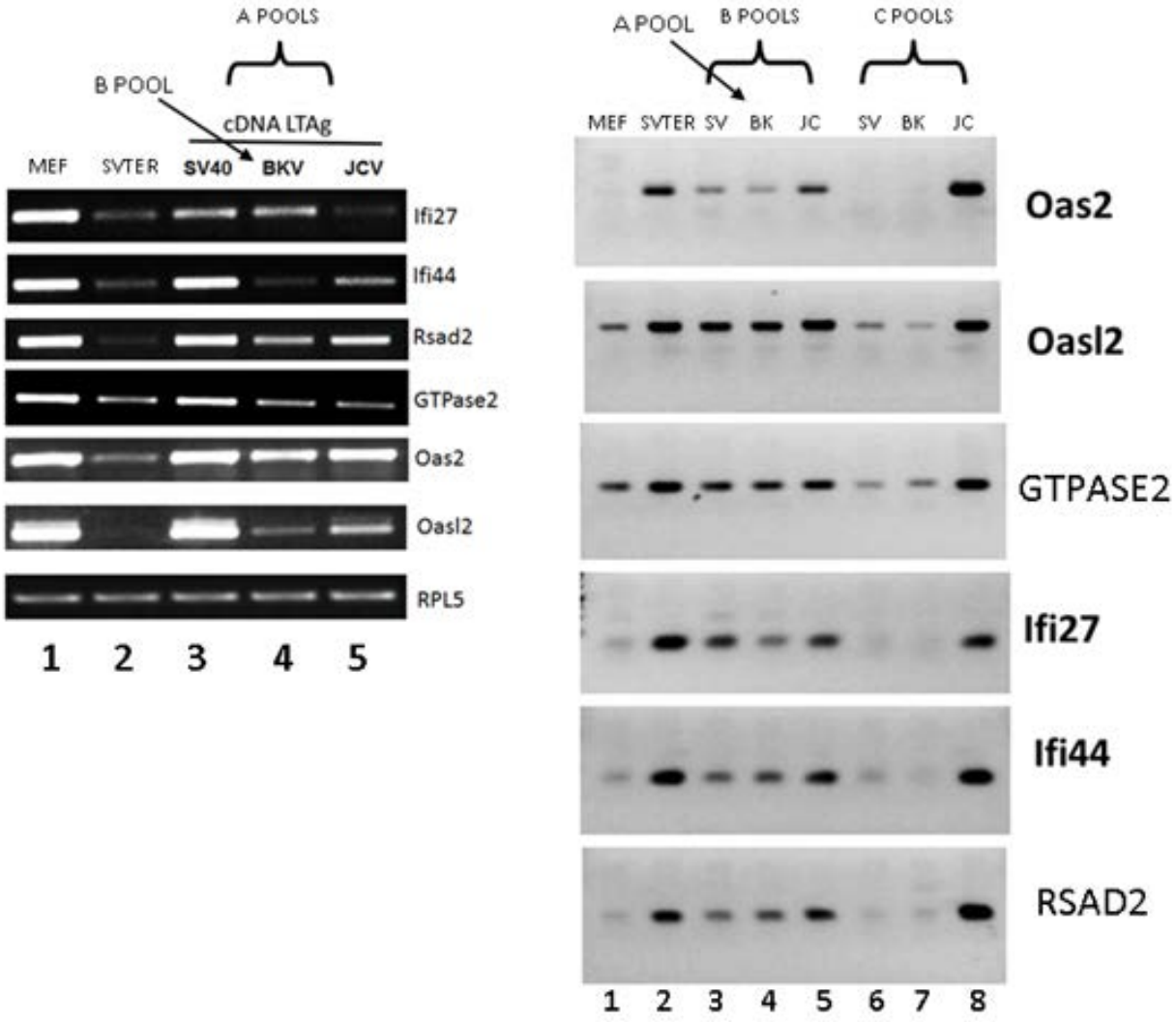
In three separate MEF pools, western blots showed JCV able to upregulate total STAT1 in all three pools tested (**Figure 10B**). Both SV40 and BKV showed upregulated total STAT1 in at least two of the three pools tested (**Figure 10A**). However, in PCR for ISG transcript levels, SV40 and BKV only showed upregulation in two of three pools tested (**Figure 11**). JCV showed all three pools upregulating ISGs. I conclude that pools of T antigen expressing MEFs lacking small T antigen were capable of upregulating the interferon response.

Results from figures 10 and 11 indicate that the MEFs pools tested expressing constructs encoding the cDNA of the viral LTAg, are capable of large T antigen expression and other T antigen splice products, but do not express small T antigen. Additionally, these pools are still capable of upregulating the interferon response. Together, these results suggest that small T antigen is not necessary in the T antigen induced upregulation of the interferon response.



Panel A shows the protein levels of total STAT1 for three pools each of the different viral LTAg tested. **Panel B** shows T antigen expression in each MEF pool tested, (A,B, or, C). The last lane of **panel B** shows SV40 genomic early region expression for comparison.

Figure 10: Large T antigen cDNA expression



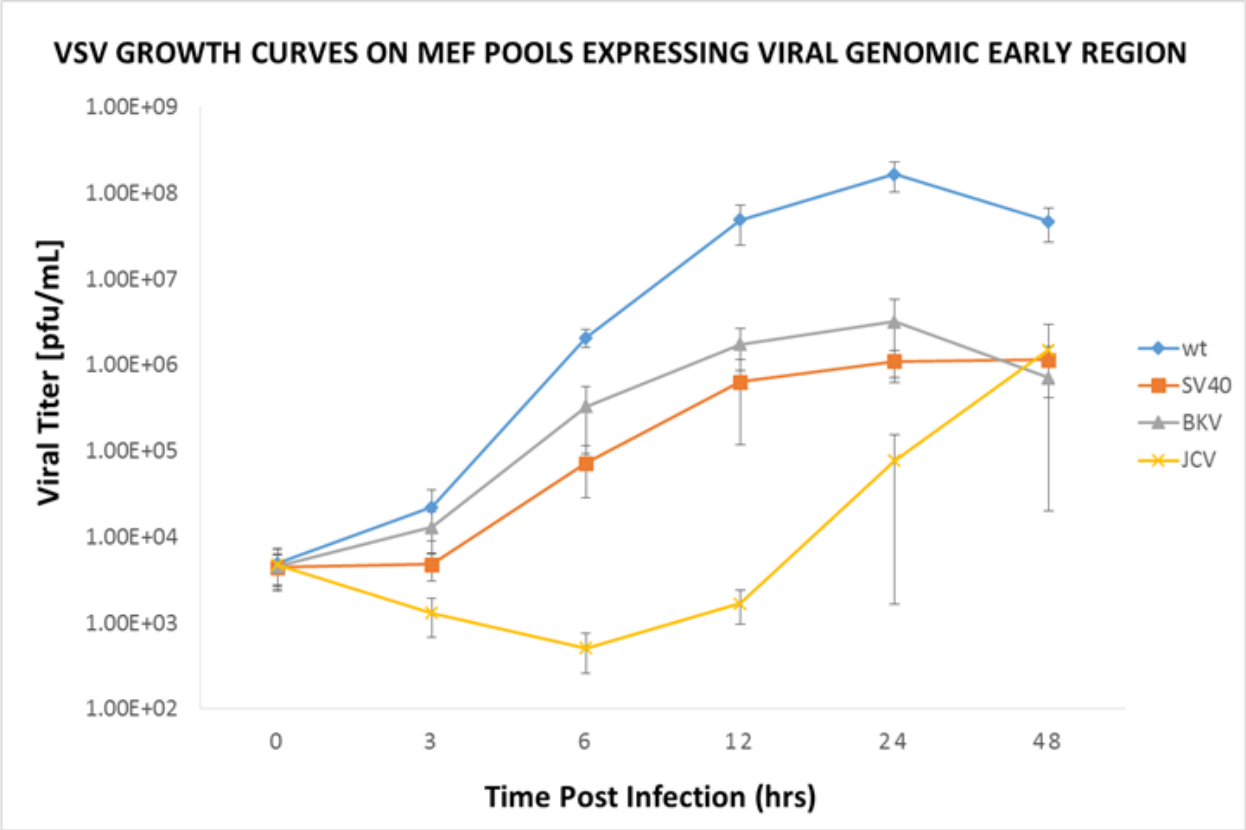
Both panels show ISG transcript levels across the different MEF pools (a, b, c) expressing the LTAg cDNA from the corresponding virus. The first two lanes in both panels are either naïve MEFs (MEFs), or MEF pools expressing the genomic early region of SV40 (SVTer).

