Respiratory Syncytial Virus Based Vectors for the Treatment of Cystic Fibrosis

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

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The Integrated Biomedical Science Graduate Program

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Abstract

Cystic fibrosis (CF) is the most common lethal recessive genetic disease in the Caucasian population. It is caused mutations in the CF transmembrane conductance regulator (CFTR) gene which lead to the production of a partially or completely non-functional protein. Lack of CFTR in epithelial cell membranes results in abnormal ion transport and dehydration of mucosal surfaces. In the respiratory tract this leads severe lung disease, the leading cause of morbidity and mortality in the CF population. Since CFTR was first cloned in 1989, a major goal of CF research has been to develop effective gene therapy for CF lung disease. Respiratory syncytial virus (RSV) naturally infects the ciliated cells that line the human respiratory tract, cells that express CFTR and play a major role in determining the hydration of the airway mucosal surface. In addition, although an immune response is mounted against RSV, this does not prevent subsequent infections suggesting that an RSV-based vector might be effectively re-administered a requirement since the apical ciliated cells of the respiratory tract are terminally differentiated and have a defined life span. To test whether the large, 4.5 kb, CFTR gene could be expressed by a recombinant RSV and whether infectious virus could be used to deliver CFTR to ciliated airway epithelium derived from CF patients, we have inserted the CFTR gene into four sites in the recombinant, green fluorescent protein-expressing (rg)RSV genome to generate virus expressing four different amounts of the CFTR protein. Two of these rgRSV-CFTR viruses were capable of expressing CFTR with little effect on viral replication. rgRSV-delivered CFTR was functional in primary CF airway epithelial cultures. Infection with these viruses resulted in chloride channel
function similar to that seen in non-CF cultures and correction of additional characteristic defects seen in CF airway epithelia. Unfortunately, most ciliated airway cells infected with RSV die within 5 days. To create an improved RSV-based vector we generated an RSV “replicon” by removing the three glycoprotein genes from a full-length RSV cDNA. The replicon is able to replicate in cells without killing them or producing infectious virus, allowing the isolation and expansion of replicon-containing cells. Providing the viral glycoproteins in trans enables the production of “one-step virus” (OSV) which is capable of delivering a replicon to fresh cells where it produces its encoded proteins, but again cannot spread. We inserted the CFTR gene into the RSV replicon genome in four positions. All of these replicons were capable of producing the CFTR protein. When CFTR-expressing replicon OSV were used to infect primary human airway epithelial cultures, replicon containing cells remained within the cultures for 12-20 days, indicating that replicon containing cells have a survival advantage over those infected with complete virus. Our results indicate that RSV is capable of expressing CFTR, supplying enough functional CFTR to primary CF airway cells to correct their physiologic defects and with modification can remain in primary airway cells for a prolonged period of time. Collectively, our data support further investigation of RSV as a potential vector for CF gene therapy.
Dedication

This work is dedicated to my family. To my mother, Judy, for providing me with an amazing role model, for always believing in me and for supporting me on all my decisions in life. To my father, Bob, for always being there for me, no matter where, no matter what time, and for always knowing exactly what to say or do. To my husband, Steve, for being my rock, for understanding me when no one else could and for being the love of my life.

I would also like to dedicate this work to the father of the Integrated Biomedical Graduate Program, Dr. Allan Yates (1943-2010). His passion for knowledge and his ability to motivate students to achieve their best is unmatchable and he will surely be missed.
Acknowledgments

Thank you to the General Mason Scholarship Fund of The Research Institute at Nationwide Children's Hospital for funding my graduate work.

Thank you to Dr. Mark Peeples for all his guidance and support in my scientific career. Thanks to all the members of the Peeples' laboratory particularly Barb Newton, Steve Kwilas, Arife Unal and Katelyn Leahy for their practical assistance. Thank you to John Riordan at UNC for the monoclonal antibody to CFTR and to everyone in the Pickles lab at UNC for their assistance with CFTR functional testing.

I would also like to thank the members of my Dissertation Committee for all of their personal and professional help throughout my graduate career. I can't imagine a better set of mentors.

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                Medicine, The Ohio State University

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transmembrane conductance regulator corrects the bioelectric phenotype of human cystic

Fields of Study

Major Field:  Integrated Biomedical Science

Minor Fields: Molecular Virology and Gene Therapy, Translational Research
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### Abbreviations

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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>A549</td>
<td>Immortalized alveolar epithelial cell line</td>
</tr>
<tr>
<td>AAV</td>
<td>Adeno-associated virus</td>
</tr>
<tr>
<td>ABC</td>
<td>Adenosine-5'-triphosphate binding cassette</td>
</tr>
<tr>
<td>Ad</td>
<td>Adenovirus</td>
</tr>
<tr>
<td>AMPK</td>
<td>Alpha1-AMP activated protein kinase</td>
</tr>
<tr>
<td>ASL</td>
<td>Airway surface liquid</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine-5'-triphosphate</td>
</tr>
<tr>
<td>AUG</td>
<td>Start codon sequence</td>
</tr>
<tr>
<td>BALB/c</td>
<td>Inbred mouse strain</td>
</tr>
<tr>
<td>BAVD</td>
<td>Bilateral absence of the vas deferens</td>
</tr>
<tr>
<td>BME</td>
<td>Beta-mercaptoethanol</td>
</tr>
<tr>
<td>BRSV</td>
<td>Bovine respiratory syncytial virus</td>
</tr>
<tr>
<td>Ca^{2+}</td>
<td>Calcium</td>
</tr>
<tr>
<td>CaCC</td>
<td>Calcium activated chloride channels</td>
</tr>
<tr>
<td>CAL</td>
<td>Cystic fibrosis transmembrane conductance regulator associated ligand</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CBAVD</td>
<td>Congenital bilateral absence of the vas deferens</td>
</tr>
<tr>
<td>CCA</td>
<td>Chimpanzee coryza agent</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
</tbody>
</table>
CF  Cystic fibrosis
CFPAC-1  Cystic fibrosis pancreatic duct cell line
CFRD  Cystic fibrosis-related diabetes
CFTR  Cystic fibrosis transmembrane conductance regulator
CHIP  Carboxy-terminus of heat shock protein 70 interacting protein
CHO  Chinese hamster ovary cells
Cl⁻  Chloride
CMV IE  Human cytomegalovirus immediate early promoter
CMV_{\text{min}}  Minimal cytomegalovirus immediate early promoter
CO₂  Carbon dioxide
COPD  Chronic obstructive pulmonary disease
cp52  Respiratory syncytial virus mutant resulting from repeated cold passage
cpRSV  Cold passaged respiratory syncytial virus
cptsRSV  Cold passaged temperature sensitive respiratory syncytial virus
C-terminus  Carboxy terminus
CTLs  Cytotoxic T lymphocytes
CX3C  Chemokine motif
°C  Degrees Celsius
DC  Dendritic cells
DC-chol/DOPE  DC-cholesterol and dioleoyl-phosphatidylethanolamine
DIOS  Distal intestinal obstruction syndrome
DMEM  Dulbecco’s modified Eagle medium
DNA  Deoxyribonucleic acid
<table>
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<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tr>
<td>DOTAP</td>
<td>N-(1-(2,3-dioleothyloxy)propyl)-N,N,N-trimethyl-ammoniummethylsulphate</td>
</tr>
<tr>
<td>Dpn I</td>
<td>Restriction endonuclease from <em>Diplococcus pneumonia</em></td>
</tr>
<tr>
<td>dsRNA</td>
<td>Double stranded ribonucleic acid</td>
</tr>
<tr>
<td>EDMPC</td>
<td>1,2-dimyristoyl-sn-glycero-3-ethylphosphocholine, chloride salt</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine-tetraacetic acid</td>
</tr>
<tr>
<td>EIAV</td>
<td>Equine infectious anemia virus</td>
</tr>
<tr>
<td>ENaCs</td>
<td>Epithelial sodium channel</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>F</td>
<td>Fusion protein</td>
</tr>
<tr>
<td>F0</td>
<td>Precursor form of the fusion protein</td>
</tr>
<tr>
<td>F1</td>
<td>Larger subunit of the fusion protein formed after furin cleavage</td>
</tr>
<tr>
<td>F2</td>
<td>Smaller subunit of the fusion protein formed after furin cleavage</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>fcs</td>
<td>Furin cleavage site</td>
</tr>
<tr>
<td>FEV$_1$</td>
<td>Forced expiratory volume in 1 second</td>
</tr>
<tr>
<td>FI-RSV</td>
<td>Formalin inactivated respiratory syncytial virus</td>
</tr>
<tr>
<td>FIV</td>
<td>Feline immunodeficiency virus</td>
</tr>
<tr>
<td>G</td>
<td>Respiratory syncytial virus attachment glycoprotein</td>
</tr>
<tr>
<td>G1349D</td>
<td>Cystic fibrosis mutation</td>
</tr>
<tr>
<td>G551D</td>
<td>Cystic fibrosis mutation</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>GE</td>
<td>Gene end</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>GS</td>
<td>Gene start</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanine triphosphate</td>
</tr>
<tr>
<td>HAE</td>
<td>Well differentiated culture of human airway epithelial cells</td>
</tr>
<tr>
<td>HBD</td>
<td>Heparan binding domain</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HCO$_3^-$</td>
<td>Bicarbonate</td>
</tr>
<tr>
<td>HD</td>
<td>Helper-dependent</td>
</tr>
<tr>
<td>HeLa</td>
<td>Human cell line derived from an ovarian carcinoma</td>
</tr>
<tr>
<td>HEP-2</td>
<td>Human epithelial cell line type-2</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>HMPV</td>
<td>Human metapneumovirus</td>
</tr>
<tr>
<td>hpi</td>
<td>Hours post infection</td>
</tr>
<tr>
<td>hr</td>
<td>Hour</td>
</tr>
<tr>
<td>HRA</td>
<td>Heptad repeat A</td>
</tr>
<tr>
<td>HRB</td>
<td>Heptad repeat B</td>
</tr>
<tr>
<td>HS</td>
<td>Heparan sulphate</td>
</tr>
<tr>
<td>Hsc70</td>
<td>Heat shock conjugate 70</td>
</tr>
<tr>
<td>Hsp70</td>
<td>Heat shock protein 70</td>
</tr>
<tr>
<td>IB3-1</td>
<td>Cystic fibrosis immortalized bronchial cell line</td>
</tr>
<tr>
<td>ICAM</td>
<td>Inter-cellular adhesion molecule</td>
</tr>
<tr>
<td>ICOR</td>
<td>Outwardly rectifying intermediate conductance chloride channel</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
</tbody>
</table>
IG  Intergenic region
IgA  Immunoglobulin A
IgG  Immunoglobulin G
IKKε  IκB kinase
IL  Interleukin
IRF3  Interferon regulatory factor 3
JAK  Janus kinase
kDa  Kilodalton
L  Large polymerase protein
Le  Leader sequence at the 3’ end of the paramyxovirus genome
LRI  Lower respiratory infection
M  Respiratory syncytial virus matrix protein
M2-1  Respiratory syncytial virus protein
M2-2  Respiratory syncytial virus protein
MAb  Monoclonal antibody
MCC  Mucociliary clearance
Mg²⁺  Magnesium
MI  Meconium ileus
min  Minute
MIP  Macrophage inhibiting protein
ml  Milliliter
mm  Millimeter
MOI  Multiplicity of infection
<table>
<thead>
<tr>
<th>Term</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MSCs</td>
<td>Mesenchymal stem cells</td>
</tr>
<tr>
<td>MVA-T7</td>
<td>Modified vaccinia virus, Ankara strain, engineered to produce T7 polymerase</td>
</tr>
<tr>
<td>N</td>
<td>Nucleoprotein</td>
</tr>
<tr>
<td>Na⁺</td>
<td>Sodium</td>
</tr>
<tr>
<td>NBD</td>
<td>Nucleotide binding domains</td>
</tr>
<tr>
<td>NDV</td>
<td>Newcastle disease virus</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer cells</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>NPD</td>
<td>Nasal potential difference</td>
</tr>
<tr>
<td>NS1</td>
<td>Respiratory syncytial virus nonstructural protein 1</td>
</tr>
<tr>
<td>NS2</td>
<td>Respiratory syncytial virus nonstructural protein 2</td>
</tr>
<tr>
<td>nt</td>
<td>Nucleotide</td>
</tr>
<tr>
<td>N-terminus</td>
<td>Amino terminus</td>
</tr>
<tr>
<td>ORCC</td>
<td>Outwardly rectifying Cl⁻ channels</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>P</td>
<td>Respiratory syncytial virus phosphoprotein</td>
</tr>
<tr>
<td>p.i.</td>
<td>Post-inoculation</td>
</tr>
<tr>
<td>PA</td>
<td>Pseudomonas aeruginosa</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCL</td>
<td>Periciliary layer</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>PDZ</td>
<td>Postsynaptic density protein-95, disc-large and ZO-1 domain</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PEI</td>
<td>Polyethyleneimine</td>
</tr>
<tr>
<td>pep 27</td>
<td>Peptide fragment excised by the furin cleavage of the RSV F protein</td>
</tr>
<tr>
<td>PERT</td>
<td>Pancreatic enzyme replacement therapy</td>
</tr>
<tr>
<td>pfu</td>
<td>Plaque forming units</td>
</tr>
<tr>
<td>pH</td>
<td>Hydrogen ion concentration</td>
</tr>
<tr>
<td>PI</td>
<td>Pancreatic insufficiency</td>
</tr>
<tr>
<td>PIV1</td>
<td>Parainfluenza 1</td>
</tr>
<tr>
<td>PIV3</td>
<td>Parainfluenza type 3</td>
</tr>
<tr>
<td>PIV5</td>
<td>Parainfluenza type 5</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PKG</td>
<td>Protein Kinase G</td>
</tr>
<tr>
<td>PLL</td>
<td>Poly-L-lysine</td>
</tr>
<tr>
<td>Poly (A)</td>
<td>Polyadenylate</td>
</tr>
<tr>
<td>PP2A</td>
<td>Protein phosphotase</td>
</tr>
<tr>
<td>PP2C</td>
<td>Protein phosphotase</td>
</tr>
<tr>
<td>PS</td>
<td>Pancreatic sufficiency</td>
</tr>
<tr>
<td>PVM</td>
<td>Pneumovirus of mice</td>
</tr>
<tr>
<td>R domain</td>
<td>Regulatory domain</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated on activation normal T cell expressed and secreted</td>
</tr>
<tr>
<td>RFP</td>
<td>Red fluorescent protein from the Discosoma species of coral</td>
</tr>
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</table>

