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# Interferon Resistance and Efficiency of HCV Replicon Establishment in Huh7 Cells Expressing Simian Virus 5 V Protein

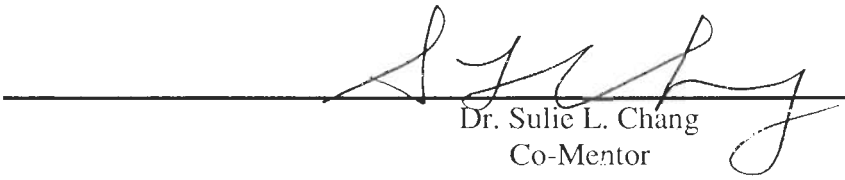
by

**Nicole E. Wagner**

Submitted in partial fulfillment of the requirements for the
degree of Master of Science in Microbiology from the
Department of Biology of Seton Hall University

September, 2005

**APPROVAL PAGE**



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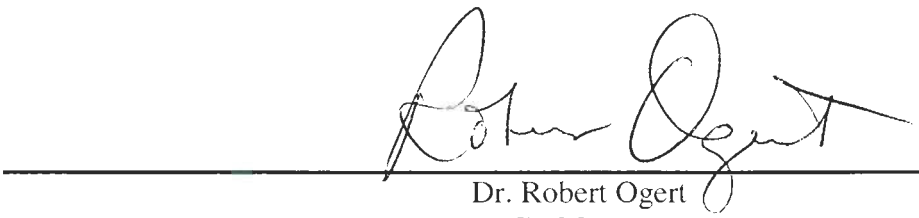

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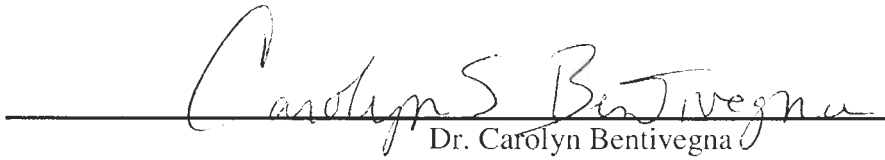

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Finally, I want to thank those very special people in my life that always encouraged me and gave me a lift or a push when I needed it most. My parents, John and Diane Vantuno, never let me give up, and provided many meals when I didn't have time to cook for myself. My siblings, John and Gina, and my countless wonderful friends always let me know how proud they were of me for pursuing my degree. Last but never least, my dear husband Stephen is an inspiration to me, and I'm grateful every day that he is my partner in life. I dedicate this thesis to our unborn twins, who are without a doubt the greatest achievements of my life.

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## ABSTRACT

The interferon (IFN) response is a cell's natural defense against viral infections. The V protein of Simian Virus 5 (SV5) shuts down the IFN response by targeting STAT1, a vital component in both the IFN-α/β and the IFN-γ pathways, for proteasome-mediated degradation. It has been suggested that cells engineered to stably express SV5 V protein can be a useful tool for studying viruses that are difficult to grow in vitro. Hepatitis C virus (HCV) is a major health concern, with as many as 200 million people infected worldwide. Advancements in HCV studies have been slow due to its inability to replicate in tissue culture. The development of an HCV replicon system is an important milestone in the field, but only a very few isolates have been successfully established as replicons in the human hepatoma cell line Huh7. Here we describe the generation of a Huh7 cell line that stably expresses the SV5 V protein. The cell line is non-responsive to IFN-α and IFN-γ. When treated with IFN-α, the cells are shown to yield higher titers of EMCV as compared to control. We demonstrate that the cells are more permissive to establishment of an HCV replicon. This study shows that an IFN non-responsive Huh7 cell line expressing SV5 V protein can be a useful tool in the field of HCV research.

# INTRODUCTION

Interferons (IFNs) are cytokines produced and secreted by eukaryotic cells in response to viral infections and increased dsRNA in the cell (Type I IFNs), or to mitogenic or antigenic stimulation of the cells (Type II IFNs). Type I IFNs include IFN-α, IFN-β, IFN-ε, IFN-κ, IFN-ω, IFN-δ, and IFN-τ; the only Type II IFN is IFN-γ. The main role of IFNs is to activate an antiviral state in the cell, though they have several other functions, including regulation of cell growth and immunomodulatory activity (Stark et al., 1998; Samuel, 2001; Pestka et al., 2004).

IFNs interact with specific cell surface receptors to initiate a signaling pathway, ultimately resulting in the transcription of IFN-stimulated genes (ISGs). Type I IFNs bind to a specific receptor consisting of two subunits, IFNAR1 and IFNAR2. The intracellular domains of these subunits interact with Janus tyrosine kinases (JAKs) Tyk2 and Jak1. Binding of a Type I IFN to the receptor causes cross-phosphorylation and activation of Tyk2 and Jak1. The activated JAKs then phosphorylate signal transducer and activator of transcription 1 (STAT1) and STAT2. The phosphorylated STAT1 and STAT2 form a heterodimer, translocate to the nucleus, and associate with IFN regulatory factor 9 (IRF9) (also called p48) to form a complex known as IFN-stimulated gene factor 3 (ISGF3). ISGF3 binds to specific sequences in the host genome called IFN-stimulated response elements (ISREs) and activate transcription of IFN stimulated genes (ISGs), such as the dsRNA protein kinase PKR or 2'-5'-oligoadenylate synthetase (OAS), to induce an antiviral state within the cell. In a similar fashion, IFN-γ binds to a receptor

consisting of the subunits IFNGR1 and IFNGR2, which interact with Jak1 and Jak2. The JAKs cross-phosphorylate and activate each other, then phosphorylate STAT1. Phosphorylated STAT1 forms a homodimer called gamma activate factor (GAF), translocates to the nucleus, and binds to gamma activated sequence (GAS) to activate transcription of ISGs (Stark et al., 1998; Samuel, 2001; Pestka et al., 2004).

Most DNA and RNA viruses have evolved various ways to block a cell's IFN response. Some of these strategies include secreting a soluble homologue of the IFN α/β receptor which competes with the cellular receptors for IFN-α/β binding (poxviruses); expressing a dsRNA binding protein that sequesters dsRNA in the cell and preventing induction of the IFN pathway (poxviruses); decreasing the expression of proteins such as Jak and IRF9 that are vital to the IFN pathway (herpesviruses); expression of VAI viral RNA that binds to, but does not activate, PKR (adenoviruses); expression of a protein that inhibits the cellular production of IFN-α/β (some bunyaviruses); blocking a cell's secretory pathway, thus inhibiting IFN α/β secretion (poliovirus); and degrading vital components of the IFN pathway (paramyxoviruses) (Garcia-Sastre, 2002; Gotoh et al., 2002; Katze et al., 2002).

Simian Virus 5 (SV5), a negative-stranded RNA paramyxovirus, expresses two proteins, P (44 kDa) and V (24 kDa), from the P gene The proteins share the same N-terminal 164 amino acids, but their C-terminals differ due to a polymerase "stutter" during transcription that adds two non-templated G residues to the V mRNA (Thomas et al., 1988). The V protein gets packaged in the SV5 virion (Paterson et al., 1995), so it is present in the host cell immediately upon viral infection. Though many functions have

been associated with the V protein (Precious et al., 1995; Randall and Bermingham, 1996; Lin and Lamb, 2000; He et al., 2002; Poole et al., 2002; Andrejeva et al., 2004; Sun et al., 2004), the main function of V protein is to target STAT1 in the host cell for proteasome-mediated degradation (Didcock et al., 1999; Andrejeva et al., 2002a). By degrading the host's STAT1, SV5 removes a key factor in both the IFN-α/β and the IFN-γ pathways, thus inhibiting the IFN response of the host cell (Didcock et al., 1999; Andrejeva et al., 2002a).