Figure 11: ISG Transcription in Large T cDNA pools

5.5 POLYOMAVIRUS T ANTIGEN INDUCES AN ANTI-VIRAL STATE

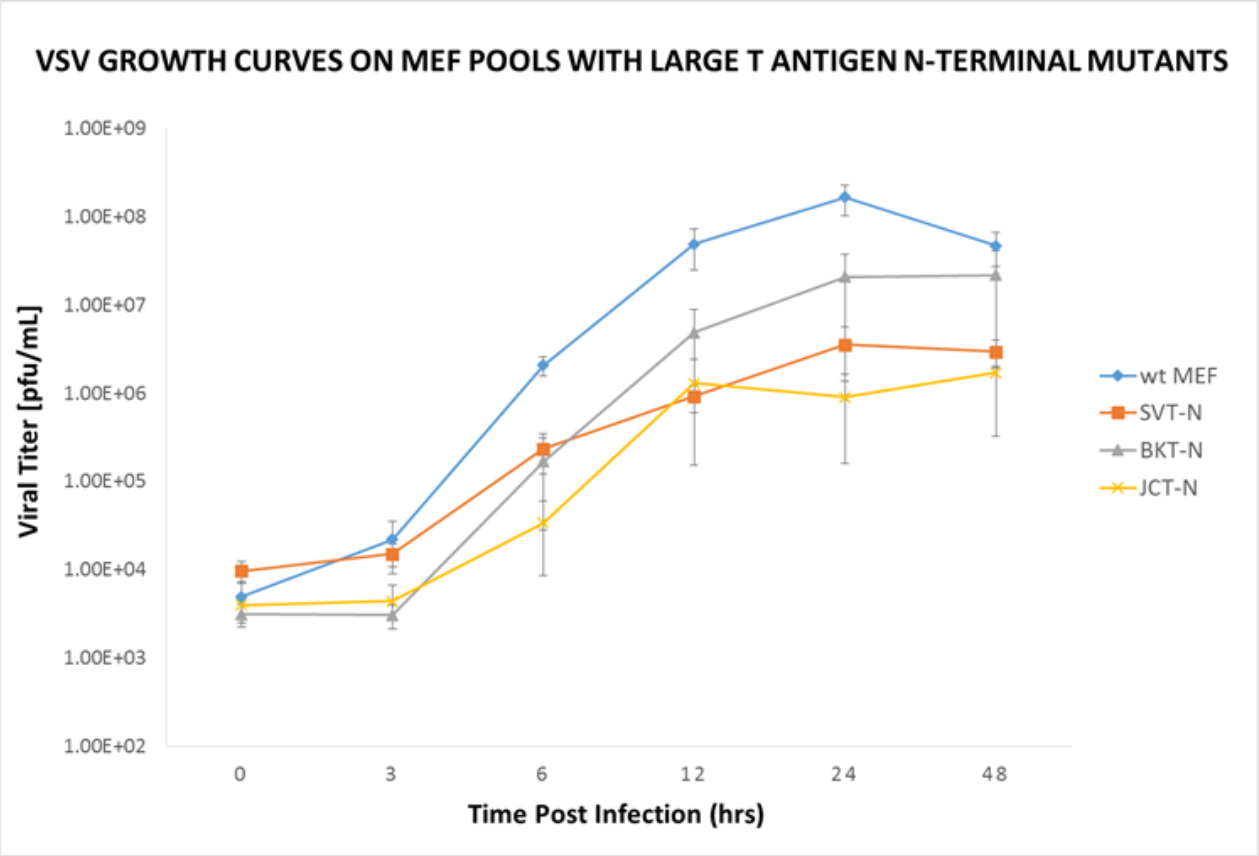
The interferon response is normally upregulated upon pathogen detection, and the response results in a defensive state for the cell against the pathogen. In this study, the pathogen VSV served as a measure of the antiviral state. VSV is a positive sense RNA virus. RNA viruses have been shown to be susceptible to ISG induction (e.g. OAS2) (**Silverman 2007, Lin 2009, Fensterl 2012**). To determine if large T antigen expression induces an antiviral state I tested pools of MEFs expressing the SV40, BKV, or JCV early regions for the ability to support VSV growth. VSV growth curves were prepared by titration of supernatant harvested from MEF pools at various time points. Infections in separate MEF pools expressing the different polyomavirus T antigens showed the production of VSV virus to be attenuated by typically 10-100 fold, with few exceptions. Growth curve data is illustrated in **Figures 12** and **13**, for the genomic early region expressing pools, and N-terminal truncations mutant pools, respectively. However, both of these figures exhibit the identical data (i.e., the same data) for the growth of VSV on wild-type MEFs. The data for VSV growth on wild-type MEFs were used in both figures for comparison to either the genomic early region expressing pools, or the N-terminal truncations mutant pools. **Figure 12** shows that wt MEFs produced 1e8pfu/mL of VSV at about 12-48hrs. However, VSV growth plateaus at ~1e6pfu/mL around 12-48 hours in MEF pools expressing the genomic early regions of SV40 or BKV. JCV MEF pools appears to exhibit slower production of VSV relative to the other pools. However, JCV pools still eventually produced the same titer at 48hrs as SV40 and BKV (**Figure 12**). Similar to the results shown in figure 12, MEF pools expressing the N-terminal truncation mutant of the large T antigen produce VSV titers of ~1e6pfu/mL at 12-48hrs (**Figure 13**). However, one pool of BKV N-terminal truncation MEFs exhibited slightly higher viral titers (~1e7-1e8pfu/mL) between 12-48hrs (**Figure 13**).