**xx**
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>rgRSV</td>
<td>Recombinant green fluorescent protein expressing respiratory syncytial virus</td>
</tr>
<tr>
<td>RhoA</td>
<td>Ras homolog gene A</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNP</td>
<td>Ribonucleoprotein</td>
</tr>
<tr>
<td>ROMK2</td>
<td>Renal potassium channel</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RSV</td>
<td>Respiratory syncytial virus</td>
</tr>
<tr>
<td>RSV-IVIG</td>
<td>Respiratory syncytial virus intravenous immunoglobulin</td>
</tr>
<tr>
<td>(r)tTA</td>
<td>Reverse tetracycline transactivator</td>
</tr>
<tr>
<td>RW30</td>
<td>Derivative of D46 containing the Renilla GFP gene and two ribozymes</td>
</tr>
<tr>
<td>SA</td>
<td>Staphylococcus aureus</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>sG</td>
<td>Soluble G protein</td>
</tr>
<tr>
<td>SH</td>
<td>Respiratory syncytial virus small hydrophobic protein</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SIV</td>
<td>Simian immunodeficiency virus</td>
</tr>
<tr>
<td>SNAREs</td>
<td>Soluble N-ethylmaleimide-sensitive factor attachment protein receptors</td>
</tr>
<tr>
<td>SR19 T7</td>
<td>Sindbis virus replicon expressing T7 polymerase</td>
</tr>
<tr>
<td>STAS</td>
<td>Anti-sigma antagonist</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal Transducers and Activators of Transcription</td>
</tr>
<tr>
<td>SV40</td>
<td>Simian virus 40</td>
</tr>
<tr>
<td>SV5</td>
<td>Simian virus 5 (also known as PIV5)</td>
</tr>
</tbody>
</table>
T7  T7 bacteriophage
T7 polymerase  Bacteriophage RNA polymerase
tet  Tetracycline
TetR  Tetracycline response element
TGFβ  Transforming growth factor beta
TLR  Toll-like receptor
TMD  Transmembrane domain
TNFα  Tumor necrosis factor alpha
Tr  Trailer sequence at the 5’ end of the paramyxovirus genome
TrC  Trailer complement sequence at the 3’ end of the paramyxovirus antigenome
TRN-CFTR  Truncated form of CFTR that terminates after the R domain of the protein
ul  Microliter
UTP  Uridine triphosphate
Vero  Immortalized cell line derived from African green monkey kidney cells
α  Alpha
β  Beta
ΔF508  Delta 508 cystic fibrosis mutation
λ  Lamda
µg  Microgram
µM  Micromolar
CHAPTER 1: CYSTIC FIBROSIS

History

Cystic fibrosis (CF) is most common autosomal recessive genetic disease in the Caucasian population. It is caused by a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. The first mention of a disease that resembles CF occurred in the 18th century when there was mention of a childhood illness where “the child will soon die whose brow tastes salty when kissed”, these children were thought to be bewitched [1]. In the early 20th century a disease consisting of pancreatic dysfunction and meconium complications began to be associated with symptoms of pulmonary disease [2-4]. It wasn’t until 1930 that this disease was recognized as being separate from celiac disease but the first mention of “cystic fibromatosis with bronchiectasis” did not occur until 1936 [5, 6]. Pathological features of CF were not appreciated, though, until 1938 when mucus plugging of pancreatic ducts was observed and the term “cystic fibrosis of the pancreas” was coined [7, 8]. Children with cystic fibrosis of the pancreas died from meconium complications or malnutrition; the life expectancy of these children was less than 6 months. In the mid 1940s, a general state of thickened mucus in most exocrine glands was observed in these patients causing its name to be changed to mucoviscidosis [9]. In 1946 it was determined that this disease followed an autosomal recessive form of inheritance [10]. One of the most important observations was made during a heat wave in New York in 1948 when many of the children admitted to the hospital with heat shock had mucoviscidosis [11]. This led to the discovery in 1953 that dehydration was occurring in these patients due to increased salt
excretion in the sweat [12, 13]. Shortly thereafter it was appreciated that there was widespread abnormalities in all exocrine glands leading to the second and last name change for this disease now known as cystic fibrosis. In 1959, the sweat test, the most common and reliable test for CF, was standardized [14]. The 1950s also brought about the organization of specialized centers for the treatment of patients with CF and the founding of the Cystic Fibrosis Foundation in 1955. Additional disease characteristics of CF were also discovered in the 1950s involving intestinal epithelium, intrahepatic bile ducts and the gall bladder. In the 1960s, altered electrolyte absorption in the sweat duct and altered fluid and bicarbonate (HCO₃⁻) secretion in the pancreas was observed in CF patients [15-18]. It was not until the 1980s, however, that a chloride ion (Cl⁻) transport defect was observed in the sweat duct and airways of CF patients [19-22]. In 1989 the gene involved in the development of CF, CFTR, was discovered via positional cloning [23-25].

**Epidemiology**

CF is occurs with an incidence of ~1 in every 2,500 live Caucasian births in the United States. The frequency is lesser in the Latin American population (1 in 4,000-10,000) and even more so in the African and Asian American populations (1 in 15,000-20,000 and 1 in >30,000, respectively) [26]. Studies have indicated that 1 in 25 individuals of European descent carry a mutation in at least one of their CFTR alleles. This high frequency of CFTR mutation is too high to be explained by spontaneous mutation. It has therefore been suggested that at some time in human evolution carrying a mutation in CFTR offered a selective advantage. An increased resistance to pathogen induced diarrheal diseases such as that of enterotoxic *Escherichia coli*, *Vibrio cholerae* or *Salmonella typhi* have been suggested
as a benefit of heterozygocity [27-29]. A decreased resistance to heat may have excluded CFTR mutations from warmer climates.

The median age of survival for an individual diagnosed with CF in the United States in 1938 was only months, in 1969 it was 14 years and in 2000 it was 32 years. This increase in life expectancy was mostly due to the advances in medical care received by CF patients in lieu of a CF cure. The life expectancy of a CF patient differs by country depending on its socioeconomic status and availability of centralized care. The current mean survival for individuals with CF born in the US is ~37 years while the United Kingdom predicts the median age of survival of babies born with CF in the 21st century to be over 50 years of age [30, 31]. The life expectancy of a CF patient also depends on their disease status; some patients still die very young while others live into their 60s.