Cellular proteins have specific half-lives that vary anywhere from minutes to days, and even to years. The ubiquitin-proteasome proteolytic pathway is a complex system in cells that controls protein degradation. The proteolytic process can be broken down into two steps: 1. tagging the substrate with ubiquitin molecules, and 2. degradation of the tagged protein by the 26S proteasome complex. Ubiquitin is a 76-residue polypeptide that is attached to a substrate protein in a three-step process. First, an ATP-dependent E1 ubiquitin activating enzyme makes a high-energy thiol ester intermediate (E1-S~ub). Next, an E2 ubiquitin conjugating enzyme transfers the activated ubiquitin from E1 to the substrate (which is bound to E3). Finally, the E3 ubiquitin protein ligase covalently attaches the ubiquitin molecule to the bound substrate. Additional ubiquitin molecules are attached to the previous ubiquitin to form a chain, which is recognized by the downstream 26S proteasome complex. The ubiquitinated protein is degraded, and the ubiquitin molecules are recycled in the cell (Weissman, 2001; Glickman and Ciechanover, 2002). Cullin proteins are found in multisubunit RING finger E3

complexes and interact with linker proteins that recruit the substrate recognition components (Weissman, 2001).

STAT proteins normally have long half-lives in cells (Siewert et al., 1999). When cells are infected with SV5, V protein is immediately introduced into the cell from the viral package. V protein is also expressed early from the viral genome. STAT1 is quickly degraded in infected cells, while STAT2 levels remain unchanged (Didcock et al., 1999). At very low cellular levels of SV5 V protein, STAT1 is shown to be ubiquinated (Ulane and Horvath, 2002). The V protein has been shown to interact with the damage specific DNA binding protein DDB1 (Lin et al., 1998). This V-DDB1 interaction is required for STAT1 degradation (Andrejeva et al., 2002b). The V-DDB1 complex has been shown to co-precipitate with Cullin 4a (Cul4a), which is associated with E3 ubiquitin ligase activity (Precious et al., 2005). As further evidence that V protein induces proteasome-mediated degradation of STAT1, cells that are treated with the proteasome inhibitors MG132 or lactacystin before SV5 infection do not show STAT1 degradation upon infection (Didcock et al., 1999).

Human cell lines have previously been engineered to constitutively express SV5 V protein (Young et al., 2003). These cells do not respond to interferons due to their lack of cellular STAT1. Cells that do not respond to interferons can not establish an effective antiviral state. When these cells were infected with various viruses, it was observed that most viruses grew to higher titers and formed larger plaques than in cells that did not express SV5 V protein. Attenuated and slow-growing viruses especially showed an increase in viral titers and improved plaque formation in SV5 V protein cells. The

authors proposed that IFN-nonresponsive SV5 V protein cell lines could be used to study slow-growing attenuated or recombinant viruses, as well as viruses that have so far been refractory to replication in vitro, such as caliciviruses and certain hepatitis viruses.

The hepatitis C virus (HCV), a 9.6 kb plus-stranded RNA virus in the *Flaviviridae* family, was identified in 1989 as the causative agent of a disease then known as non-A, non-B hepatitis (Choo et al., 1989). Approximately 200 million people are infected with HCV worldwide. In most cases the infection is persistent, and long-term infections often lead to the development of cirrhosis and hepatocellular carcinoma (Poynard et al., 1997). HCV infection is the main indication for liver transplantation. Current therapy for HCV infection is a combination of a conjugated IFN-α and ribavirin. This treatment has sustained viral responses of 85-90% in genotypes 2 and 3, but only about 45% in the more prevalent genotype 1. The study of HCV and the development of selective antiviral drugs to treat HCV infection have been hindered by the lack of cell culture models and the inability to grow the virus in vitro. Some progress has been made since the development and subsequent improvements of genetically modified HCV mini-genomes (replicons) that self-amplify to high levels in the human hepatoma cell line Huh7 (Lohmann et al., 1999; Bartenschlager et al., 2003). Replicon RNA contains a neomycin resistance gene upstream from the HCV genes, and is maintained in cells using G418-supplemented medium. Treatment of replicon-harboring cells with IFN-α inhibits replication of the HCV RNA (Blight et al., 2000), and prolonged treatment can "cure" the cells of the replicon RNA (Blight et al., 2002). To date, only four different HCV isolates function in the replicon system, including two from genotype 1b (Con1 and HCV-N)

(Lohmann et al., 1999; Guo et al., 2001), one from genotype 1a (H77) (Blight et al., 2003), and one from genotype 2a (JFH-1) (Kato et al., 2003). Establishment of more HCV subtypes in a replicon system has proved difficult. Establishment of replicons has also been shown to depend on the permissiveness of the Huh7 cell line (Blight et al., 2002; Lohmann et al., 2003).

As suggested by Young and coworkers (2003), a cell line that is IFN-nonresponsive could be a valuable tool for studying hepatitis viruses. Because HCV replicons are IFN-α sensitive, we hypothesized that an IFN-nonresponsive human hepatoma cell line would be more permissive to HCV replicon establishment. Therefore, a Huh7-SV5V cell line was generated. STAT1 degradation was examined, and the cell line was characterized for its nonresponsiveness to IFN-α and IFN-γ. An EMCV viral yield assay assessed the cells' sensitivity to IFN-α treatment during a single-round viral infection. Finally, the Huh7-SV5V cells were tested for their permissiveness for HCV replicon establishment using the genotype 1b Con1 replicon.

## MATERIALS AND METHODS

**Cell culture**. Huh7 and HeLa-R19 cells were grown in Eagle's minimum essential medium, supplemented with 10% fetal bovine serum and sodium pyruvate. All cells were negative for mycoplasma contamination as screened by the MycoAlert mycoplasma detection assay (Cambrex). Huh7-FlpIn cells were grown in the presence of 500 µg/ml Zeocin (Invitrogen). Huh7-V and Huh7-CAT cells were grown in the presence of 100 µg/ml Hygromycin B (Invitrogen).

**Cloning of the V protein gene**. Simian Virus 5 (SV5) strain 2WR was obtained from ATCC. Genomic RNA was isolated using QIAamp Viral RNA mini kit (Qiagen). The V gene was amplified using C. therm. Polymerase for reverse transcription in two-step PCR (Roche), using the following primers: 5'-AATAAgctagcGGTGCCAACAGCGCAATCC-3' and 5'-CCGAggtaccCATCCCTGCTTTAAGTCC-3' (lower-case sequences are the NheI and KpnI restriction sites, respectively; underlined sequences correspond to the SV5 V protein gene). The resulting 840bp fragment was isolated, digested with NheI and KpnI, and cloned into the NheI and KpnI sites of the plasmids pcDNA5/FRT (Invitrogen) and pcDNA3.1/Zeo(+) (Invitrogen). DNA sequence was confirmed using Beckman-Coulter CEQ 2000XL DNA Analysis System.

**Generation of stable cell lines**. Cells expressing the SV5 V protein gene or the chloramphenicol acetyltransferase (CAT) gene were isolated using the Flp-In System (Invitrogen). Briefly, Huh7 cells were tranfected with ScaI-linearized pFRT/*lac*Zeo plasmid, grown in the presence of 500 μg/ml Zeocin, and resistant colonies were isolated. The colonies were screened for β-galacatosidase activity using both the β-Gal Assay Kit (Invitrogen) and the β-Gal Staining Kit (Invitrogen). Positive clones (100% β-gal expression) were then screened for the *lac*Zeo gene insertion. Genomic DNA was isolated from each clone using the DNeasy Tissue kit (Qiagen), digested with HindIII, 15 μg/sample was separated in a 0.7% TAE agarose gel, transferred to BrightStar-Plus positively charged nylon membrane (Ambion) via vacuum transfer, and UV crosslinked to the membrane using a Stragene Stratalinker. Probe was generated from a HindIII/SacI fragment of the *lacZ* gene, biotinylated using the BioPrime DNA Labeling System (Invitrogen). Development of the Southern blot was performed using North2South Chemiluminescent Hybridization and Detection Kit (Pierce).