To address the possibility that the number of cells infected during the VSV growth curve assays influenced virus production, statistical analyses were performed. That is, could the reduced viral output observed in T antigen expressing cells instead be attributed to how many cells were infected, and not the influence of T antigen? While conducting the VSV growth curves described above, the number of cells infected varied between some experiments. Specifically, the number of cells infected across experiments, both with and without a polyomavirus T antigen, had a mean of ~9.5e5 cells, a median of 8.5e5cells, and a range of 2.35e6 cells. However, the MOI of 0.1 was adjusted according for each assay based on the number of cells counted at the time of infection. The viral titer at 24 hours (peak of virus production) for each experiment was correlated to the initial number of cells infected. Only a very weak negative correlation could be observed between the number of cells at the time of infection and the ultimate viral output at 24hrs (Correlation = -0.19). If it is assumed there is no influence by T antigen expression on the output of virus, this statistical observation confirms that the number of cells infected did not influence the production of virus. Thus, the expression of T antigen must be responsible for the attenuated level of virus production in T antigen MEFs compared to wt MEFs.



Early region pools are either SV40 (red), BKV (grey), or JCV (gold). The X-axis indicates time points of collection post infection. Viral titer is indicated on the Y-axis. Standard error of the mean is included at each data point.

Figure 12: Titration of VSV growth on wt MEFs or MEF pools expressing the genomic early region

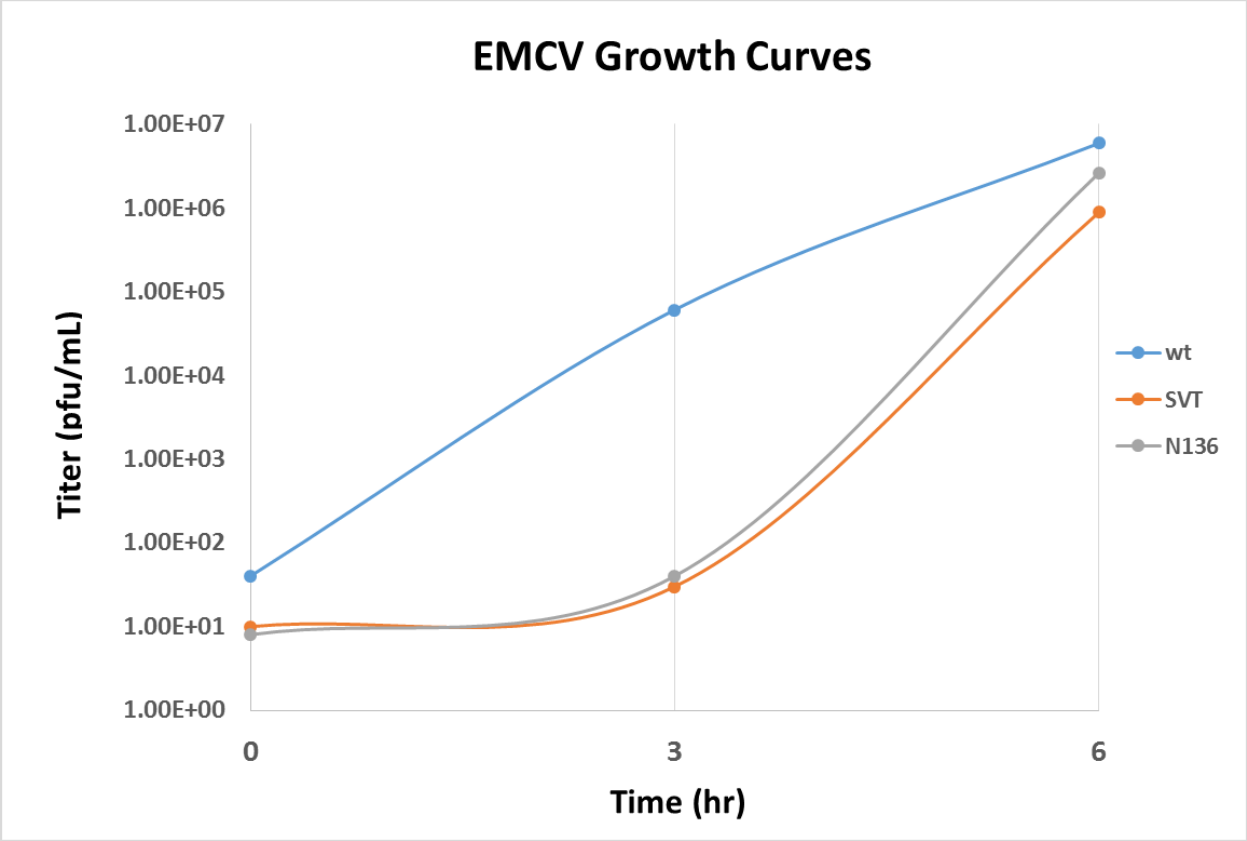


Pools are either SV40 LTAg (red), BKV LTAg (grey), or JCT LTAg(gold). The X-axis indicates time points of collection post infection. Viral titer is indicated on the Y-axis. Standard error of the mean is included at each data point.

Figure 13: Titration of VSV growth on wt MEFs or MEF pools expressing the amino terminal truncation of large T antigen

The antiviral state produced in T antigen expressing MEFs is not limited to VSV, and was extended to encephalomyocarditis virus (EMCV). In order to determine if T antigen could attenuate EMCV growth additional growth curves were performed. Wild type, SV40 genomic early region expressing MEFs, and N136 MEFs, were infected at an MOI of 0.005 over a time course of six hours. At six hours post infection peak titers for both the T antigen pools and wild type pools were reached. However, at 3 hours post infection a 3000-fold reduction in viral titer was observed in both T antigen expressing pools when compared to wild type MEFs (**Figure 14**).

Based on the evidence from both VSV and EMCV viral growth curves, it can be concluded that the T antigen induced interferon response in MEFs results in an antiviral state.