A CF diagnosis is made based on clinical symptoms of the disease along with the presence of biochemical and genetic markers [32]. The most widely used test for CF is the quantitative pilocarpine iontophoresis sweat chloride test. Pilocarpine is used to stimulate sweating on a small area of the forearm; the sweat is collected via a closed capillary system then tested for its concentration of Cl− ions. Generally concentrations above 60mmol/L are indicative of CF, concentrations above 30mmol/L and 40mmol/L are suggestive of CF in infants less than 6 months old and older adults, respectively [33]. For those who do not have a conclusive sweat Cl−, nasal potential difference, rectal mucosa biopsy, semen analysis and fecal concentrations of chymotrypsin and pancreas specific elastase offer alternative diagnostics [34-37]. Fifty percent of CF patients are diagnosed before 6 months of age, ~90% are diagnosed before their 8th birthday [31]. Newborn screening was just recently adopted by
all 50 states in the US so the percentage of individuals diagnosed early is sure to rise.

Newborn screening is done via the Guthrie blood spot method. The amount of immunoreactive trypsinogen (IRT) is measured in a dried blood spot taken from an infant. Increased levels of IRT are indicative of pancreatic injury and are increased in CF patients regardless of their pancreatic sufficiency. If the IRT test is positive, it can be repeated in 3-4 weeks to confirm (IRT/IRT, sensitivity >87%) or a genotype analysis can be performed (IRT/DNA, sensitivity >99%) [26]. Scanning for the 40 most common CFTR mutations can identify >90% of individuals with CF but a smaller panel including only the most common regional mutations may also be of benefit [38, 39]. Early diagnosis results in improved nutrition and growth which is associated with a better prognosis in CF patients indicating a benefit of newborn screening [40-43]. Early diagnosis also allows for early disease intervention. Early diagnosis may however pose the risk of exposing young CF patients to debilitating bacterial infections when they attend specialized treatment centers and come into contact with patients with more advanced disease. The benefit of improved nutrition has thus far outweighed this risk of exposure, however, due to the increased level of cleanliness maintained in specialized CF treatment centers [44]. Regardless of the result of the newborn screen, a sweat test must also be performed to confirm the diagnosis of CF.

The Cystic Fibrosis Transmembrane Conductance Regulator

It was determined early on that CF tissue behaved as if it was impermeable to Cl⁻ [19-22]. It was also observed that Cl⁻ secretion could be activated by cAMP in normal but not CF airway tissue [45, 46]. These observations led investigators to suggest that the CFTR gene encoded a Cl⁻ channel or an ion channel conductance regulator. This gene was located on the long arm of chromosome 7 (7q31) and occupied 250 kb of DNA containing 27 exons [47]. The
gene encoded a 6.1kb transcript that produced a 1480 amino acid protein of ~170 kDa in its mature form. The CFTR protein is a multi-domain integral membrane glycoprotein shown to function primarily as a low conductance, cAMP-activated, phosphorylation and ATP dependent Cl⁻ channel [48-55]. However, CFTR also plays very important roles in the regulation of other ion channels and intracellular membrane transport. CFTR is expressed apically in the luminal epithelial cells lining most exocrine glands but most importantly in the airways, small intestine, vas deferens and the ducts of the pancreas [56-59]. CFTR has also been found in cardiac myocytes and in cells of the kidney and hypothalamus but its function in these organs is unknown as they are not highly involved in the pathogenesis of CF [60-64]. CFTR is expressed both apically and basolaterally in the epithelial cells lining the sweat gland and sweat ducts [65].

CFTR is a member of the ATP binding cassette (ABC) family of membrane transporters (ABC-C7). It is most closely related to the multidrug resistance proteins 1-9 and the sulfonylurea receptors 1 and 2, which regulate inwardly rectifying potassium channels. However, CFTR is the only ABC protein that actually functions as an ion channel and contains a regulatory (R) domain. In addition to the R domain CFTR contains two transmembrane domains (TMDs), each consisting of six membrane spanning helices, and two nucleotide binding domains (NBDs), large hydrophilic domains capable of binding and hydrolyzing ATP [25]. Another difference between CFTR and the rest of the ABC transporter family is the lack of sequence homology between its two NBDs, they are only 27% identical, as opposed to the nearly homologous NBDs of other ABC transporters [66]. Three extracellular loops and two intracellular loops connect the helices of each TMD. The C terminus of CFTR contains a PDZ (postsynaptic density protein-95, disc-large and ZO-1)
domain binding site which facilitates its interactions with other proteins and the
cytoskeleton. The general structure of CFTR consists of N terminus-TMD1-NBD1-R-TMD2-
NBD2-C terminus [67]. The number of CFTR molecules that compose a functional channel is
not known with certainty, however. Many studies have shown that CFTR can form dimers,
some of which exhibit altered electrophysical characteristics [68-71]. Some studies have
suggested that dimerization is part of the normal activation process of CFTR and that PDZ
interactions may be required [72-74]. The more accepted idea, however, is that CFTR exists
and functions as a monomer [75-78]. The low resolution crystal structure of CFTR depicts it
as a monomer and no other ABC transporters containing 2 TMDs and 2 NBDs exist as
dimers [79, 80].

**CFTR Biosynthesis**

CFTR expression is time and tissue specific due to alternate start sites and alternative
splicing [81, 82]. Alternatively spliced forms of CFTR include exon 4, exon 5 (heart
specific), exon 9 (expressed more in vas deferens than in airways), intron 10, exon 12, and
TRN-CFTR (truncated form of CFTR that terminates after the R domain of the protein due to
the insertion of a premature stop codon) [63, 83-88]. TRN-CFTR is expressed specifically in
the kidney and gradually replaces wild type CFTR during development; its gating is normal
but it incorporates into the plasma membrane less frequently resulting in decreased Cl-
secretion [89].

Coincident with translation CFTR is inserted into the membrane of the endoplasmic
reticulum (ER) for post-translational modification. Transport through the ER and Golgi
apparatus results in the addition of two complex N-linked oligosaccharide groups at
positions 894 and 900 on the 4\textsuperscript{th} extracellular loop, the first loop of TMD2 of CFTR. During translation, the CFTR polypeptide backbone (~130 kDa) is transported through the Sec61 translocon into the ER. In the ER, CFTR receives two immature sugar chains on its 4\textsuperscript{th} extracellular loop becoming the immature, core glycosylated CFTR protein (~150 kDa) [90-92]. ER luminal chaperones calnexin and calreticulin and cytoplasmic chaperone heat shock protein 70 (Hsp70) help this protein attain its correctly folded, protease-resistant form before exiting the ER [93-95]. Additional chaperones and co-chaperones also interact with CFTR as it is being processed to appraise and assist in folding and assembly [96-99]. After attaining its proper conformation, CFTR disassociates from its chaperones. CFTR is then transported to the Golgi, via the CopII export system, where its glycosylation is modified and it becomes the mature ~170 kDa CFTR protein which is transported to the membrane [100]. The Sec24 coat protein of CopII vesicles is thought to recognize an ER exit motif in the CFTR NBD1 [101]. An intact C terminus is required for transport to the membrane and appropriate apical targeting [102, 103]. The intracellular loops also appear to be important for processing and trafficking to the membrane [104-106]. Proper glycosylation is not, however, required for proper processing or function of CFTR [107, 108]. Although passage through the Golgi is required for the maturation of CFTR's glycosylation and its expression at the cell membrane, it has been suggested that CFTR becomes functional in the ER [109].

Complex quality control mechanisms are involved in CFTR processing which is monitored throughout its course. Appropriate domain structure and interdomain interactions are required for proper maturation of CFTR. Quality control machinery evaluates CFTR on local and global structural maturation. Only 25-50\% of core glycosylated CFTR attains the correctly folded, protease resistant form that is transported to the Golgi; the remainder is
deglycosylated, exported from the ER and rapidly degraded via the ubiquitin dependent cytosolic 26S proteosome complex [92, 110, 111]. The amount of CFTR that eventually matures is cell type specific. Co-chaperones heat shock conjugate 70 (Hsc70) and carboxy-terminus of Hsp70 interacting protein (CHIP) interact with CFTR in the ER to target the immature protein to ubiquitin conjugating enzymes [112, 113]. The level of CHIP expression in a tissue has been shown to correlate with the amount of ubiquitinated CFTR indicating that the cell type specific levels of CHIP expression may play a role in the amount of mature CFTR expressed in that tissue [113]. Improperly folded CFTR could also be targeted for degradation because of a prolonged association with calnexin [114]. During processing, CFTR is also required to associate with the Hsp90 network which is responsible for refolding already folded proteins; it has been suggested that this may play a role in CFTR’s inefficient maturation [115, 116]. Another suggestion is that CFTR is ubiquitinated before translation is complete and that it is the balance between deubiquitination and ubiquitin chain elongation while the protein is in the ER that ultimately determines the fate of the protein upon exit from the ER [117, 118]. Indeed, additional studies indicate that both co- and post-translational processes dictate the ability of CFTR to attain conformational maturation. NBD1 was shown to attain a protease resistant form during translation, NBD2 however, does not and it was suggested that the proper maturation of NBD2 is dependent on proper maturation of NBD1 [119]. Proper maturation of NBD2, however, does not seem to be required for CFTR to leave the ER as a CFTR mutant truncated after TMD2 is able to attain mature glycosylation [120]. Another suggestion is that there is an earlier ER checkpoint such that if NBD1 is improperly folded, this potentiates ubiquitin chain elongation and increases the chance of degradation of the immature protein [121]. This could be the case if a mutation hides the ER export motif in NBD1 [101]. It is still not known
however if improperly folded CFTR presents negative stimuli resulting in degradation or if it hides the positive stimuli needed to progress to the Golgi resulting in ER retention and eventual degradation. Interdomain interactions are very important to the maturation and function of CFTR, as expressing separate halves of CFTR results in the formation of an active channel and co-expression of portions of CFTR can rescue the expression of mutant CFTR proteins [122-124]. An additional checkpoint has been suggested to monitor the association of the CFTR TMDs. If the TMDs do not associate properly, the protein is ubiquitinated by an alternate complex [125, 126]. Still other quality control mechanisms and degradation pathways may also exist [111, 127]. Some studies have indicated that quality control machinery only controls CFTR maturation on a structural level and while this usually results in the production of functional channels, some mutant channels that lack the ability to function are efficiently produced while other mutant CFTR channels that are known to possess some functional capability are usually degraded [109, 128].

Once at the plasma membrane, CFTR undergoes cycles of endocytosis and exocytosis via clathrin-coated vesicles, possibly through an interaction between an internalization signal at the C-terminus of CFTR and AP-2 or α-adaptin [129-132]. Indeed, mutations in the C terminus of CFTR have been shown to affect the stability of CFTR in the membrane [133, 134]. It is believed that this process of “recycling” is somewhat controlled by cAMP as increases in cAMP result in increased expression of CFTR in the cell plasma membrane through an inhibition of internalization [135-138]. The microtubule and actin networks have both been shown to be required for CFTR recycling [139-142]. Actin filaments are also required for cAMP stimulation of CFTR function by mechanisms other than increasing expression at the cell surface [143-145]. Dimerization of CFTR has been suggested to inhibit
its internalization and thus increase its activity by increasing its resident time in the membrane [136, 138]. Thus the regulation of CFTR expression and maturation are very complex involving alternate transcription start sites, alternative splicing, the balance between maturation and degradation, the availability of chaperones, possible dimerization and the degree of recycling. The average half life of CFTR is ~16 hours [92].