A Huh7-FlpIn clone with a single *lac*Zeo gene insert was chosen and co-transfected with the plasmid pOG44 and either pcDNA5/FRT/SV5V or pcDNA5/FRT/CAT. Cells were grown in the presence of 50 μg/ml Hygromycin B, and resistant colonies were isolated. The colonies were screened for Zeocin sensitivity and lack of β-gal activity. One positive clone was chosen for each, and total RNA was isolated using RNeasy Mini Kit (Qiagen), followed by treatment with DNA-free DNase Treatment and Removal Reagent (Ambion). RNA expression for each gene was analyzed by RT-PCR using RETROscript Reverse Transcription for RT-PCR with

random decamer primers (Ambion), followed with Platinum PCR SuperMix (Invitrogen) and the following primers: SV5 V protein forward 5'-GCTTCTCCCCAGATGAGATC-3', reverse 5'-GATAGGATTCTCTCTGGGTT-3'; CAT forward 5'-GCCAATCCCTGGGTGAGTTTC-3', reverse 5'- GAAACTCACCCAGGGATTGGC-3'.

**Immunoblots**. Prior to generating stable cell lines, the activity of the cloned SV5 V protein was confirmed by STAT1 and STAT2 immunoblots. HeLa-R19 cells were plated at $1.5x10^5$ cells per well in BIOcoat collagen-coated 6-well plates (Becton-Dickinson) and incubated overnight. Cells were then transfected using SuperFect transfection reagent (Qiagen), according to the manufacturer's protocol. Each well was transfected with 2 μg of either pcDNA5/FRT/CAT control plasmid (Invitrogen), pcDNA5/FRT/SV5V, or pcDNA3.1/SV5V. Cells were incubated with transfection complexes for 2 hours, washed with DPBS, and incubated for 24 hours in medium supplemented with 2% FBS plus either mock treatment or treated with 1000 IU/ml of human IFN-a2b (R&D Systems). After 24 hours, cells were washed with DPBS and lysed with 300 μl per well of Novex tris-glycine SDS sample buffer 2X (Invitrogen) supplemented with 50 μl per ml of 2-mercaptoethanol. Samples were boiled for 10 minutes, separated on 4-12% NuPAGE Bis-Tris gels (Invitrogen) in NuPAGE MOPS reducing running buffer (Invitrogen), and transferred to PVDF membranes in NuPAGE transfer buffer, reducing conditions plus 20% methanol (Invitrogen). STAT1 and STAT2 proteins were detected with Stat1 p84/p91 (C-136) and Stat2 (A-7) monoclonal

antibodies, respectively (Santa Cruz Biotechnology). Blots were developed for chemiluminescence using the WesternBreeze Chemiluminescent Kit-Anti-Mouse (Invitrogen) followed by colorimetric detection using Western Blue stabilized substrate for alkaline phospatase (Promega).

The generated stable cell lines Huh7-V and Huh7-CAT were plated in medium supplemented with 1% FBS, at $1.5x10^5$ cells per well in BIOcoat collagen-coated 6-well plates (Beckton-Dickinson). Cells were either mock-treated, or treated with 2000 IU/ml of PEG-Intron (Schering-Plough) for 3, 6, 9, 24, and 48 hours. Cells were washed with DPBS and lysed with 300 µl per well of Novex tris-glycine SDS sample buffer 2X (Invitrogen) supplemented with 50 µl per ml of 2-mercaptoethanol. Samples were boiled for 10 minutes. Separation of proteins and detection of STAT1 and STAT2 were carried out as described above.

**IFN activation assays.** Huh7-V and Huh7-CAT cells were plated at $1.5 \times 10^4$ cells per well in BIOcoat collagen-coated 96-well plates (Becton-Dickinson). After cells attached to plates, they were transfected using SuperFect transfection reagent (Qiagen), according to the manufacturer's protocol. Each well was transfected with 10 ng of pRL-CMV *Renilla* Luciferase control reporter vector (Promega) plus 500 ng of either pCIS-CK negative control, pISRE-luc, or pGAS-luc PathDetect *cis*-reporter plasmids (Stratagene). The IFN-α/β responsive plasmid pISRE-luc contains five tandem repeat sequences of the IFN-stimulated response elements (ISRE) fused to the firefly luciferase gene. The IFN-γ responsive plasmid pGAS-luc contains four tandem repeat sequences of

the IFN regulatory factor 1 (IRF-1) gamma activated sequence (GAS) fused to the luciferase gene. Cells were incubated with transfection complexes for 3 hours, washed with DPBS, and incubated overnight in medium supplemented with 2% FBS.

Cells were either mock treated, or treated with 1000 IU/ml of human IFN-α2b or 1000 IU/ml of human IFN-γ (R&D Systems). At 4 and 8 hours post IFN treatments, media was aspirated from wells and cells were lysed in 50 µl Passive Lysis Buffer (Promega). 40 ml of lysates were transferred to white MicroLiteI plates (DYNEX) and assayed using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol. Plates were read on a DYNEX MLX Microtiter Plate Luminometer, 10 second read/reporter/well. Data were normalized by calculating the (firefly RLU)/(*Renilla* RLU) ratios. Activation of the reporters was compared to the mock treated samples.

**Viral yield assay**. Encephalomyocarditis virus (EMCV) was kindly provided by Peter Buontempo (Schering-Plough). Huh7-V and Huh7-CAT cells were plated in medium supplemented with 1% FBS, at 1.5 x 105 cells/well in BIOcoat collagen-coated 6-well plates (Beckton-Dickinson). Cells were either mock-treated, or treated with 1000 IU/ml of PEG-Intron (Schering-Plough) overnight. Cells were then infected with EMCV at an m.o.i. of 0.5 $TCID_{50}$ for one hour at 37°C. Virus was then washed off of cells with DPBS, and cells were incubated for 5 hours at 37°C, either with or without PEG-Intron, to allow for a single round of viral replication. Cells were washed with DPBS, and 1 ml of medium supplemented with 1% FBS (without Hygromycin B) was added to each well.

The plates were frozen at -80°C and allowed to go through 3 rounds of freeze/thaw to release EMCV particles. Supernatants from the plates were clarified by spinning at 10,000xg for 10 minutes. Ten-fold serial dilutions were made of the EMCV yields, from $10^{-2}$ to $10^{-10}$. HeLa-R19 cells were plated in medium supplemented with 1% FBS, at 8000 cells/well in BIOcoat collagen-coated 96-well plates and infected with 100 µl of the serial dilutions of EMCV. The infected cells were incubated at 37°C for 72 hours. The media was aspirated, and viable cells were stained as follows. Cells were incubated at 37°C for 3 hours in 1 mg/ml Methylthiazolyldiphenyl-tetrazolium bromide (MTT) (Sigma-Aldrich) in DPBS. MTT was aspirated and cells were solubilized with 100 ml 1:1 N,N-dimethylformamide (Acros) : water plus 20% sodium dodecyl sulfate (SDS) (FisherBiotech). Plates were read on a DYNEX MRX Absorbance Reader, 405 nm wavelength. Data were analyzed for $TCID_{50}$ values using the Reed Muench fifty percent endpoint determination method. Statistical significance of the resulting $TCID_{50}$ values was determined using the Student T test of significance, one-tailed, homoscedastic.