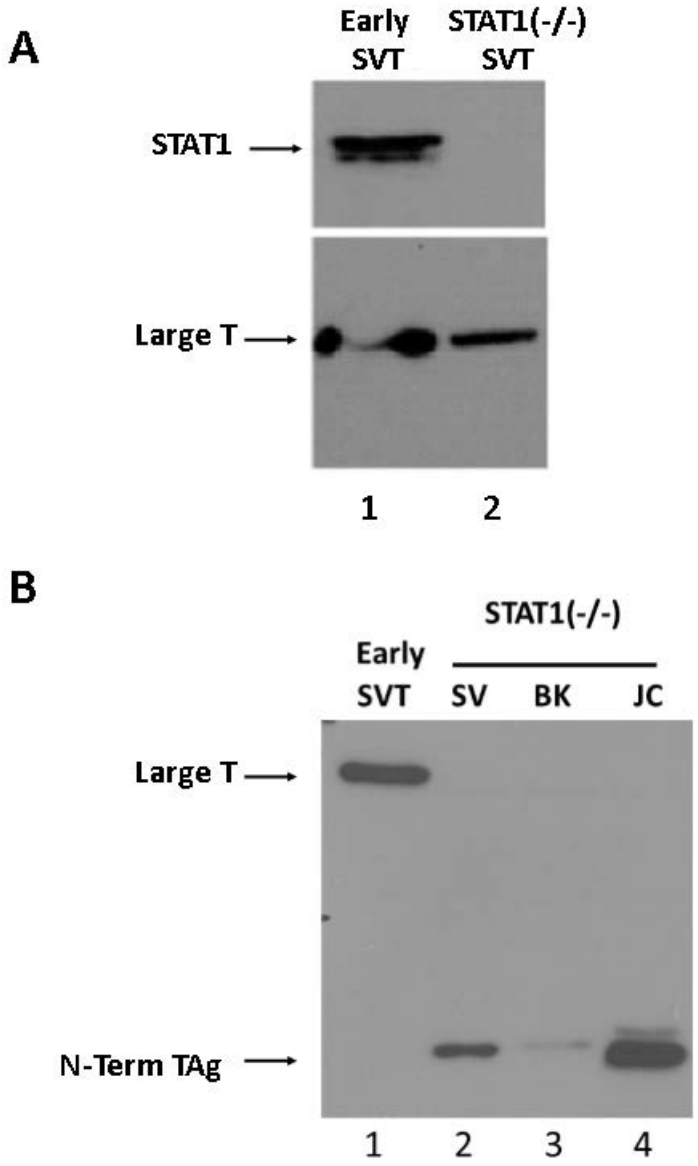
EMCV growth was examined on wt MEFs, MEF pools expressing SVT genomic early region, or MEF pools expressing the N-terminal truncation mutant of T antigen (N136). The X-axis shows the time points of collection post infection. Viral Titer (pfu/mL) is indicated along the Y-axis.

Figure 14: Titration of EMCV growth

5.6 STAT1 TRANSCRIPTION FACTOR IS NECESSARY FOR THE INDUCTION OF THE ANTIVIRAL STATE

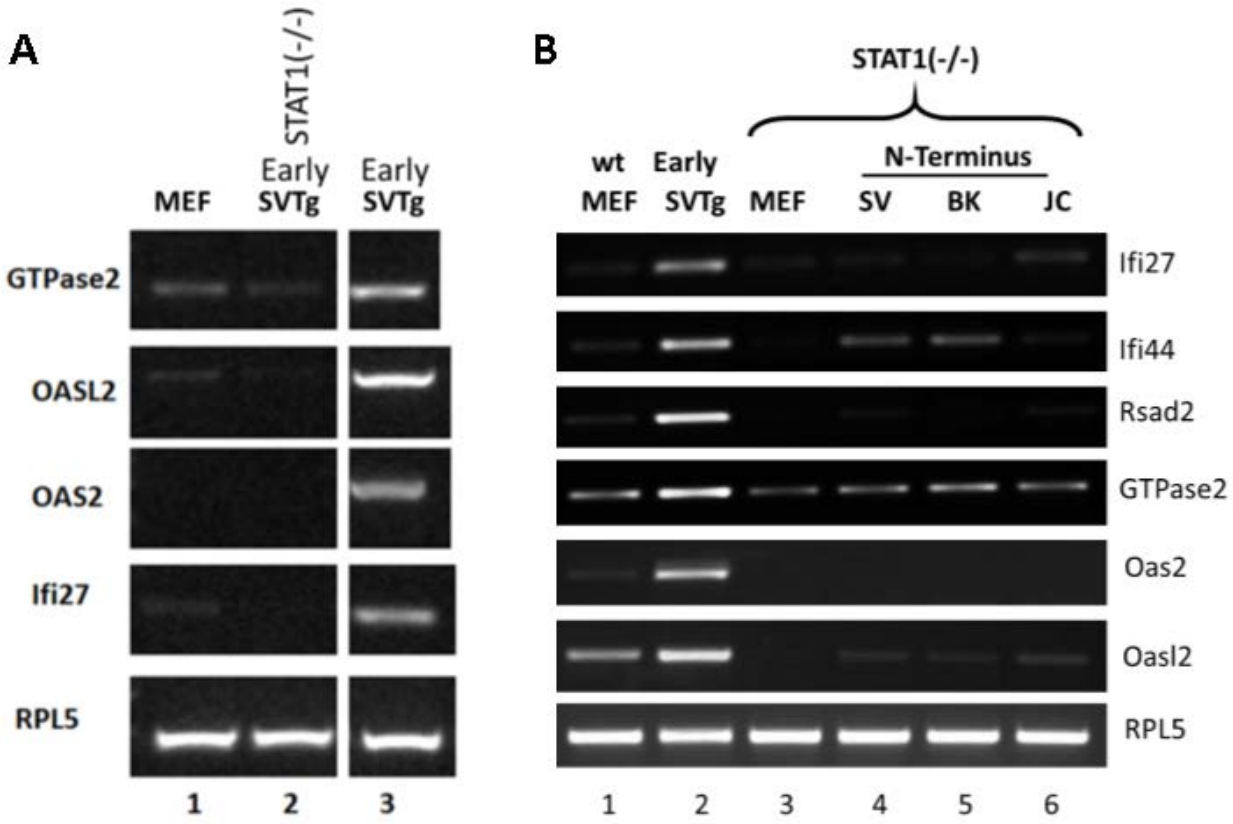
To determine if the upregulation of ISGs by T antigen requires STAT1, pools of STAT1 knockout MEFs expressing T antigen were examined. The STAT1 transcription factor is an important mediator in IFN signaling pathways. It was shown that IFN signals (e.g IFN-α,β) are not produced during T Antigen expression in MEFs, but the interferon response is still upregulated (**Rathi 2010**). Therefore, it was hypothesized that T Antigen may be reliant on type I or II IFN pathway mediators for ISG induction. To determine the possible importance of STAT1, T Antigen-expressing pools were generated from MEFs generated from STAT1(-/-) mice. As described earlier, whole-cell protein extract and total RNA from these pools were examined. Pools were expressing T antigen as expected (**Figure 15**). Results showed none of the viral T antigens able to induce uprgeulation of ISG (**Figure 16**). **Figure 15** indicates that all pools not upregulating ISGs are still expressing the viral T antigens. In conclusion, STAT1 transcription factor is necessary for the upregulation of ISGs by T antigen.