**CFTR as a Cl⁻ Channel**

The primary function of CFTR is as a low conductance (6-10 pS) channel that is selective for anions [48, 146]. The anion selectivity is as follows: Br⁻ ≥ Cl⁻ > I⁻ > F⁻, however, CFTR conducts other ions much less frequently than it conducts Cl⁻ [147]. CFTR is a non-rectifying channel with a linear current-voltage relationship [46, 54, 146]. CFTR is not a pump but a channel that allows anions to move according to their electrochemical gradient mediating the passive diffusion of Cl⁻ through epithelial cell membranes. This function of CFTR makes it a key player in determining the intracellular/extracellular ion concentration which ultimately controls the fluid flow across the epithelial cell membrane. CFTR is chiefly responsible for fluid and electrolyte secretion in the airway, intestine, pancreas and sweat gland secretory coil and fluid and electrolyte absorption in the sweat gland duct. For CFTR to function as a Cl⁻ channel it must first be phosphorylated in a cAMP-dependent manner at its R domain then bind and hydrolyze ATP at its NBDs.

Phosphorylation of the CFTR R domain controls the activity of the CFTR Cl⁻ channel.

Phosphorylation of the R domain of CFTR can be accomplished by multiple kinases but the most well defined is cAMP-activated, protein kinase A (PKA). There are nine potential PKA sites in the R domain (eight serines and one threonine), six of which are known to be
phosphorylated in vivo [148-151]. The exact sequence of the R domain is unimportant for channel activation as long as the phosphorylation sites are intact [152]. Phosphorylation of sites S660, SS75, S813 seem to be very important to channel activation and phosphorylation of sites S768 and S737 seem to be inhibitory by impeding the phosphorylation of additional sites [150, 153-155]. CFTR has been isolated in multiple phosphorylation states leading to the suggestion that phosphorylation is incremental; phosphorylation at one site may influence further phosphorylation [155-157]. The degree to which the R domain is phosphorylated is thought to play a role in the ability of the NBDs to facilitate channel gating [154, 157-159]. It is thought that phosphorylation of the R domain results in conformational changes, due to the addition of negative charges, that either bring the NBDs together increasing their ability to bind ATP or allow for more extensive conformational changes between the NBDs and TMDs upon ATP binding thus facilitating channel opening [160-162]. In support of this concept, mutation of the phosphorylation sites to aspartate resulted in a permanently open channel in the presence of ATP [153, 154]. Removal of the C terminus of the R domain also resulted in a constitutively open channel possibly by allowing the NBDs to more freely associate. Removing the entire R domain, however, resulted in only a partially active channel indicating that it plays an additional role in gating [163, 164]. Of note, mutation of all of the PKA sites resulted in a protein that could still be activated by PKA suggesting there are additional weaker sites or additional mechanisms of PKA activation [165].

Purified R domain undergoes conformational changes upon sequential phosphorylation possibly with phosphorylation of distinct sites affecting conformation in specific ways [160]. The degree of phosphorylation correlates with the open probability of the channel.
suggesting that phosphorylation can fine tune gate activity [158, 166]. An unphosphorylated R domain has been shown to be inhibitory toward CFTR channel opening [167]. It does not inhibit ATP binding or hydrolysis so it is thought that perhaps in its unphosphorylated state the R domain does not allow the conformational changes to occur in the NBDs and TMDs that are required for channel opening [168, 169]. It has also been suggested that, in addition to mediating conformational changes at the NBDs, phosphorylated R domain is able to interact with the N terminus of CFTR further stimulating channel opening [170, 171]. The conformation of the NBDs is also thought to influence the phosphorylation status of the R domain [172, 173]. Some studies have observed activation of CFTR by ATP in the absence on R domain phosphorylation [174-176]. The physiologic relevance of these in vitro observations is unknown, however, and it is widely accepted that CFTR channel gating requires cAMP-dependent phosphorylation of the R domain and ATP binding and hydrolysis at the NBDs.

As mentioned previously, the CFTR R domain can be phosphorylated by kinases other than PKA. PKC can phosphorylate two sites in the R domain and has been shown to slightly activate the CFTR channel [149, 165, 177, 178]. However, initial phosphorylation by PKC can enhance and is possibly required for phosphorylation of the R domain by PKA [149, 177-181]. Calcium/calmodulin dependent kinase I has been shown to phosphorylate the R domain in vitro, however, it is not clear whether this occurs in vivo [149]. CFTR can also be phosphorylated and activated by p60^c-src [182]. cGMP dependent PKG I and II are also able to phosphorylate the R domain, but only PKG II has been shown to phosphorylate it in vivo [183-185]. In the intestinal epithelium and pancreas, the hormone guanylin can stimulate the production of cGMP which in turn stimulates the PKG II pathway which may play a role
in the activation of CFTR in these organs [186]. At high enough levels, cGMP can also stimulate PKA [187-189]. It is this possible role for cGMP in the activation of CFTR that supported a protective role for CFTR mutations in heterozygotes. Bacterial enterotoxins induce cGMP which would activate CFTR in the intestinal epithelium leading to fluid secretion resulting in severe diarrhea. In the absence of fully functioning CFTR, this phenomenon could be reduced [28, 187, 189].

The R domain of CFTR can also be dephosphorylated by protein phosphatases. PP2A seems to function in the sweat duct and cardiac myocytes while PP2C acts in the airway and intestines [178, 190-192]. The level of intracellular potassium can inhibit certain phosphatases thereby indirectly affecting CFTR function [193]. When CFTR is dephosphorylated it is in a quiescent state, unable to function until it has been resphophorylated indicating the CFTR can be regulated by both cellular kinases and phosphatases [194].

After phosphorylation, the actual opening and closing of the CFTR channel is dependent on ATP binding and hydrolysis at the NBDs. Nucleotide triphosphates other than ATP can activate the CFTR channel but much less efficiently (ATP > GTP > ITP = UTP > CTP) [195]. The NBDs contain sequences that are required for ATP binding and hydrolysis, including the Walker A sequence (required for ATP binding), the Walker B sequence (required for Mg\(^{2+}\) binding) and the signature or linker sequence (required for ATP hydrolysis) [196]. Originally, it was believed that each NBD had its own ATP binding site and that binding and hydrolysis of ATP to NBD1 opened the channel while binding and hydrolysis of ATP at NBD2 closed the channel [158, 197-201]. This gating scheme was proposed as a result of
studies in which mutations to either NBD inhibited its ability to hydrolyze ATP [195, 202, 203]. In addition, studies with non-hydrolyzable ATP analogs, phosphate analogs, temperature and Mg²⁺ have supported the idea that hydrolysis is required for opening of the CFTR channel [201, 204-207]. Additional studies suggested that hydrolysis is rate limiting for both closing and opening the CFTR channel [117, 158, 194, 200].

Alternate studies suggested an interaction between the NBDs was required for efficient binding and hydrolysis of ATP. These and more recent studies have indicated that the NBDs come together to form two ATP binding sites each composed of sequences belonging to each NBD. Nucleotide binding site 1 (NBS1) is composed of the Walker A sequence of NBD1 and the signature sequence of NBD2, while NBS2 is composed of the Walker A sequence of NBD2 and the signature sequence of NBD1[66, 208, 209]. Thus when bound to ATP the NDBs form what could be considered a heterodimer. NBS1 has been shown to be a stable ATP binding site at which hydrolysis does not occur frequently [168]. ATP binding and hydrolysis do, however, readily occur at NBS2 [210]. ATP binding at either site can be the rate limiting step for channel opening suggesting that both ATPs must be bound for CFTR to open [211, 212]. Based upon these observations a modified scheme for CFTR gating has been proposed [66]. After R domain phosphorylation induces conformational changes that bring the NBDs in closer proximity, an ATP is able to bind to NBS1. Binding of this ATP stimulates additional conformational changes that result in binding of ATP at NBS2 which then stimulates channel opening by altering the conformation of the TMDs. Additional phosphorylation of the R domain may aid in the binding of ATP at NBS2 [158, 197, 198]. The signature sequence may play a role in linking the conformational changes at the NBDs to conformational changes of the TMDs [67, 213]. The intracellular loops of the TMDs may
also interact with the NBDs facilitating channel opening [104, 106, 214, 215]. The ATP at NBS2 is then hydrolyzed to disrupt the tight interaction of the NBDs and close the CFTR channel. It is this formation and disruption of the NBD heterodimer, through ATP binding and hydrolysis, that creates the conformational changes in the TMDs needed to open and close the CFTR Cl⁻ channel leading some to term CFTR as a “hydrolyzable-ligand gated channel” [208, 209, 216]. Mutations at NBS2, that prevent ATP hydrolysis, result in channels that are open longer supporting the hypothesis that hydrolysis of this ATP causes channel closing [198, 199, 206, 208, 212]. However, this scheme still does not fit with the many studies that suggest that hydrolysis is required for opening, as well as closing, of the CFTR channel. In addition, other studies suggest that ATP hydrolysis is not required for channel closing [207, 217].

Some studies have suggested that CFTR channel activity can occur in the absence of ATP binding/hydrolysis when the R domain is phosphorylated [196, 218, 219]. CFTR's ability to function in the absence of ATP hydrolysis may be dependent on the concentration of ATP or Mg²⁺, phosphorylation status, temperature or interactions with additional proteins [196, 197]. The contradictions among the results of these studies could result from mutations in one domain affecting the function of a different domain due to the tight interaction between all the CFTR domains. Also, results may vary depending on the in vitro system used.

While the R domain and NBDs control the activation and gating of CFTR, it is the TMDs that form the pore of the CFTR ion channel. While multiple studies have shown that when expressed separately either TMD can reconstitute a membrane pore, it is thought that both TMDs must be present to form a functional anion selective channel [122, 220-222]. Amino
acids in the 1st, 2nd, 3rd, 5th, 6th, 11th and 12th helices have been shown to be present in the pore [223-228]. Amino acids K95, R334, K335, R347 and R352 are thought to line the pore and may be important for anion selectivity and channel conductance [229-232]. Sequences that do not line the pore may also assist in determining anion selectivity [233]. An interaction between helix 1 and helix 6 in TMD1 with helix 12 in TMD2 would suggest they play important roles in formation of the pore [122, 225, 234]. Studies have indicated that the pore is ~5.3 Å in diameter through the center with larger intracellular and extracellular vestibules [224, 229, 235]. The extracellular loops may contribute to this shape [231, 236]. The shape of the CFTR pore may also contribute to its anion selectivity [237]. It is thought that anions collect near the positively charged regions at the ends of the TMDs and flow through the channel once it is open [238]. The degree of Cl− flow through the CFTR channel would depend on the frequency and duration of channel opening.