**HCV replicon colony forming efficiency.** Huh7 cells harboring the HCV Con1 (genotype 1b) replicon were kindly provided by Andrea Hart (Schering-Plough). Total RNA was isolated from subconfluent replicon cells using RNeasy Mini Kit (Qiagen). Huh7-V and Huh7-CAT cells were transfected with HCV replicon total RNA by electroporation. Briefly, cells were grown to subconfluence, trypsinized, washed twice in DPBS, and resuspended in DPBS (with calcium and magnesium) to $1.25 \times 10^7$ cells per ml. 400 µl of cell suspension ($5 \times 10^6$ cells) was mixed with 0, 2.5, 5, or 10 µg of HCV

replicon total RNA in a 0.4 cm electrode gap Gene Pulser Cuvette (BioRad). Cells were electroporated using a GenePulser II (BioRad) set to 250 V, 950 μFd, and infinite resistance. The electroporated cells were resuspended in 20 ml medium containing 100 μg/ml of Hygromycin B, and 10 ml of the cell suspension was plated onto BIOcoat collagen-coated 10-cm plates (Beckton-Dickenson). 24 hours after electroporation, cells were washed with DPBS, and selective medium was added back containing 100 μg/ml Hygromycin B plus 0.5 mg/ml G418. Plates were fed with selective medium two times per week for 2-3 weeks until resistant HCV replicon colonies were visible. Plates were then washed with DPBS, stained for one minute with crystal violet stain (3% v/v formaldehyde, 30% v/v ethanol, 160 mg/ml NaCl, 480 mg/ml crystal violet), washed with water, and air-dried. Colonies were counted using a colony counter pen (Bel-Art).

## RESULTS

**The cloned SV5 V protein degrades STAT1, but not STAT2, in transfected HeLa-R19 cells.** To confirm that the cloned SV5 V protein is active, the levels of STAT1 and STAT2 in transiently transfected HeLa-R19 cells were examined by immunoblot analysis using antibodies reactive to either STAT1 (Figure 1a) or STAT2 (Figure 1b). After transfection, the cells were either mock-treated, or treated with human IFN-α2b for 24 hours. While STAT1 is not easily detected in mock-treated cells (Figure 1a; lanes 1-3), it is readily detected with IFN-α2b treatment in the cells expressing CAT (Figure 1a; lane 4). The detectable levels of STAT1 decreased in the presence of SV5 V protein after IFN-α2b treatment (Figure 1a; lanes 5-6), indicating SV5 V protein-mediated STAT1 degradation is occurring. The loss of detectable STAT1 is not 100%, which is due to a less than 100% transfection efficiency of the HeLa-R19 cells. There were no obvious STAT1 degradation products detected, as would be the case with a protein sequence-specific endoprotease such as caspase (King and Goodbourn, 1998). This result was also seen by Didcock and coworkers (1999), and indicates that the SV5 V protein targets STAT1 for proteasome-mediated degradation. Expression of the SV5 V protein did not affect STAT2 levels in the HeLa-R19 cells (Figure 1b). Similar results were observed with transfected Huh7 cells (data not shown), but the extent of STAT1 degradation was not as dramatic due to the lower transfection efficiency of Huh7 cells as compared to HeLa-R19 cells.

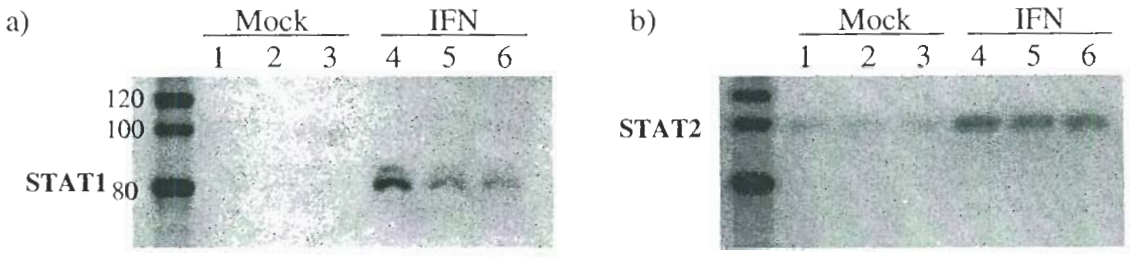


Figure 1. STAT1, but not STAT2, is degraded in cells transfected with a SV5 V protein expression plasmid. HeLa-R19 cells were transiently transfected with the plasmids pcDNA5/FRT/CAT (lanes 1, 4), pcDNA5/FRT/SV5V (lanes 2, 5), or pcDNA3.1/SV5V (lanes 3, 6). The cells were either mock-treated (lanes 1-3) or treated with IFN-α2b (lanes 4-6) for 24 hours. STAT1 (a) and STAT2 (b) were detected in whole-cell lysates by immunoblot analysis.

**Huh7-FlpIn host cell line was established.** For the successful generation of stable human liver expression cell lines using the Invitrogen Flp-In System, Flp-In host cells must be generated from a human liver cell line. Huh7 cells were transfected with ScaI-linearized pFRT/*lac*Zeo plasmid, and zeocin-resistant colonies were isolated. Genomic DNA preps of several clones were isolated and screened by Southern blot analysis to test for single integration of the pFRT/*lac*Zeo DNA. A single integration is desirable, as multiple integrations can lead to genomic recombination during the second stage of generating Flp-In stable cell lines. The clone Huh7-FlpIn-3.3 screened positive for a single integration of the DNA (data not shown). The Huh7-FlpIn-3.3 clone was analyzed for β-galactosidase activity (Figure 2) to screen for expression of the lacZeo fusion protein from the integrated DNA. Parental Huh7 cells did not have detectable β-galactosidase activity (Figure 2a), while the Huh7-FlpIn-3.3 clone showed a significant β-galactosidase specific activity of 47 pmoles ONPG hydrolyzed/mg protein. This specific activity is similar to that of Huh7 cells transiently transfected with the expression plasmid pcDNA3.1/*lacZ* (data not shown). Cells were also stained in monolayers for β-galactosidase (Figure 2b). Huh7 cells were negative for β-galactosidase, while 100% of the Huh7-FlpIn-3.3 cells stained positive (blue), indicating both successful stable integration of and expression from the pFRT/*lac*Zeo DNA.

**Huh7-SV5V and Huh7-CAT stable cell lines were generated.** Following the Invitrogen Flp-In protocol for generating stable cell lines, the host clone Huh7-FlpIn-3.3 was co-transfected with the plasmid pOG44, which expresses the Flp recombinase enzyme, and either pcDNA5/FRT/SV5V (coding for the SV5 V protein) or