Quantification of viral (VSV) production revealed no appreciable difference between that produced from STAT1(-/-) MEFs and STAT1(-/-) MEFs expressing T antigen (**Figure 17**). The levels of VSV production on wild-type MEFs and MEFs expressing the genomic early region from SV40 from **Figure 12** are included for comparison (**Figure 17**). The data from **Figure 17**, in conjunction with examination of ISGs (**Figure 16**), suggests STAT1 is necessary for the upregulation of ISGs by T antigen expression in MEFs, and that generation of the antiviral state is also dependent on STAT1. I conclude that the antiviral state generated by the SV40 T antigen is not maintained with the loss of STAT1.



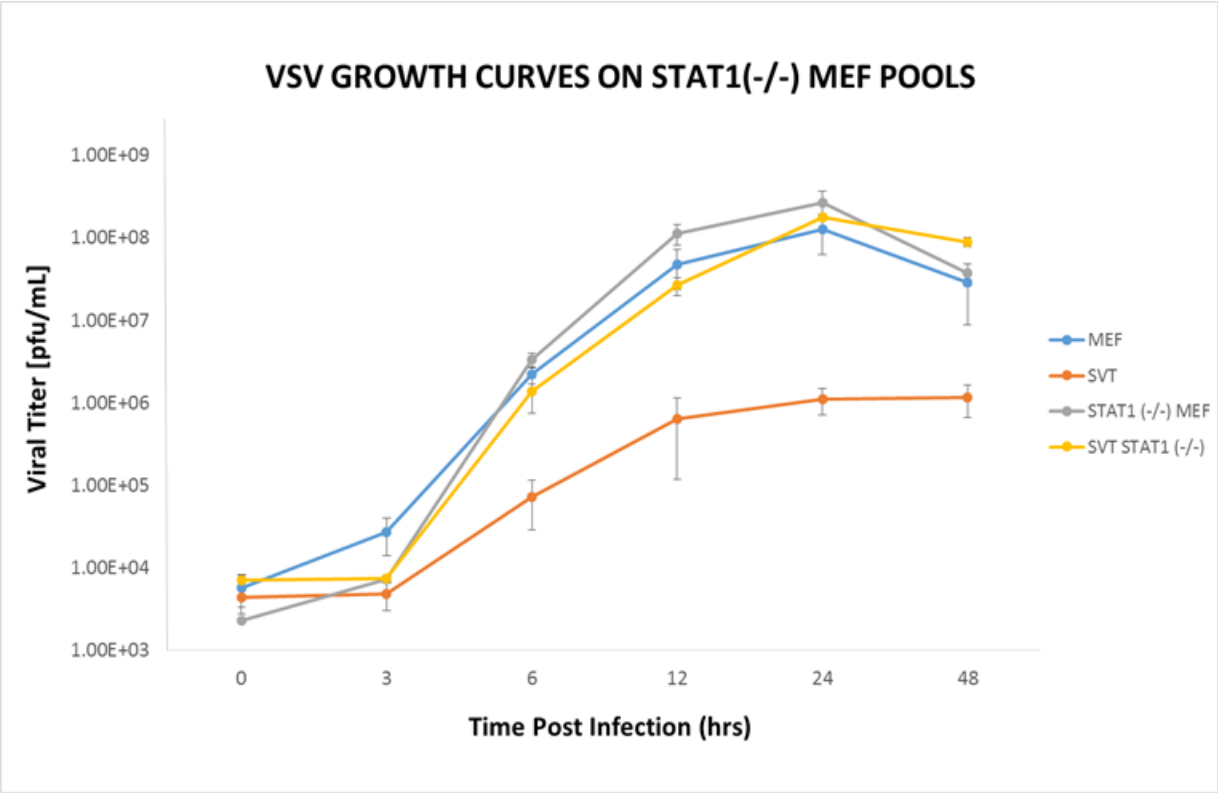
Panel A shows the genomic early region of SV40 in STAT1(-/-) MEF pools (lane2). Normal MEF pools of SV40 genomic early region are included in lane 1. Panel B expression of the N-terminal truncation mutants for SV40, BKV, and JCV in STAT1(-/-) MEF pools (lanes 2-4).

Figure 15: Levels of T Antigen expression in STAT1(-/-) MEF pools



Pools expressing the genomic early region of SV40, **Panel A** (lane 2)**. Panel B** depicts the ISG transcript levels in N-terminal truncation mutant expression STAT1(-/-) MEF pools (lanes 4-6). Lane 3 of panel B depict STAT1(-/-) MEFs without T Antigen.