**CFTR as a Conductance Regulator**

In addition to functioning as a Cl− channel, CFTR plays a pivotal role as a conductance regulator by controlling the activity of other ion channels. CFTR has been shown to alter the activity of numerous ion channels but the most important channels with regards to CF disease are the epithelial sodium channel (ENaC) in the lung and the anion exchanger responsible for HCO3− secretion in the intestines and pancreas.

ENaCs are apically expressed in the epithelium of a variety of organs including the kidney, colon, lung and sweat gland. ENaCs are responsible for the reabsorption of Na+ from the lumens of these organs in order to maintain the salt balance and water homeostasis of the fluids that bathe these epithelial surfaces. ENaCs are activated by the concentrations of
various molecules in these fluids including ATP, adenosine, mineralcorticoids and serine proteases [239-241]. The interaction of CFTR and ENaC is most important in the lung where together they maintain the level and viscosity of the airway surface liquid (ASL) but this will be discussed more thoroughly in a later section. It was noticed early on that in the absence of CFTR, CF lung epithelial cells displayed increased Na\(^+\) absorption through ENaCs [20, 242-244]. Studies indicated that in the absence of CFTR, cAMP was able to stimulate ENaC function, while in the presence of CFTR, cAMP was inhibitory to ENaC function suggesting that activation of CFTR led to inhibition of ENaC [242, 245, 246]. These results also suggested that in the absence of this CFTR-induced inhibition, ENaC could exhibit hyperabsorption of Na\(^+\). Later studies indicated that CFTR directly downregulated ENaC function, by affecting its activation by PKA, and that this downregulation was enhanced upon activation of CFTR [247-249]. Mutant CFTR is unable to inhibit ENaC function [250, 251]. Possible mechanisms of this inhibition include: direct interaction [250, 252, 253], interaction through PDZ domain binding partners [254, 255], interaction via actin [256, 257], control of ENaC recycling [136, 137, 258] and control of intracellular Cl\(^-\) concentration [259, 260]. Studies have indicated that NBD1 of CFTR is required for the downregulation of ENaC [250, 261]. CFTR inhibition of ENaC function has been most thoroughly studied in the airways however it is thought to occur in the majority of tissues that express both of these channels except the sweat ducts. In the sweat duct, activation of CFTR also stimulates ENaC function [262]. This altered interaction is probably due to altered functions of CFTR and ENaC in this absorptive epithelium. The cause of this difference is unknown but is thought to be related to tissue specific regulation of CFTR or tissue specific expression of CFTR-interacting proteins.
In addition to controlling the activity of ENaCs, CFTR can activate the secretion of HCO$_3^-$ in the small intestines. If the activity of ENaCs, HCO$_3^-$ acts to neutralize stomach acid so that digestion and nutrient absorption of digestive enzymes from the pancreas and small intestine can operate properly. HCO$_3^-$ secretion is defective in the intestines, as well as the airways, of CF patients [263-265]. Some studies have indicated that CFTR is capable of secreting HCO$_3^-$ [263, 266, 267]. However, the permeability of HCO$_3^-$ through CFTR is only 13-26% that of Cl$^-$, making this an improbable scenario [268, 269]. One suggestion is that CFTR's conductance of HCO$_3^-$ may be dependent on how CFTR was activated [270].

In pancreatic duct cells, HCO$_3^-$ is secreted in exchange for Cl$^-$, which is then secreted via CFTR. However, HCO$_3^-$ secretion can be activated by cAMP regardless of intracellular Cl$^-$ concentration indicating that modification of intracellular Cl$^-$ concentration is not the mechanism by which CFTR activates HCO$_3^-$ secretion [267]. Also, mutated CFTR can cause a decrease in secreted HCO$_3^-$ regardless of its ability to conduct Cl$^-$ [271, 272]. Alternatively, the CFTR R domain can bind to the sulfate transporter and anti-sigma antagonist (STAS) domain of some members of the SLC26 family of anion exchange channels that mediate HCO$_3^-$ secretion suggesting this as the mechanism by which CFTR controls HCO$_3^-$ secretion [273, 274]. In addition to the intestines and pancreas, CFTR-mediated HCO$_3^-$ secretion is also observed in the submucosal glands and epithelium of the respiratory tract [267, 275, 276].

CFTR may play a role in the regulation of multiple other ion channels which may or may not affect the clinical manifestation of CF. Outwardly rectifying Cl$^-$ channels (ORCC) also malfunction in CF tissue due to defective regulation by PKA [277, 278]. Proper regulation of ORCC may be dependent on the presence of CFTR, specifically the NBD1 and R domains [221, 279-281]. One suggestion is that activation of CFTR triggers simultaneous ATP export.
from the cell which then activates ORCC through a purinergic receptor [282, 283]. Some studies have indicated that CFTR can activate ATP secretion through a separate channel [284, 285]. Other studies suggest that CFTR is able to transport ATP itself, however, this assertion is controversial [286-291]. CF tissue has been shown to have a lower level of ATP secretion, however contradictory data also exists [286, 290, 291]. It has been suggested that CFTR regulation of ATP release may depend on additional factors making it cell type dependent. CFTR also regulates other Cl⁻ channels in vitro, such as calcium activated Cl⁻ channels (CaCC) and volume regulated Cl⁻ channels possibly through ATP release as discussed above [282, 292-294]. It is thought that CFTR may play a role in regulatory volume decease. During hypotonic stress, the release of ATP results in the activation of Cl⁻ secretion which coincides with water secretion and a reduction in cell volume thus supplying water to the extracellular environment. This process is reduced in the absence of CFTR. CFTR could play a role in regulatory volume decrease by secreting Cl⁻ itself or by mediating ATP release which would then stimulate additional Cl⁻ channels [295, 296].

CFTR can also activate, as well as confer glibenclamide sensitivity to, the outwardly rectifying intermediate conductance (ICOR) Cl⁻ channels [297]. Similarly, CFTR can increase the sensitivity of the renal potassium channel, ROMK2, and the inwardly rectifying potassium channel, Kir6.1, to glibenclamide [298-300]. The physiologic importance of this activity is currently unknown. CFTR can also decrease the conductance of ROMK1 channels and alter their sensitivity to ATP [248].
**Additional CFTR Functions**

Approximately half of the total CFTR in a cell is present in the plasma membrane, the other half is present in subapical vesicles, in addition to the ER and Golgi [301, 302]. The presence of CFTR in vesicles may in part be due to its trafficking to and recycling from the membrane however, CFTR is also thought have functional roles in intracellular compartments.

As previously mentioned, CFTR is thought to attain functionality in the ER. It has been suggested that CFTR transport of Cl⁻ could create a driving force for acidification of the trans-Golgi and vesicles including prelysosomes [303-305]. Acidification of these compartments is important for glycosylation and protein trafficking. Indeed, alkalization of these compartments and an alteration in glycosylation patterns of membrane bound glycoproteins and secreted mucins is seen in patients with CF [306-312]. Specifically, there is a reduction in sialylated glycoproteins and an increase in the presence of sulfated and fucosylated glycoproteins. This alteration correlates with decreased acidification of the trans-Golgi. The enzymes involved in sialylation, sulfation and fucosylation are located in the trans-Golgi and optimally function at different pH ranges [313-316]. Alkalization of the trans-Golgi favors the activation of sulfating and fucosylating enzymes. Alternate studies, however, have refuted the role of CFTR in Golgi/endosome acidification as well as the presence of altered glycoproteins in CF patients [317-320]. The importance of this potential altered glycosylation pattern will be discussed further in a later section.

Secretory responses are decreased in the submandibular salivary glands of CF patients [321]. Also, cAMP can stimulate exocytosis in normal but not CF tissue [322]. These observations led to the suggestion that CFTR may mediate cAMP-dependent regulation of
vesicle trafficking and exocytosis. Indeed, the trafficking of lipids is impaired when mutant CFTR is present and the rate of vesicle fusion with the epithelial cell plasma membrane is increased in the presence of activated CFTR [323, 324]. The mechanism by which CFTR influences vesicle trafficking and exocytosis is unknown, however. It has been suggested that CFTR may interact with a protein within the vesicle membrane or that CFTR dependent alteration of vesicle pH may alter its plasma membrane fusion kinetics [316].

Interactions of CFTR with Other Proteins

CFTR interacts with multiple intracellular proteins through its PDZ binding domain, R domain and its N and C termini. It is assumed that different CFTR-protein interactions form and dissociate depending on their requirement for CFTR activity. Lack of inter-protein interaction with the NBDs and TMDs supports their major involvement in the primary CFTR function as a Cl- channel. The purpose of some of the following interactions is unknown, however because CF is such a complex disease it is assumed that the lack of any one of these interactions may play some role in disease manifestation.

CFTR interacts with multiple proteins via its PDZ domain. One of the most thoroughly studied CFTR interactions via its PDZ binding domain is that with the sodium-hydrogen exchanger regulatory factor (NHE-RF or EBP50) [325]. The presence of NHE-RF can increase the open probability of CFTR. NHE-RF also binds ezrin, a PKA anchoring protein, and it has been suggested that binding NHE-RF serves to bring CFTR in closer proximity to PKA [326, 327]. Indeed, loss of the PKA-ezrin interaction results in loss of CFTR activation by cAMP [328]. NHE-RF also facilitates an interaction of CFTR with the β2 adrenergic receptor which allows CFTR function to be stimulated directly by adenylate cyclase [327,
CFTR interacts with two additional proteins via its PDZ binding domain: CFTR associated protein 70 (CAP70) and the CFTR associated ligand (CAL) [330]. CAL is thought to inhibit plasma membrane expression of CFTR by increasing its degradation upon endocytosis [331]. CAP70 has only been studied in the context of CFTR dimerization and it is not known what other purpose this interaction may serve [72, 73]. The PDZ binding domain of CFTR is also thought to facilitate its interaction with the cytoskeleton [332].

A region in the C terminus of CFTR that is not the PDZ domain has been shown to interact with the alpha1-AMP activated protein kinase (AMPK). AMPK is a serine/threonine kinase that responds to changes in the intracellular AMP/ATP ratio by inactivating metabolic enzymes that consume high amounts of ATP. It has been suggested that phosphorylation by AMPK may inactivate CFTR or alter its functioning to a mode of operation that does not require ATP during times of ATP depletion [333, 334]. An interaction with AMPK may also alter the trafficking of CFTR. The C terminus, but not the PDZ domain, of CFTR also mediates interactions its with clathrin and myosin VI [335, 336].