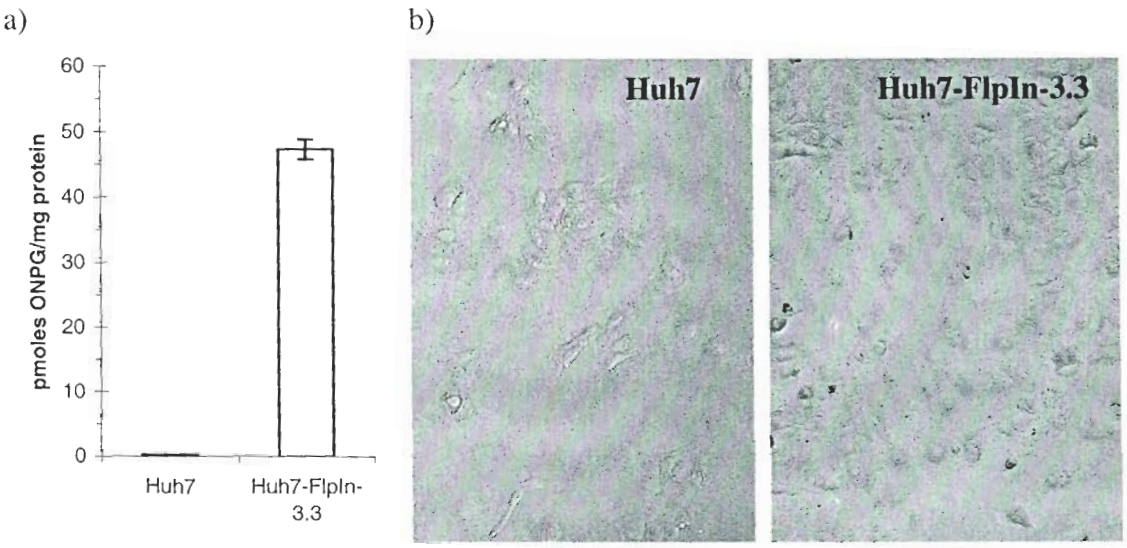


Figure 2. Huh7-FlpIn-3.3 cells exhibit β-galactosidase activity. (a) Huh7 and Huh7-FlpIn-3.3 cells were lysed by repeated freeze-thaw cycles and assayed for β-galactosidase specific activity, expressed as nmoles ONPG hydrolyzed/mg protein. (b) Photomicrographs showing monolayers of the same cells stained for β-galactosidase. Positive cells stained blue.

pcDNA5/FRT/CAT (coding for the control protein chloramphenicol acetyltransferase [CAT]). Colonies resistant to hygromycin were isolated. To rule out the possibility of a new random integration of the FRT/SV5V or FRT/CAT, as opposed to the desired FRT site-specific recombination, clones were screened for Zeocin sensitivity and lack of β-galactosidase activity (data not shown). One positive clone each was chosen for SV5 V protein and CAT to continue analyses.

Direct detection of SV5 V protein by immunoblot analysis in the Huh7-SV5V clone is not possible, as there are no commercially available antibodies to SV5 V protein, and no antigenic fusion tags were included during the cloning of the gene. Therefore, expression of the SV5 V protein was confirmed by detection of the mRNA. Total RNA from both clones was isolated, treated with DNase, and analyzed by RT-PCR for the presence of either SV5 V protein mRNA or CAT mRNA (Figure 3). CAT mRNA was detected only in the Huh7-CAT clone (Figure 3a), and SV5 V protein mRNA was detected only in the Huh7-SV5V clone (Figure 3b), indicating specificity of the RT-PCR reactions and no cross-contamination of the clones. PCR products were detected only in the reactions that were treated with reverse transcriptase, indicating that the resulting PCR product is from mRNA and not DNA contamination of the RNA preparation.

**Huh7-SV5V cells do not have detectable levels of STAT1.** To confirm activity of the SV5 V protein constitutively expressed in the Huh7-SV5V cell line, the levels of STAT1 and STAT2 were examined by immunoblot analysis (Figure 4). Huh7-CAT and Huh7-SV5V cells were either mock treated, or treated with PEG-Intron (pegylated human IFN-α2b) for up to 48 hours. In the control Huh7-CAT cells, STAT1 (Figure 4a) and

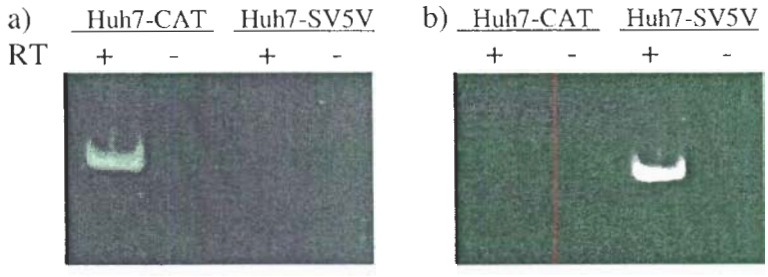


Figure 3. The mRNA species of either CAT (a) or SV5 V protein (b) was detected by RT-PCR analysis of total RNA from the stable clones Huh7-CAT and Huh7-SV5V. Total RNA was isolated from the cells and DNase-treated. RNA was then subjected to RT-PCR, either with (+) or without (-) reverse transcriptase (RT) in the reactions to rule out DNA contamination of the RNA samples.

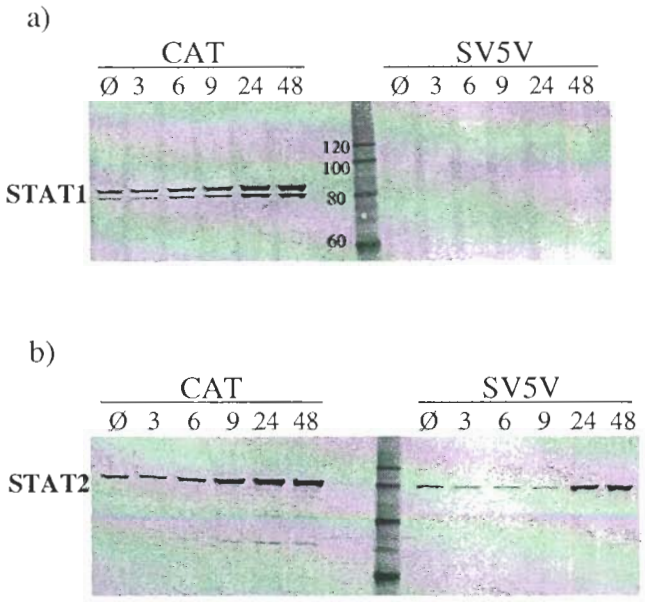


Figure 4. STAT1, but not STAT2, is degraded in cells with stable expression of the SV5 V protein. Huh7-SV5V and Huh7-CAT cells were either mock-treated, or treated with PEG-Intron for 3, 6, 9, 24, and 48 hours. STAT1 (a) and STAT2 (b) were detected in whole-cell lysates by immunoblot analysis.

STAT2 (Figure 4b) basal levels (mock-treated) were low but detectable, and increased upon PEG-Intron treatment by 24 hours. The Huh7-SV5V cells did not have detectable levels of STAT1, even after 48 hours of PEG-Intron treatment (Figure 4a). The basal STAT2 levels in the Huh7-SV5V cells appeared to be lower than in the Huh7-CAT cells (Figure 4b), but increased significantly by 24 hours after PEG-Intron treatment.

**Huh7-SV5V cells have impaired activation of IFN-α/β- and IFN-γ-responsive plasmids.** STAT1 is an integral component of both the IFN-α/β and IFN-γ signaling pathways. Because the Huh7-SV5V cells do not have detectable STAT1 levels, their ability to activate either of these pathways was assayed (Figure 5). Using the Stratagene PathDetect systems for measuring activation of the IFN-α/β (pISRE-luc) or IFN-γ (pGAS-luc) pathways (Figure 5a), the control Huh7-CAT cells showed strong activation after treatment with IFN-α2b (Figure 5b) and with IFN-γ (Figure 5c) at both four and eight hours post treatment, with even higher activation after eight hours. The Huh7-SV5V cells did not demonstrate activation of either IFN pathway after treatment with either IFN-α2b (Figure 5b) or IFN-γ (Figure 5c).