Figure 16: ISG transcript levels for STAT1(-/-) MEF pools

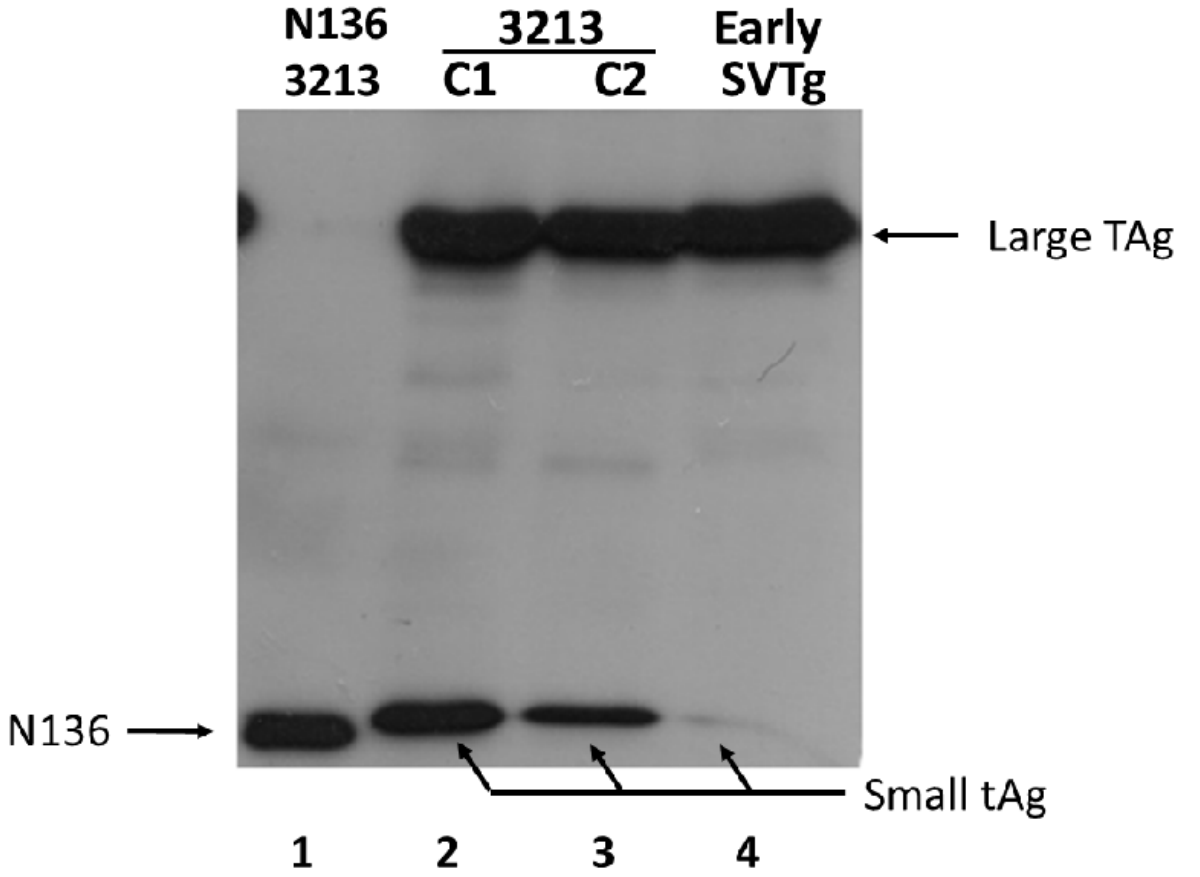


SV40 early region STAT1(-/-) MEFs (gold), or normal MEF pools expressing the genomic early region SV40 (red). Wild-type MEFs are indicated in (blue), and STAT1(-/-) MEFs are indicated in (grey). The X-axis indicates time points of collection post infection. Viral titer is indicated on the Y-axis. Standard error of the mean is included at each data point.

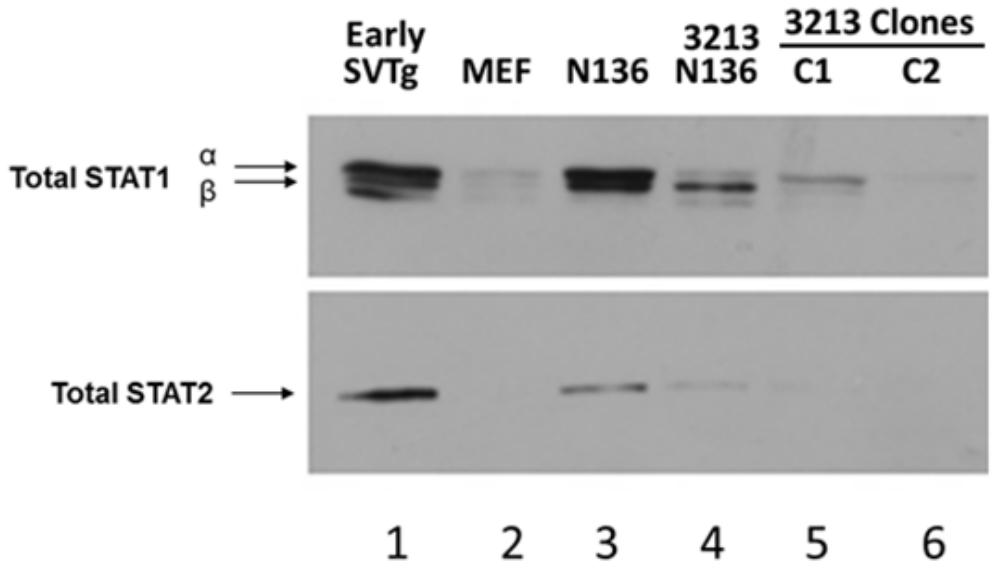
Figure 17: Titration of VSV growth on STAT1(-/-) MEF pools

5.7 T ANTIGEN BINDING OF RETINOBLASTOMA PROTEIN FAMILY MEMBERS MAY BE IMPORTANT TO UPREGULATION OF THE IFN RESPONSE

Because the truncated amino terminal mutant of LTAg from SV40, BKV, and JCV can upregulate the IFN response, it was hypothesized that binding of pRB family members, may be responsible for the upregulation of the interferon response. Two full length SV40 LTAg expressing clones, and a pool expressing SV40 N136, all unable to bind pRB were examined. The N136 pRB binding mutant (3213) is illustrated in **Figure 6**. Binding of pRB is abrogated by mutations at the E107K and E108K sites. The full length LTAg pRB binding mutant carries the same mutations, (diagram not shown). The expression of T antigen in the pRB binding mutants is shown in **Figure 18**. The evidence in **Figure 19** shows that with the inability to bind pRB via the LXCXE motif, T antigen fails to upregulate total levels of STAT1 or STAT2 to that of its unmutated form. This data requires further substantiation, but may be indicative of T antigen/pRB binding interaction as necessary for the upregulation of the IFN response. In conclusion, the binding of pRB by T antigen may be necessary to upregulate the interferon response.



Lane 1 shows N136 3213 mutant **lane 2 and 3** show clones of genomic full length T antigen 3213 mutants.
Lane 4 shows genomic early region expression of SV40 for comparison.
Figure 18: T Antigen expression in 3213 mutants



Lanes 1 and 3 represent MEF pools from genomic early region from SV40 and the LTAg amino truncation mutant (N136), respectively. Lane 2 is wt MEF. Lane 4 represents MEF pool of N136 truncation with pRB binding mutations. Lanes 5-6 are MEF clones expressing full length LTAg with pRB binding mutations.

Figure 19: Western blot for total STAT1 or STAT2 in 3213 mutants

6.0 DISCUSSION

Induction of an antiviral state is generated as a result of type I and II interferon signaling pathways. These pathways upregulate products (ISGs) that inhibit virus growth by blocking viral mRNA translation and/or nucleic acid replication. The pathways are initiated by binding of IFN signal to their cognate receptors resulting in subsequent activation/dimerization of STAT transcription factors (referenced above). STAT dimers translocate to the nucleus and promote the transcription of ISGs (referenced above). The STAT1 transcription factor has been shown to be critical to the induction of an antiviral state (**Horvath 1996**).

This study has shown that Polyomavirus T antigens upregulate the cellular interferon response in primary mouse cells, and that the induction results in an antiviral state. This study has shown that multiple polyomavirus T antigens are capable of upregulating the interferon response. It was hypothesized that because the genomic early regions of polyomaviruses SV40, BKV, and JCV encode similar T antigen proteins, the interferon response upregulated by SV40 T antigens (**Rathi 2010**) would also be observed in cells expressing the other polyomavirus T antigens. The data shown in **Figure 5** shows this, and thus extends on the previous observations made by Rathi and others. **Figures 13 and 14** show reduced VSV production in MEFs expressing the different polyomavirus early regions or large T antigen amino truncation mutants, respectively. The reduced virus proliferation in T antigen expressing pools compared to wt MEFs may be attributed to the upregulation of the interferon response and subsequent ISG production. This likely results in the establishment of a cellular environment that is inhibitory to virus growth. This study only examined a few of the hundreds of genes upregulated by interferon signaling, it is unclear exactly which ISGs are at play in the phenotype. However, it is reasonable to conclude that the ISG profile

examined could be at least in part responsible for the antiviral state as their antiviral activities have been documented (**Sadler 2008, Horvath 1996, Hallen 2007, Balan 2006, Lin 2009, Moal 2012**).