CFTR interacts with multiple soluble N-ethylmaleimide-sensitive factor attachment protein receptors or SNARE proteins. CFTR interacts with SNARE proteins via its R domain and N terminus. It is thought that these interactions mediate CFTR trafficking. The R domain can to bind to syntaxin 8 which impairs CFTR trafficking [337]. The interaction of SNARE proteins with the R domain of CFTR has led to the suggestion that phosphorylation status may be important to proper trafficking and recycling [254]. CFTR interacts with syntaxin 1A and the heterodimer of synaptosomal-associated proteins 25 and 23 (SNAP25/23) via its N terminus [330, 338]. Syntaxin 1A inhibits CFTR trafficking and cAMP-dependent activation
of Cl− transport [339, 340]. The interaction of CFTR with the SNAP25/23 heterodimer works cooperatively with CFTR’s interaction with syntaxin 1A to reduce cAMP mediated Cl− transport [341]. The N terminus of CFTR also interacts with filamin, an interaction thought to be required for appropriate recycling of the CFTR channel [142].

Contradictory results concerning proposed CFTR functions may be due to the possibility that CFTR participates in different activities in different cell types due to the presence or absence of different accessory proteins.

**CFTR Mutations in the Human Population**

Over 1500 mutations in CFTR have been described leading to varying manifestations of CF disease. Most mutations are very rare, less than 10 occur with a frequency >1% however the frequency of a given mutation can vary among different populations [342]. Virtually all individuals diagnosed with CF have mutations in both of their CFTR alleles. Individuals diagnosed with CF can express two different CFTR mutations (CF heterozygotes) which may play a role in the wide range of CF clinical manifestations. Complex alleles or those containing two mutations in the CFTR gene are rare [343, 344]. Mutations of all types are found throughout the gene but mutations in different parts of the CFTR protein can result in different functional consequences. Mutations in the NBDs or R domain usually lead to defects in biosyntheses or altered gating kinetics of the CFTR channel whereas mutations in the TMDs usually result in altered permeability or conduction of the CFTR channel.

Mutations in CFTR have been divided into six classes based upon the amount of residual CFTR activity when the mutation is present. An additional class has been suggested to address the ever increasing evidence of CFTR regulatory functions, as mutations that affect
the Cl- channel activity of CFTR may or may not affect its other functions and vice versa. Mutations associated with the most severe forms of disease would be expected to inhibit multiple functions of CFTR. Due to the complexity of CFTR maturation and function, however, mutations in one domain can affect the maturation or activity of other domains in multiple ways and thus can be categorized into more than one class.

Class I mutations include nonsense, frame shift and splice site mutations that result in the lack of CFTR synthesis. All the mutations in this class lead to premature termination of transcription resulting in unstable mRNA transcripts or aberrant truncated proteins that undergo degradation. This results in little or no CFTR production and thus loss of all CFTR functional activities causing these mutations to be associated with the most severe forms of CF. Less than 7% of CFTR mutations carried by CF patients belong to this class however this class of mutations is prevalent among those of Ashkenazi Jewish descent [345].

Class II mutations include missense mutations and amino acid deletions that cause improper processing or maturation of the CFTR protein. Class II mutations account for ~85% of all mutations carried by CF patients and occur in the NBDs, TMDs and intracellular loops [104-106, 108, 214]. Defects in maturation are most frequently seen when the mutation occurs in NBD1 indicating the importance of proper folding in this region of the protein. Like class I mutations, class II mutations are also associated with little or no CFTR expression at the cell surface resulting in loss of all CFTR functional activities and thus are also associated with the more severe forms of CF. Expression of CFTR harboring a class II mutation can be tissue specific however, possibly due to the cell type specific expression levels of the CFTR co-chaperone CHIP.
The most frequent CFTR mutation, a deletion of three base pairs in the gene resulting in a deletion of the amino acid phenylalanine at position 508 in NBD1 of the protein (ΔF508) accounting for ~70% of all carried CFTR mutations belongs to this class. The ΔF508 mutation is present in at least one allele of ~90% of CF patients [342]. The ΔF508 mutation interferes with the protein’s ability to attain its properly folded, protease resistant form in the ER [90, 119]. This inability to attain the proper structure results in prolonged association with ER chaperones, prevents further maturation and trafficking to the membrane and causes rapid degradation [90-92, 95]. The ΔF508 mutation is thought to have only minor effects on NBD1 structure however it reduces the kinetics of NBD1 folding [346, 347]. It is thought that the presence of ΔF508 must prevent further folding or interdomain interactions required for CFTR maturation [119, 348]. Small amounts of ΔF508 are able to escape degradation and show some function in CF epithelia [349, 350]. If ΔF508 is rescued from degradation upon leaving the ER however, it still shows a 5-20 fold increase in degradation in post-ER compartments, most likely due to decreased stability of the protein containing this mutation [351]. If ΔF508 is able to make it all the way to the plasma membrane it exhibits reduced function partly due to accelerated endocytosis and failure to recycle (half life = 20-40 minutes) [352-356].

Class III mutations include missense mutations and amino acid substitutions that result in aberrant regulation of CFTR channel gating. Mutations in this class are also most frequently found in the NBDs. These mutations usually interfere with ATP binding at the NBDs resulting in greatly decreased channel activation. Most of these mutations result in the production of non-functional CFTR, however, because CFTR is produced and is present in the cell membrane some of these mutations retain functional capabilities. Less than 3% of
CFTR mutations carried by CF patients belong to this class, however, the second most frequent CF-causing CFTR mutation, G551D, is a class III mutation. G551D occurs in the Walker A sequence of NBD1, its analogous mutation, G1349D, occurs in the Walker A sequence of NBD2. Both of these mutations inhibit ATP binding at their respective NBD resulting in little or no CFTR function [357].

Class IV mutations also include missense mutations and amino acid substitutions, however, these mutations result in altered conductive properties of the CFTR channel. Mutations in this class are most frequently found in the TMDs. CFTR proteins with class IV mutations exhibit normal PKA and ATP regulation but display decreased Cl⁻ conductance. CFTR containing class IV mutations is produced, is present in the cell membrane and retains some functional properties and therefore is usually associated with a less severe CF phenotype [358]. A455E is an example of a class IV mutation that exhibits partial activity leading to a milder CF phenotype [359].

Class V mutations are mostly splice site or promoter mutations that cause reduced synthesis of CFTR. In cells containing these mutations, some normal splicing is still able to occur so some normal CFTR is still being produced [360, 361]. Class V mutations can also cause small defects in CFTR maturation resulting in reduced synthesis or decreased stability of the CFTR channel in the membrane, however, CFTR channels with these mutations exhibit normal or even increased Cl⁻ transport once in the membrane [362]. In the case of class V mutations, only the organs that require a large amount of CFTR are usually affected [362, 363]. Individuals harboring class V mutations usually present with the following CF associated manifestations: congenital bilateral absence of the vas deferens, obstructive
azoospermia, disseminated bronchiectasis, allergic bronchopulmonary aspergillosis, hypertrypsinemia or chronic pancreatitis [364-367].

Class VI mutations include missense mutations and amino acid substitutions similar to class III and IV mutations. Mutations in this class, however, do not affect CFTR gating or ion permeability, instead they prohibit proper recycling of the CFTR molecule leading to decreased stability in the plasma membrane and increased turnover [134]. Class VI mutations often occur in the C terminus of CFTR. The net result of class VI mutations is lower levels of functional CFTR in the membrane and thus reduced CFTR activity.

As mentioned previously, a seventh class of mutations (class VII) has also been suggested. Class VII mutations do not affect the ability of CFTR to transport Cl⁻ but instead affect the ability of CFTR to interact with additional proteins and to fulfill its function as a conductance regulator [330]. Mutations in the C terminus of CFTR, where many of these interactions occur, often result in more mild forms of CF indicating that these interactions are not required for the most essential functions of CFTR.

**CF Disease Manifestations**

The basic defect in CF is one of ion transport, defective cAMP mediated Cl⁻ transport is found in every tissue affected by CF. However, this defect leads to the accumulation of viscous solutions in the ducts of exocrine glands which is the main disease causing modality in CF. The decrease in Cl⁻ secretion, due to the absence of CFTR, results in an imbalance of ion concentrations across the epithelial cell plasma membrane. This imbalance of ions causes an alteration in the flow of water across the membrane, dehydrating the fluids that
bathe these epithelial cells. These fluids become more viscous eventually clogging the ducts. The exceptions to this rule are ducts which contain only a minor amount of mucus, such as the sweat glands, and therefore show little of the pathology associated with the other organs affected by CF. Symptoms caused by the increase in mucus viscosity appear throughout the life of a CF patient and the timing and severity of the symptoms can vary from patient to patient.

**Airways**

Pulmonary disease is the leading cause of morbidity and mortality in the CF population; >80% of CF patients die of pulmonary failure [31]. The lungs of CF patients are structurally normal at birth however a cycle of dehydrated, thickened mucus in the airways, chronic infection and inflammation eventually results in irreversible lung damage and respiratory failure. CFTR is expressed by a variety of cells in the respiratory tract and is believed to play important and diverse roles in each cell type in order to maintain normal lung physiology. It is the lack of CFTR in each of these cell types that contributes to the development of CF pulmonary disease. CFTR expression has been detected in alveoli however the distal lung is mostly spared from the pathologic changes that occur with CF lung disease. CF lung disease is one of progressive lung function decline with episodes of disease exacerbation. Symptoms of CF airway disease include: chronic cough, airway obstruction, repeated bacterial infections, recurrent sinusitis and nasal polyps, often requiring surgery [368]. Exacerbations are characterized by chest pain, fever, cough, fatigue, and changes in sputum, eating habits or pulmonary function. Exacerbations are brought about by infection with a new pathogen or an increase in the bacterial burden or invasiveness of persistent infections. The forced expiratory volume in 1 second (FEV₁) is the most reliable and most
frequently used pulmonary function test to access pulmonary status of a CF patient [369, 370]. After most exacerbations, CF patients return to their former pulmonary function values, however, this is not always the case. The cumulative effects of multiple exacerbations contribute to pulmonary function decline.