**Yield of EMCV from Huh7-SV5V shows a reduced sensitivity to PEG-Intron treatment.** Since it has been shown that the Huh7-SV5V cell line lacks detectable STAT1 and does not respond to IFN treatment, the cells will be assayed for their ability to support viral replication in the absence or presence of IFN. Encephalomyocarditis virus (EMCV), a cardiovirus, infects Huh7 cells. Huh7-CAT and Huh7-SV5V cells were either mock treated or treated with 1000 IU/ml of PEG-Intron overnight. The cells were then infected with EMCV, and the virus was allowed to undergo a single round of

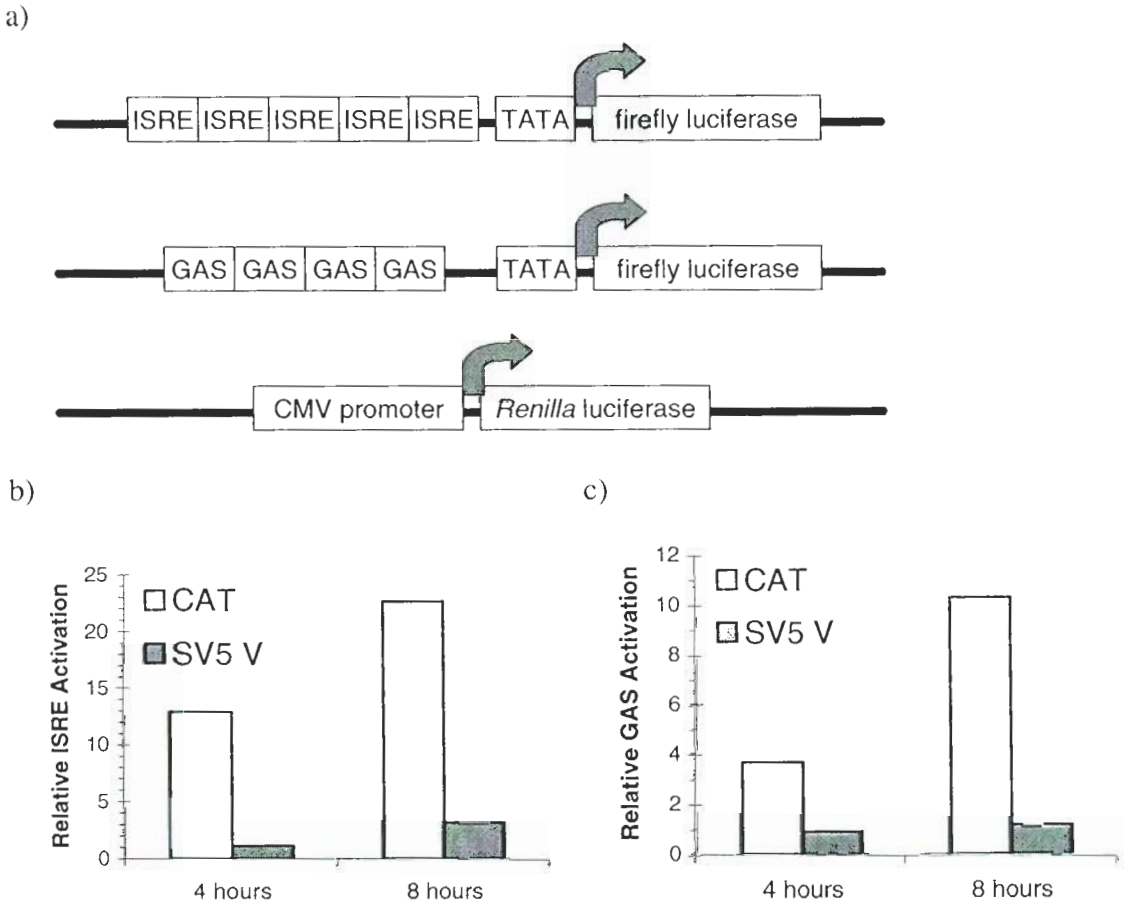


Figure 5. Cells expressing the SV5 V protein block both IFN-α/β and IFN-γ signaling. Huh7-CAT and Huh7-SV5V cells were transfected with either the IFN-α/β responsive plasmid pISRE-luc or the IFN-γ responsive plasmid pGAS-luc, together with the control plasmid pRL-CMV (a). 18 hours later, cells were either mock-treated or treated with 1000 IU/ml of IFN-α2b (b) or IFN-γ (c). Four and eight hours later, cellular lysates were analyzed for firefly luciferase (IFN activation) and *Renilla* luciferase (control); firefly luciferase activity was normalized to *Renilla* luciferase activity, and relative activation was expressed as a ratio of IFN-treated/mock-treated activities.

replication. The yield of EMCV from the cells was determined by titering on HeLa-R19 cells (Figure 6). In the mock treated cell lines, there was no significant difference between the yields from the Huh7-CAT cells vs. the Huh7-SV5V cells. When the cells were treated with PEG-Intron, viral titers from both cell lines did decrease significantly compared to the mock treated cells ($p<0.05$) (Figure 6). In addition, the titer of EMCV yield from the Huh7-SV5V PEG-Intron treated cells is significantly higher than that of the Huh7-CAT PEG-Intron treated cells ($p<0.05$). Therefore, the Huh7-SV5V cells are less sensitive to PEG-Intron treatment than the control cells in a quantitative viral infection assay.

**Huh7-SV5V cells are more permissive to HCV replicon establishment.** The Huh7-SV5V cells have been shown to be nonresponsive to IFN. Because the introduction of viral RNA can induce an IFN response in cells, it is possible that the Huh7-SV5V cells will be more permissive to the establishment of an HCV replicon RNA. Total RNA was isolated from an established HCV Con1 (genotype 1b) replicon cell line. The total RNA includes full length, functional HCV replicon RNA (Figure 7). Huh7-CAT and Huh7-SV5V cells were transfected with 0, 2.5, 5, or 10 μg of total RNA, then selected for G418 resistance. After 2-3 weeks, G418 resistant colonies, indicative of HCV replicon establishment, were stained with crystal violet (Figure 8a-f) and counted (Figure 8g). There were consistently more colonies in the Huh7-SV5V cells than in the Huh7-CAT cells (Figure 8g). No spontaneous colonies grew in the 0 μg RNA electroporations (data not shown). For electroporations of 2.5, 5, and 10 μg of total RNA, 20, 71, and 159 colonies grew in the Huh7-CAT cells, respectively, and 95, 157,

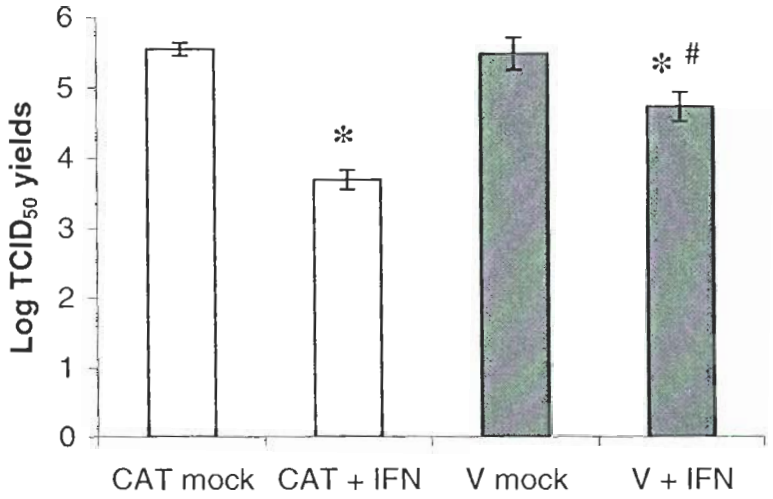


Figure 6. EMCV viral yield from Huh7-SV5V cells is not as sensitive to PEG-Intron treatment as from the control Huh7-CAT cells. Cells were either mock-treated or treated with 1000 IU/ml of PEG-Intron overnight. Cells were infected with EMCV at an m.o.i. of 0.5 $TCID_{50}$ for one hour, virus was washed off, and cells were incubated at 37°C for another 5 hours to allow for a single round of replication of EMCV. Cells were lysed by repeated freeze-thawing, and the supernatants containing EMCV were clarified and titered on HeLa-R19 cells. TCID50 values were determined using the Reed Muench fifty percent endpoint determination method. * = significant compared to mock treatment; # = significant compared to CAT + IFN.