The MEF pools expressing the genomic early region from SV40, BKV, and JCV produce a variety of T antigen splice products. The data from **Figure 4A** shows expression of both LTAg and smaller T antigen products that could be stAg or 17kT. Further analysis depicted in **Figure 4B** shows transcription of the LTAg clearly and a relatively small presence of stAg. Additional examination has shown small T antigen transcript made in all genomic early region pools (data not shown). Interestingly, BKV showed a high level of LTAg transcript but no expression of the LTAg product. The likely explanation for this observation is that there is a high proportion of BKV truncated TAg made. Because the position of the forward and reverse primers are upstream of the truncated TAg introns, any truncated TAg transcript made would be indistinguishable from large T antigen transcript, as visualized on an electrophoresis gel.

This study found that the amino terminal truncation mutant of the LTAg is necessary and sufficient for the upregulation of the interferon response, but the carboxy terminal mutant is neither. The data from **Figure 8** shows the ISGs upregulated to the level of the full length T antigen. However the data in **Figure 9** reveal that the C257 mutant is unable to induce the induction of ISGs. Together, these data suggest that the activity in the amino terminus of the large T antigen is responsible for the induction of the interferon response.

The binding of pRb family members may be necessary for upregulation of the interferon response by T antigen. Because the activity of T antigen necessary for upregulation of the interferon response can be narrowed to within the first 136 amino acids, the elimination of activities within the truncation mutant sequence should abrogate the ability to induce the

interferon response. The data from **Figure 19** shows with the loss of pRB binding there is also a failure to upregulate STAT1/2 levels.

Elimination of the small T antigen does not prevent the upregulation of the interferon response. At least two of the three pools expressing polyomavirus LTAg cDNA (or 3/3 for JCV) examined were still able to upregulate the interferon response. Although the structures of SV40 small T relative to JCV and BKV small T are very different, loss of these proteins did not prevent the MEF pools to still upregulate the response in either case. One interesting question that remains is if the small T antigen expressed alone can upregulate the interferon response.

The expression of STAT1 transcription factor is critical to the upregulation of the interferon response and the establishment of the antiviral state. As shown previously (**Horvath 1996**), STAT1 is necessary for the generation of an antiviral state. In agreement with those previous observations, this study shows that the T antigen induction of the antiviral state depends on STAT1 transcription factor (**Figure 17**). This data also suggests the possibility that T antigen is acting upstream of the STAT1 transcription factor.

Many questions still remain about the mechanism behind the induction of the antiviral state. Because the phenotype seems to be activated with at least the expression amino terminus truncation mutant, the activity of T antigen necessary should be limited to this region. The known activity of the region involves the binding of the J domain to the cellular Hsc70 protein to incite release of the cellular E2F transcription factors bound to the viral LXCXE motif. The evidence provided in **Figure 19** indicates that pRB binding may be necessary for at least the upregulation of STAT1/2 total protein levels. This evidence requires further substantiation to conclude on the apparent inability of pRB binding mutants to upregulate the interferon response. Interestingly, examination of clones with cellular E2F knockouts expressing the genomic early region of SV40

or SV40 N136, failed to upregulate total STAT1 (data not shown). Although additional replicates have yet to be tested, or have ISG transcripts been examined, this evidence may be indicating that somehow the cellular E2Fs, after they are released from pRBs by T antigen, play a role in upregulating the interferon response.

Alternative mechanisms for the upregulated interferon response phenotype are also possible. A recent study by (**Ferrero 2014**) elucidated the mechanism behind a similar phenotype in human BJ-hTERT cells. They found that the large T antigen mediated DNA damage response activates IRF1 signaling inducing the production of IFNβ and subsequent transcription of ISGs (**Ferrero 2014**). However, because the previous study by Rathi et al. in 2010 indicated that MEFs expressing T antigen did not show interferon signal production it can be concluded that mouse cells follow a different pathway for the upregulation of the interferon reponse. This study attempted to identify other candidate interferon signals that maybe responsible for activating the interferon response, in addition to confirming the previous result by Rathi and others in 2010. An RT-PCR was performed using primers designated for α and β inteferons as described previously (**Rathi 2010**). Those experiments failed to show interferon production in any pool tested (data not shown). Alternatively one interferon β signal not tested which does bind to the type one interferon receptor, interferon β2 (a.k.a. IL-6), was examined. That experiment showed a uniform level of the factor produced across MEFs and MEFs expressing T antigen. Thus, it seemed as though there was no upregulation of the signal with the expression of T antigen versus wt MEFs (data not shown). However, this does not rule out the possibility that IL-6 transcripts are expressed at a higher level in T antigen expressing cells. Another recent study implicates T antigen as a mediator of AKT phosphorylation (**Yu 2008**). That study suggested that SV40 large T antigen interacted directly with insulin receptor substrate-1 (IRS-1), which binds to PI3K, which phosphorylates

AKT. With the elimination of IRS-1 T antigen mediated activation of AKT was also lost. Most interestingly, they showed that T antigen with a mutation for pRB binding was unable to bind IRS-1 and activate AKT (**Yu 2008**). The study by Yu and others is most provocative in light of the results produced from this study. Further investigation into the possible connection between IRS-1/ T Antigen binding may prove fruitful for understanding the concurrent upregulation of the interferon response.

T antigen upregulation of the interferon response is not necessary for cellular transformation. Polyomavirus T antigen is well known for its studies on cellular transformation and tumorigenesis (**Damania & Pipas 2009**). It was also hypothesized at the beginning of this study that T antigen's upregulation of the interferon response may be related to the onset of cellular transformation. Thus, because it is well known that LTAg and truncations of LTAg will transform primary MEFs in vitro, a preliminary experiment was conducted to determine if T antigen expressing MEF pools unable to upegulate ISGs would also fail to transform. An anchorage independence assay of a pool of SVTg early region STAT1(-/-) MEFs were examined alongside STAT1(-/-) MEFs alone. The SVTg early region STAT1(-/-) MEFs were able to clearly transform the cells in culture and grow independent of anchorage while the STAT1(-/-) MEFs did not. Based on this evidence it appears that STAT1 and ISG upregulation are not related to T antigens ability to transform cells.