CFTR is expressed at very low levels in the ciliated cells of the respiratory tract [371-373]. However, it is the lack of CFTR in these cells that causes one of the most serious problems in the CF lung, the presence of dehydrated, viscous airway surface liquid (ASL). The ASL is a 7-30 µm thick, isotonic fluid layer that covers the respiratory epithelium [374]. It is composed of two layers: the periciliary layer (PCL) and the mucus layer. The top mucus layer is a tangled, thickened gel of variable thickness composed of high molecular weight mucins that traps inhaled particles. The bottom PCL is a low viscosity, aqueous gel that provides that right environment for cilia to beat in a coordinated fashion and is generally the height of fully extended cilia, ~7 µm [375, 376]. Beating of the cilia in the PCL moves the mucus layer from the distal lung to the mouth allowing the removal of foreign material via expectoration or swallowing, a process termed mucociliary clearance (MCC) [377]. MCC is considered one of the primary defense mechanisms of the airway because it removes pathogens caught in the mucus layer without triggering an immune response. The hydration status of the PCL and mucus layer is paramount for effective ciliary movement and MCC [378]. ENaCs, which are also expressed in ciliated airway epithelial cells, work cooperatively with CFTR to maintain the intracellular/extracellular ion balance and thus the hydration of the ASL [379]. ENaCs actively absorb Na⁺ which coincides with the removal of water from the extracellular environment. CFTR allows the flow of Cl⁻ to maintain the intracellular/extracellular ion balance but as the ASL approaches its physiologic level, CFTR
begins to inhibit ENaC function therefore allowing more Cl\(^-\) and water secretion to maintain the proper level of ASL hydration and isotonicity [380-383]. Molecules in the ASL, particularly adenosine and ATP, partially regulate the activities of CFTR and ENaC and maintenance of ASL hydration depends on proper function of both ENaC and CFTR channels [241, 384].

CF airways exhibit reduced Cl\(^-\) secretion, increased Na\(^+\) absorption and dehydrated ASL [19, 242, 385-387]. This can be measured as a more electronegative nasal potential difference though the ion composition of the ASL is not altered [388]. Absence of functional CFTR in the ciliated epithelium results in the unrestrained activity of ENaCs and thus increased Na\(^+\) and water absorption, as it follows its osmolar gradient. The inability to secrete Cl\(^-\) and water once the ASL begins to be depleted leads to dehydration of the ASL [389, 390]. This continued absorption of water from the ASL has two immediate consequences: first, the PCL is collapsed and cilia are unable to beat, second, the mucus layer is dehydrated becoming thick and viscous. The outcome of these events is that MCC essentially ceases resulting in mucus stasis and airway obstruction [391]. Loss of the PCL also allows the mucins in the mucus layer to come in contact with the surface of the epithelial cells facilitating mucus adhesion and eventually mucus plaque formation. Mucus plugs and the inability to clear mucus is seen early in CF infants [392, 393]. The inability to clear pathogens due to ineffective MCC and the formation of mucus plaques that provide unique niches for bacterial growth result in the colonization of CF airways with opportunistic bacteria [394, 395].

In addition to ciliated cells, the expression of CFTR in submucosal glands may also play a role in the development of CF lung disease. CFTR is highly expressed in secretory serous
cells of submucosal glands [57, 396]. In these cells CFTR is believed to play a major role in the processing of secreted mucins. As mentioned previously, the presence of functional CFTR in the Golgi is thought to aid in acidification of this compartment and thus be required for proper proteoglycan processing. In the absence of CFTR this compartment is more alkaline favoring the activity of fucotransferases and sulfatransferases instead of sialyltransferases. This leads to an alteration in the ratio of mucins secreted including an increase in asialoproteoglycans. Bacteria that infect the CF airway have been shown to preferentially bind to asialoproteoglycans [312, 397-400]. Alteration of mucin composition may further contribute to mucus viscosity although other studies have not confirmed this assertion [401, 402]. If true, CFTR may also function in goblet cells [396]. In addition to mucins, submucosal glands are also responsible for secreting protease inhibitors and antimicrobial peptides and enzymes [403]. CFTR also stimulates submucosal gland secretion and decreased secretion is observed from glands of CF patients [404, 405]. One of the earliest changes to occur in the airways of a CF infant is hypertrophy of submucosal glands due to impaction with thick viscous secretions that they are unable to flush out [406-408]. Decreased submucosal gland secretion results in reduced concentrations of antimicrobials in the lumen of the airways leading to a further diminished ability to defend against bacterial pathogens [409, 410]. If CFTR facilitates exocytosis, this may also result in the inability of submucosal glands to secrete antimicrobial factors into the airway lumen. The composition and dehydration of CF mucus may also lead to reduced activity of the antimicrobials that do get secreted and an inability of immune cells to migrate properly [411]. Indeed, CF airway secretions exhibit reduced bactericidal activity [412]. Later in the progression of CF lung disease, submucosal glands undergo hyperplasia resulting in an increase in the secretion of mucus into the airways [393]. Submucosal glands can also
rupture causing airway wall damage. Inflammation due to the presence of bacteria also causes increased mucus secretion leading to enhanced mucus plaque formation and further obstruction of the airways. Reduced CFTR mediated $\text{HCO}_3^-$ secretion may also lead to aggregation of mucins [413].

Infection of the CF airway occurs early in infancy and continues throughout the life of a CF patient. CF patients do not have a higher risk of infection of other organs. CF patients are extremely susceptible to a very specific set of bacterial organisms for unknown reasons [414]. Initial infections are eradicated due to host response or antibiotic intervention; however, eventually the lungs of CF patients become chronically colonized with bacteria that are unable to be cleared by the host or any outside intervention. CF patients are most often initially infected with *Staphylococcus aureus* (SA) and *Haemophilus influenzae* [415]. However these recurrent infections can be cleared. Most CF patients (~98%) are infected with PA by the age of 3. Initial recurrent *Pseudomonas aeruginosa* (PA) infections can also be cleared. However, PA eventually develops a mucoid phenotype and assembles into biofilms that are difficult if not impossible to eradicate [416-418]. PA can develop antimicrobial and antibiotic resistance rapidly [419, 420]. Most CF patients (80%) are persistently colonized by PA by their mid teens [421]. By adulthood, >90% of CF patients are chronically colonized with PA, of which 70-80% is mucoid. Colonization correlates with a reduction in lung function and a reduced rate of survival; conversion to mucoid PS correlates with a further decline [422-427]. Colonization with PA occurs easily due to the reduced MCC and enhanced binding of this bacterium to asialylated proteoglycans. The unique growth environment afforded by mucus plaques in the airways of CF patients enhances its transition from a rough phenotype to a biofilm-forming, alginate producing,
mucoid phenotype [395, 428]. Mucoid PA is protected from immune factors and antibiotics and frequently causes increase inflammation [429]. PA is usually found in the mucus layer of the CF lung indicating that the altered composition of secreted mucins in the mucus layer that has the biggest impact on PA colonization [430]. PA has been shown to bind to CF epithelium more efficiently than to non-CF airway epithelium so the expression of altered cell surface glycoproteins could play a role in initial infection [431]. Areas of wounded tissue also express asialylated proteoglycans and PA has been shown to bind to cells at sites of injury [432, 433]. Some studies have found that CFTR can bind PA resulting in its internalization and eventual elimination from the airway. Therefore in the absence of CFTR PA could not be efficiently cleared from the airways by this mechanism [434, 435]. Mutant CFTR present at the cell membrane may be able to bind but not internalize PA. PA secreted virulence factors (elastases, proteases, toxins) directly damage lung tissue, interfere with the host immune response and stimulate additional mucus secretion which leads to additional bacterial growth and tissue damage [436-438]. The secretion of these virulence factors increases during exacerbations [439, 440].

Although PA may be the most important bacteria colonizing the CF airways, other pathogens also play a role in the deterioration of the CF lung. Burkholderia cepacia can have a major impact on pulmonary disease in CF patients [441, 442]. Occasionally B. cepacia infection, most commonly genovar III, can lead to a bacteremia with rapid pulmonary function decline and a high probability of death also known as “cepacia syndrome” [443, 444]. Some CF patients (10-20%) are also carriers of methicilin resistant SA (MRSA) which is associated with poorer lung function in adults, however, infection is usually brief [445, 446]. CF patients can also carry atypical mycobacterium, particularly M. avium and M.
abscessus, but the clinical impact of these infections is uncertain [447, 448]. Additional bacteria can also be present (*Alcaligenes xylosoridans, Stenotrophomonas maltophilia*) but do not seem to affect lung function [421, 449]. Many CF patients carry *Aspergillus fumigatus*, some (up to 15%) can develop a severe allergic reaction to it, allergic bronchopulmonary aspergillosis, resulting in compromised lung function but the risk varies geographically [450]. Invasive aspergillosis is rare.

It is not known for certain whether persistent infection causes increased inflammation in CF patients or if they have dysfunctional immune responses prior to infection. It is thought that CF patients do not have a systemic immune defect although slight increases in inflammation are seen in organs other than the lung. The lungs of CF infants are free from inflammation at birth and some infants who have never been infected are also free from inflammation [393, 451]. However, some studies have indicated that CF patients exhibit enhanced inflammatory responses early in life, in the absence of infection, possibly due to the absence of CFTR or to the altered state of the airway epithelium [452, 453]. The more accepted view, however, is that an exaggerated immune response occurs early in life after an initial insult and this immune response persists well beyond eradication of the infection [454-456]. This would allow inflammation to be seen in the absence of an infection. It is clear that the inflammatory response in the CF lung is exaggerated in response to infection [457-459]. CF patients exhibit imbalances in cytokine and antioxidant production both of which probably play a role in the hyper-inflammatory environment of the CF lung.