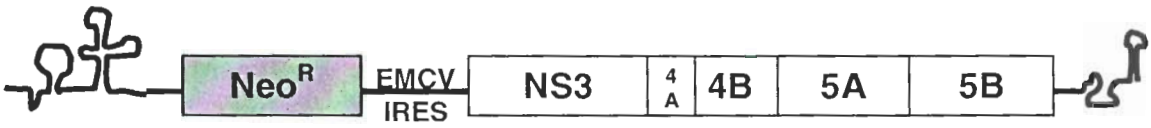


Figure 7. Diagram of HCV replicon RNA. RNA is flanked by the 5' and 3' non-coding regions (NCR) of HCV Con1 (genotype 1b). The 5' NCR is the internal ribosomal entry site (IRES) which drives expression of the neomycin phosphotransferase gene ($Neo^R$). A second IRES from EMCV drives expression of the HCV non-structural genes NS3, NS4A, NS4B, NS5A and NS5B. This RNA self-replicates in a host cell. Cells harboring the HCV replicon RNA are selected by growing in the presence of the antibiotic G418.

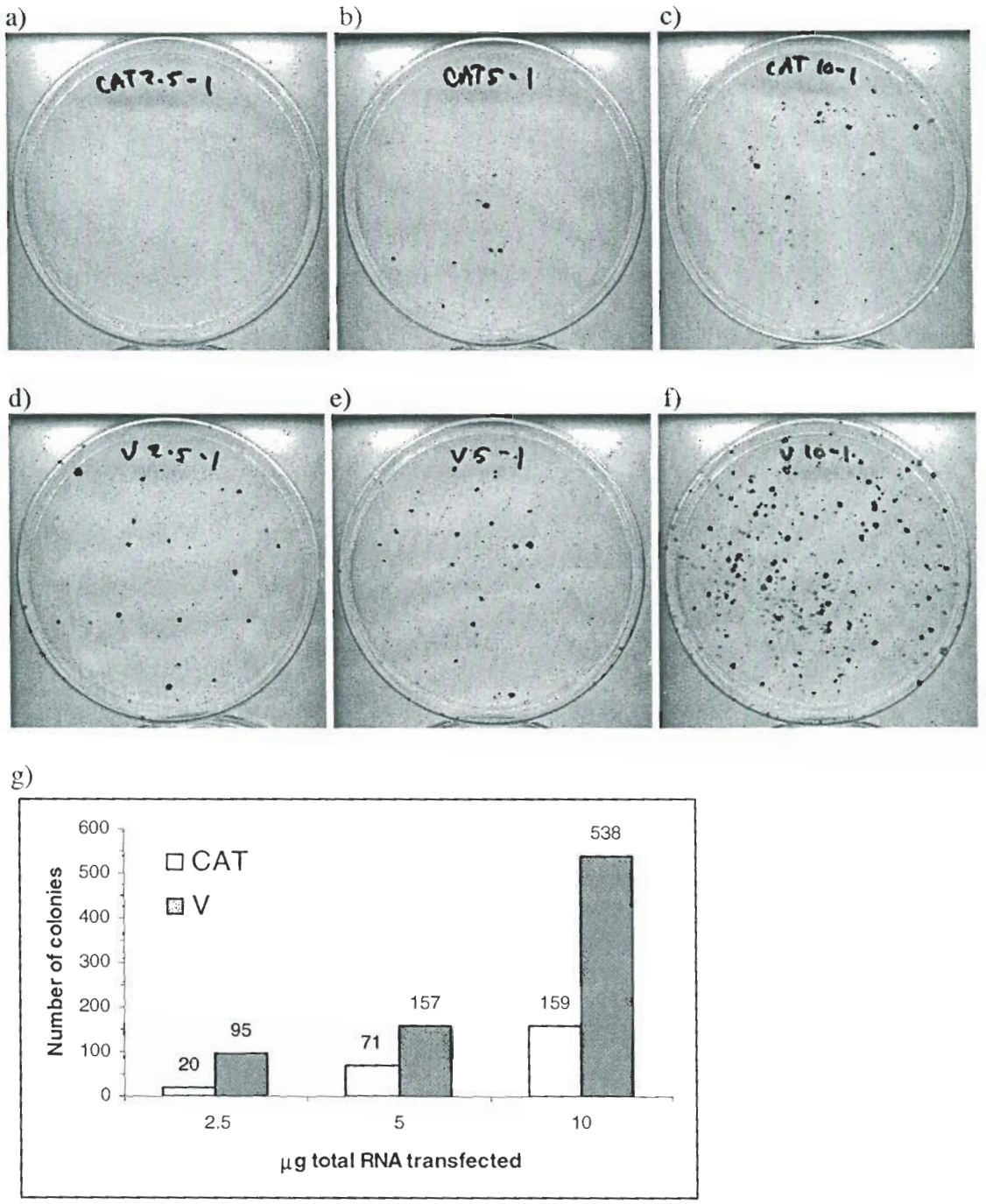


Figure 8. Huh7-SV5V cells are more permissive for HCV replicon establishment. Huh7-CAT (a-c) and Huh7-SV5V (d-f) cells were electroporated with 2.5 (a, d), 5 (b, e), or 10 µg (c,f) total RNA from an established HCV replicon cell line; colonies harboring HCV replicon RNA were selected with G418 and stained. Total colony numbers were counted on each plate (g).

and 538 colonies grew in the Huh7-SV5V cells, respectively. It was also noted that the colonies in Huh7-SV5V cells were generally larger and denser than colonies in Huh7-CAT cells (Figure 8a-f).

## DISCUSSION

The cellular interferon response is an important defense against viral infections. Either Type I IFN (such as $\alpha$ or $\beta$) or Type II IFN-$\gamma$ treatment can establish an antiviral state within a cell by specific pathways that eventually upregulate expression of ISGs such as PKR and OAS. STAT1 is a critical component of both the IFN-$\alpha/\beta$ and IFN-$\gamma$ pathways. Simian Virus 5 (SV5) expresses V protein to selectively target STAT1 in the host cell for proteasome-mediated degradation, thus shutting down both IFN pathways, and preventing the host cell from establishing an antiviral state. Young and coworkers (2003) engineered human cell lines with constitutive expression of the SV5 V protein and showed that these cell lines support replication of slow-growing and attenuated viruses to significantly higher titers. They suggested that cells expressing SV5 V protein can be useful tools for studying other difficult-to-grow viruses, such as the hepatitis viruses. We therefore engineered Huh7 cells, a human hepatoma cell line that has been shown to support replication of hepatitis C virus (HCV) replicon RNA, to express SV5 V protein.

We first cloned the gene for SV5 V protein into an expression vector and confirmed its activity. When the cloned SV5 V protein was expressed in either HeLa-R19 or Huh7 cells, STAT1 levels decreased, while STAT2 levels remained unchanged. King and Goodbourn (1998) has shown that STAT1 can be inactivated by a caspase-specific cleavage in apoptotic HeLa cells. Caspase cleavage products can be observed on an immunoblot. We did not observe any cleavage products (Figure 1a), indicating that the decrease in STAT1 levels is not a result of sequence-specific caspase cleavage, but is

instead due to proteasome-mediated degradation as reported by Didcock and coworkers (1999), and thus confirming the activity of our cloned SV5 V protein. The STAT2 levels remained unchanged in cells expressing SV5 V protein (Figure 1b), indicating that the degradation of STAT1 is SV5 V protein-specific, and not due to a more general cellular effect.