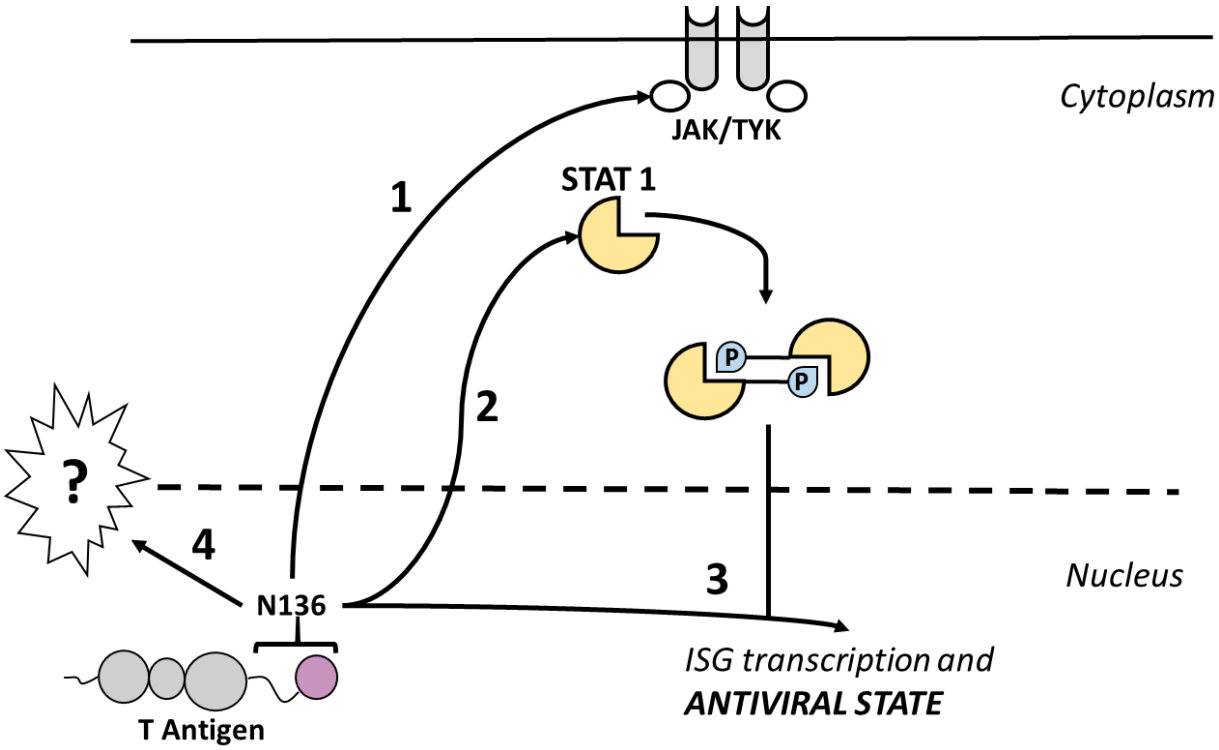
A technical challenge was faced during examination of MEF pools throughout this study. The pools are actually a "pooling" (i.e. collection) of surviving colonies following drug selection. The colonies grow at different rates and have various morphologies. During early passages there are many different types of colonies exhibiting various characteristics. As passaging proceeds, few colonies tend to dominant the pool. Eventually, at approximately passage 10+, a single

morphology dominates. This phenomenon has not seemed to result in a major shift in results when sample from early passage pools were compared to later passages, however this has not been rigorously examined.

Future studies surrounding the T antigen upregulation of the interferon response/ antiviral state will focus on resolving the complete mechanism behind how T antigen relies on STAT1 to upregulate ISG transcription without the aid of interferon production. With that in mind, the examination of IFN receptor knockout MEFs expressing T antigen would confirm an IFN independent mechanism. Additionally, some results have indicated that T antigen can interact with the membrane associated janus kinases (JAKs). JAK1 is a kinase upstream of STAT1 activation. It has been shown by immunoprecipitation that T antigen from murine polyoma virus can directly bind to JAK and alter its activity (**Weihua 1998**). However, one consideration take must be taken into account on this line of experiments and understanding the mechanism of interferon response upregulation by T antigen is the location of the T antigen protein. The large T antigen from polyomavirus is known to be located within the nucleus (**Damania & Pipas 2009**). During this study, immunofluorescence assays using a T antigen specific monoclonal antibody confirmed this result (data not shown). Thus, it is not unreasonable to hypothesize that T antigen is somehow manipulating the STAT1 pathway from within the nucleus, and not the cytoplasm.

The purpose of this study was to investigate polyomavirus T antigens and the upregulation of the cellular interferon response. The strategy utilized was to elucidate on the mechanism of T antigen's upregualtion of the interferon response, and determine possible consequences of the effect. This study demonstrates that T antigen from multiple polyomaviruses are capable of upregulating the cellular interferon response. Additionally, activity within the amino terminus of the large T antigen is necessary and sufficient to activate the interferon response. Large T antigen

is not reliant on the carboxy terminal activity, nor the influence of small T antigen. However, STAT1 was shown to be critical for ISG upregulation. Together, the activity of T antigen results in an antiviral state. **Figure 20** models the possible mechanism and relates back to the results discussed.



(1) T antigen may interact with JAK to activate STAT1. (2) T Antigen may interact with STAT1 dimers within the cytoplasm. (3) T antigen interacts upstream of STAT1 dimers within the nucleus. (4) T antigen interacts with an unknown factor that mediates STAT1 activation.

Figure 20: Possible models for T antigen activation of the Antiviral State

7.0 PUBLIC HEALTH SIGNIFICANCE

The research presented here offers an opportunity for the advancement of public health. Because the mechanism of how T antigen activates the antiviral response is not completely understood, there could perhaps be additional cellular factors are at play unbeknownst to biology, e.g., a secreted IFN-like signal. An IFN-like secreted factor that activates an antiviral response may be useful as a therapy in treatment of viral, or even, other diseases. Indeed, IFN treatment is already widely used to treat many different diseases (**Dubois 1983, Yoshida 1999, Korenman 1991, Lin 1999**). Therefore, further study into this mechanism may continue to contribute to greater understanding of virology, but also presents an opportunity for new applications in human health.

The human polyomaviruses BKV and JCV pose a significant public health threat. Both of these viruses are estimated to be prevalent in ~70% of adults, and can incite serious disease in immunocompromised patients (**Antonsson 2013, Egli 2009**). Specifically, BKV has been shown to cause neuropathy in renal transplant patients (**Kim 2004**); JCV infection can result in the incidence of progressive multifocal leukoencephalopathy (**Aksamit 1986**, **Padgett 1982**). In order to develop more effective therapies against BKV and JCV these viruses must be further examined to elucidate prospective targets for therapy. The work presented here contributes to a greater understanding of the activities of these polyomaviruses, and therefore allows new insight into possible avenues to inhibit the viruses from causing disease. Thus, this work contributes to the overall advancement of public health.

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