The cell line that has been shown to most efficiently support HCV replicons is the human hepatoma line Huh7. HCV replicons have not been established in any primary human liver cells. Date and coworkers (2004) reported that HepG2 (a human hepatocyte cell line) and IMY-N9 cells (a fusion of human hepatocytes and HepG2 cells) can support HCV replicon RNA, but with substantially lower efficiency than Huh7 cells. No other reports of cell lines other than Huh7 have been reported to support HCV replicons. We therefore chose to create a stable Huh7 cell line expressing SV5 V protein for the eventual study of HCV in a highly permissive cell line. For appropriate controls for our experiments, we also created a stable Huh7 cell line expressing CAT. The Invitrogen FlpIn system was chosen to generate these cell lines to ensure that the SV5 V and CAT genes were inserted into the host genome at the same location. By inserting our genes of interest at the same location, we can be sure that 1. any interruption of normal gene expression in Huh7 is the same in both cell lines, and 2. the expression levels of both CAT and SV5 V protein are similar in the two cell lines. Expression of both proteins was confirmed by RT-PCR of cellular RNA (Figure 3), and activity of SV5 V protein in the stable cell line was confirmed by a lack of detectable STAT1, even after IFN-α treatment (Figure 4).

It has been shown previously that IFN treatment of cells, including hepatoma cell lines, up-regulates the expression of both STAT1 and STAT2, potentially amplifying the cytokine response in the cell (Melén et al., 2000; Iolascon et al., 2004). This explains the increased levels of detected STAT1 and STAT2 proteins upon IFN treatment of both the transfected HeLa-R19 cells (Figure 1) and the stable Huh7-CAT and Huh7-SV5V cell lines (Figure 4). It was noted that, even after 48 hours of IFN-α treatment, STAT1 was not detectable in the Huh7-SV5V cells by immunoblot (Figure 4a). Therefore, any STAT1 that might be newly-expressed by a stimulated cell is quickly degraded due to the presence of SV5 V protein.

Both Type I and Type II IFNs can establish an antiviral state in a cell. The signaling pathways differ for the various IFNs (Stark et al., 1998; Samuel, 2001; Pestka et al., 2004). Therefore, it is possible to inhibit one specific IFN pathway (e.g. the IFN-γ pathway), yet the cell can still establish an antiviral state by stimulation with another IFN (e.g. IFN-α). The SV5 V protein is a particularly interesting protein because, by rapidly degrading cellular STAT1 via the proteasome, it inhibits both the IFN-α/β and the IFN-γ pathways at the same time. We confirmed that the Huh7-SV5V cell line is unresponsive to both IFN-α and IFN-γ treatment (Figure 5). This should make the cell line particularly useful for viral studies, as it has a dual IFN-nonresponsive phenotype.

We tested the Huh7-SV5V cells in an EMCV viral yield assay to see if the lack of IFN response would lead to a higher viral titer after a single round of EMCV replication. While we predicted that viral yield would be higher in our cell line both with and without IFN treatment, we did not see an increase in the viral yield from untreated cells (Figure

6). This could be due to the fact that the virus underwent only a single round of replication, and did not allow adequate time for the Huh7-CAT cells to establish an antiviral state and thus reduce the observed viral yield. It would be interesting to compare viral yield and plaque formation of EMCV in our cells after multiple rounds of viral replication, although it may still be possible that there will be no difference in EMCV viral yields in untreated cells; Young and coworkers (2003) had observed that some wild type viruses, such as the Bunyamwera virus, did not grow to significantly higher titers in their SV5 V protein cells. We did observe that, when treated with IFN-α, the Huh7-SV5V cells did have a significantly higher yield than the Huh7-CAT cells (Figure 6), indicating that the cells expressing SV5 V protein are less responsive to IFN. The viral yield was significantly lower than the untreated Huh7-SV5V cells, which may be due to stimulation of alternate pathways. This needs to be studied further.

Finally, we studied the permissiveness of the Huh7-SV5V cell line for establishment of HCV Con1 (genotype 1b) replicon RNA. Due to difficulties we encountered preparing pure HCV replicon RNA in vitro for eletroporation into our cells, we instead isolated total RNA from an established HCV replicon cell line. This ensured that we would have functionally self-replicating HCV replicon RNA in our total RNA preparation for electroporation. The Huh7-SV5V cells did prove to be more permissive to HCV replicon establishment. G418-resistant colony numbers were ~2- to ~4.5-fold higher in Huh7-SV5V cells compared to Huh7-CAT cells (Figure 8). The Huh7-SV5V colonies were also generally larger and denser than those from Huh7-CAT. The two cell lines do not differ in their growth kinetics, and they demonstrate similar transfection

efficiencies, so these are not factors that would affect the growth rates or overall numbers of replicon colonies. It is likely that the Huh7-SV5V replicon colonies are larger and denser because the replicon RNA was established more readily in the more permissive cells, and the cells could grow better earlier in the presence of G418. Also, IFN responses to viral RNA can often induce apoptosis (Stark et al., 1998; Samuel, 2001). The Huh7-CAT cells would mount an IFN response to the introduction of HCV viral RNA, and affected cells may not be as robust due to the induction of apoptosis. Huh7-SV5V cells, which cannot mount the same IFN response, would not therefore induce apoptosis, but instead continue to grow at their normal rate. Because we know that the SV5 V protein and the CAT genes were both initially inserted into the same site of the host genome, there should be no difference in the overall gene expression and resulting intracellular environment between the two cell lines, except for the effects of SV5 V protein expression on the cells' ability to respond to viral RNA via the IFN response.

Very recently, Lindenbach and coworkers (2005) described the first report of a full-length HCV genome that replicates and produces infectious viral particles in tissue culture. This development could revolutionize the field of HCV research. An important tool for the advancement of in vitro HCV replication is a highly permissive cell line. The Huh7-SV5V cell line could prove to be very useful in future studies of in vitro HCV replication, as HCV is sensitive to IFN. A cell line that cannot establish an antiviral state via either the IFN-$\alpha/\beta$ or the IFN-$\gamma$ pathways would potentially allow for greater levels of HCV infection and higher titers of HCV viral particles.

## SUMMARY

In summary, we successfully cloned the V protein gene from the paramyxovirus Simian Virus 5 (SV5). The expressed V protein was confirmed to be functional by its ability to degrade STAT1 via the proteasome. The human hepatoma cell line Huh7 was engineered to stably express either the SV5 V protein or the control CAT protein. The Huh7-SV5V cells did not have detectable levels of STAT1, even after up to 48 hours of IFN-α treatment. STAT1 is a vital component of both the IFN-α/β and the IFN-γ pathways. These cells proved to be non-responsive to both IFN-α and IFN-γ, as would be predicted in a STAT1-deficient cell line. The cells were treated with IFN-α and infected with EMCV for a single round of viral replication. The EMCV viral yield was significantly higher from the Huh7-SV5V cells as compared to the Huh7-CAT cells, indicating that the Huh7-SV5V cells are less responsive to IFN and thus cannot effectively establish an antiviral state. Finally, the cells were tested for their permissiveness to support replication of an HCV Con1 (genotype 1b) replicon RNA. The Huh7-SV5V cells proved to be ~2- to ~4.5-fold more permissive as determined by replicon colony numbers. Replicon colonies from the Huh7-SV5V cells were observed to be larger and denser than those from the control cells. These studies indicate that an IFN non-responsive human hepatoma Huh7 cell line expressing the SV5 V protein can be a potentially useful tool for future in vitro HCV studies.

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