It is well accepted that there is an imbalance in the secretion of pro and anti-inflammatory cytokines in the airways of CF patients resulting in a heightened influx of inflammatory
cells. There is an increase in the release of interleukin-8 (IL-8), IL-6, IL-1β, TNFα, leukotriene B4 and arachadonic acid metabolites and a decrease in the release of IL-10 [460-465]. This imbalance, and therefore inflammation, can become even worse during chronic infection and disease exacerbations. IL-8 is a potent neutrophil attractant produced mostly by epithelial cell. Neutrophils also secrete IL-8 so they can self-perpetuate their influx [466]. An abnormal stimulation of NFκB in the CF airways has been suggested to be the cause of this altered cytokine secretion [467-469]. IL-10 is a potent anti-inflammatory factor that plays a role in the inhibition of NFκB thus in its absence there is reduced inhibition of NFκB activation leading to excessive production of inflammatory cytokines [470-472]. Additional factors including intracellular Ca²⁺ concentration and hypoxia may also play a role in mediating the observed increase in NFκB activity and possibly IL-8 secretion as well [473, 474]. Alterations in the composition of the ASL alone may constitute an irritation that stimulates NFκB signaling. CFTR may also indirectly inhibit NFκB signaling suggesting that its absence may also play a more direct role in increased cytokine production [475]. The increased secretion of IL-8, and the large quantity of bacterial products present, causes a dramatic influx of neutrophils in to the airways of CF patients. Even patients with mild lung disease display increased IL-8 and neutrophils in their airway [476]. The concept that the absence of CFTR is somehow involved in the exaggerated immune response seen in CF patients is also supported by some in vitro studies. The overproduction of IL-8 and a submucosal accumulation of neutrophils are seen in cultured CF cells in the absence of stimulus [453, 477]. The inhibition of CFTR function alone can also cause increased NFκB activation and IL-8 secretion [478].
As mentioned previously, there is also an antioxidant imbalance in the CF airway. Infiltrating neutrophils secrete reactive oxygen species (ROS) in addition to other products; the amount of neutrophils present leads to the production of ROS that overwhelms the antioxidant defenses of the CF airway [479, 480]. In addition, CF airways are thought to have reduced antioxidant properties due to impaired absorption of antioxidants in the gut and decreased secretion of antioxidants in the airway, possibly directly due to the lack of CFTR [481-483]. There is a lack of nitric oxide in the lungs of CF patients [484-486]. In addition to being a potent antimicrobial, and therefore playing a role in the reduced clearance of pathogens from the CF lung, nitric oxide also functions as an antioxidant suggesting yet another cause for the observed antioxidant imbalance [487]. This imbalance can lead to tissue damage caused by ROS [488]. It has also been suggested that this ROS-antioxidant imbalance may play a role in activating NFκB [489].

The abundance of neutrophils in the airways of CF patients is thought to directly correlate with the amount of lung damage observed. In addition to the increased influx of neutrophils, the altered cytokine production can also cause neutrophils to live longer. Combined with the observation that neutrophils far outnumber the macrophages responsible for their clearance, this indicates that the increase in abundance of neutrophils in the CF lung is due to increased influx, decreased clearance and increased longevity [490]. In addition to toxic factors secreted by the bacteria, neutrophils secrete ROS and elastase which contribute highly to tissue damage. ASL dehydration concentrates these toxic substances at the epithelial cell surface. Neutrophil proteases directly digest lung tissue and easily overwhelm local protease inhibitors which can also be inactivated by neutrophil and bacterial products [491, 492]. Neutrophil elastase can stimulate cytokine and mucus secretion [466, 493].
Neutrophil elastase can also cause the breakdown of mucins which may further expose the airway epithelium to bacterial and neutrophilic secretions leading to increased inflammation and tissue damage. There is also a decrease in the amount of neutrophil apoptosis and an increase in the amount of necrosis [494]. Necrosis allows further release of neutrophil proteases and elastase that can cause further mucus thickening and tissue damage. Necrosis also releases long strands of high molecular weight DNA that can increase mucus viscosity [495]. Increased mucus viscosity eventually leads to more obstruction and thus more places for bacteria to colonize. The cycle of airway obstruction, chronic infection and exaggerated inflammation becomes self-perpetuating and eventually leads to permanent lung tissue damage. CF airways exhibit dilated bronchi filled with mucopurulent debris, loss of cilia, squamous cell metaplasia, delayed differentiation and airway remodeling [393, 496, 497]. Damage to epithelial tissue eventually exposes the submucosa, airway wall and supporting structures to toxic neutrophil and bacterial factors leading to irreversible tissue injury in the form of bronchiectasis and emphysema ultimately leading to organ failure. Infiltration of blood vessels into damaged tissue can lead to hemoptysis and bronchial cysts can develop predisposing the patient to pneumothorax [498].

Although there is a heightened immune response in the CF lung, this response is unable to clear pathogens from the respiratory tract further supporting the exaggerated, sustained inflammation in the lungs of CF patients [412, 499, 500]. PA actively participates in immune evasion by inactivating IL2, a potent T cell growth stimulator [501]. PA can also disrupt the immune response by cleaving cell surface markers from neutrophils that are required for their effective killing capacity. Neutrophil elastase can also interfere with the opsonophagocytosis of pathogens [502-504] Inefficient neutrophil activity can lead to more
recruitment and thus increased mucus viscosity and tissue damage. Antibodies to mucoid PA are also not effective opsonins resulting in the production of immune complexes that can further damage tissue [505]. Alveolar macrophages have been suggested to have impaired ability to digest phagocytosed material due to the lack of CFTR in their phagosome vacuoles [506].

CFTR could be considered the coordinator of water distribution and mucin secretion in the airways due to its function as a Cl\(^-\) channel and a regulator of ENaC function. The inability to secrete Cl\(^-\) alone has also been shown to cause ASL depletion, however, the hyperactivity of ENaCs is what plays a key role in mediating CF lung disease [507, 508]. This is clearly displayed in the mouse model of ENaC overexpression. The ENaC overexpressing mouse has normal lungs at birth, however, it develops spontaneous lung disease, similar to that in CF, early in life [509]. This mouse exhibits early increased Na\(^+\) absorption, ASL depletion, and reduced MCC leading to mucus obstruction of the airways and a reduced ability to clear inhaled pathogens. This mouse also displays goblet cell metaplasia, chronic neutrophilic inflammation and lung tissue damage resulting in death due to pulmonary failure. Epithelial cells in this mouse undergo swelling and necrosis which could result in stimulation of an immune response in the absence of infection.

Damage to the CF lung is heterogeneous. The upper zone of the lung is the most effected with highly reduced MCC and multiple areas of bronchiectasis. The middle zone has diminished MCC and fewer areas of tissue damage. The lower zone had some areas of decreased MCC and bronchiectasis but is largely spared. CaCC may provide some compensatory Cl\(^-\) and water, secretion in these areas, however, they are not as efficient at
secreting Cl- as CFTR and are much more vulnerable to insults [510]. CaCC are activated by an increase in extracellular ATP which can inhibit ENaCs as well [511]. Dehydration of the ASL provides the means by which the ATP concentration is increased enough to activate CaCC. It has been suggested that viral infections of these more normal areas of the lower lung is a major cause of exacerbations of lung disease in CF patients [512]. CF patients are not more susceptible to respiratory viral infections (rhinovirus, influenza, adenovirus, respiratory syncytial virus, parainfluenza viruses) however viruses can cause more severe lower respiratory tract disease in these patients resulting in increased inflammation and decreased lung function [513-518]. Viral infections can cause further reductions in the ASL covering the more healthy portions of lung by reducing the amount of extracellular ATP and therefore interfering in compensatory ion regulation [510]. Viral infections may also damage the respiratory epithelium exposing the submucosa to bacterial and inflammatory factors. Viral infections correlate with disease exacerbations and are thought to predispose and maybe even facilitate, bacterial infection of the airway [519-521]. It has been suggested that viral infections may even precede the development of lung disease in CF infants [512, 522]. Progression of pulmonary disease has been associated with frequent viral infections.

The severity of pulmonary disease in CF patients is closely related to their nutritional status [523]. Dealing with severe pulmonary disease requires more energy expenditure which makes it easier to become malnourished especially when getting the proper nutrition is already an ongoing issue for CF patients [524]. Malnutrition then leads to more severe pulmonary disease [525]. However, proper nutrition is associated with less severe pulmonary disease, reduced frequency of exacerbations and a slower decline of pulmonary function [526, 527].
Microarray analysis of gene transcription in the lungs of CF mice displayed a number of alterations in the absence of pathology. Twenty nine genes exhibited enhanced transcription while twenty five exhibited reduced transcription. Many of the alterations occurred to factors involved in the expression, processing and function of CFTR. Other alterations occurred in factors involved in protein trafficking and degradation, signal transduction, ion transport and inflammation. It was speculated that these alterations were trying to compensate for the absence of CFTR [528]

**Pancreas**

Next to the airways, the pancreas is the most affected organ in CF disease. CF patients can exhibit pancreatic sufficiency (PS), however, the vast majority (70-90%) are born with or develop pancreatic insufficiency (PI) during their first year of life [529]. Infants experiencing PI often present with failure to thrive, abdominal distension, chronic diarrhea or cholestasis. These patients display ductal obstruction with ductal and acinar atrophy and lobular fibrosis with fat infiltration and scarring [530]. Although the pancreas of CF patients is not prone to infection there is mild leukocyte infiltration [531]. PI occurs when the pancreatic ducts become obstructed, with viscous pancreatic secretions, which inhibits the delivery of pancreatic enzymes to the intestines. As in the airways, CFTR, expressed apically in pancreatic duct cells, plays a main role in determining the hydration status of pancreatic secretions and in its absence these secretions become thick and viscid leading to pancreatic duct obstruction [56]. Pancreatic duct obstruction results in an inability to transport pancreatic enzymes to the intestines of CF patients leading to malnutrition, lipid soluble vitamin deficiencies, inability to absorb essential fatty acids and steatorrhea. The ability of
pancreatic acinar cells to secrete enzymes does not seem to be compromised. Similar to the coordinated activity of CFTR and ENaC in the airways, the role of CFTR in activating HCO$_3^-$ secretion is important to the development of PI in CF patients. HCO$_3^-$ plays a major role in the alkalinization of pancreatic secretions which is necessary for the transport of pancreatic enzymes to the intestines. In the absence of CFTR, there is an inadequate amount of extracellular HCO$_3^-$ in the pancreatic ducts to effectively buffer the pancreatic secretions resulting in the precipitation and premature activation of the pancreatic enzymes [532, 533]. Obstruction of the pancreatic ducts causes these activated pancreatic enzymes to be retained in the intrapancreatic ducts which then undergo digestion. Once the enzymes have escaped the ducts they continue to destroy pancreatic tissue. Once a certain proportion of pancreatic islet cells have been destroyed, patients develop insulin insufficient diabetes. Because insulin resistance can also be present this form of diabetes is termed CF-related diabetes (CFRD) [534]. The use of corticosteroids increases the risk of developing CFRD. The prevalence of CFRD among CF patients increases with age and is present in up to 30% of patients over 25 years of age [535, 536]. The presence of CFRD is associated with poorer nutritional status, more severe pulmonary disease and poorer survival [537, 538]. Even patients with PS exhibit reduced alkalinization of their pancreatic secretions and reduced levels of digestive enzymes in their intestines [533]. These patients are also at risk of developing recurrent or chronic pancreatitis [539].

CF mutations are classified as mild or severe based on whether they result in PS or PI. Class I-III mutations result in very little or no CFTR function and are therefore associated with PI and thus considered severe mutations. Class IV-VII mutations result in some CFTR function, are associated with PS and therefore considered mild mutations [540]. Patients carrying
one severe and one mild mutation usually have PS [541]. Mutations that lead to reduced HCO$_3^-$ secretion are only seen in patients with PI and patients with these severe mutations exhibit reduced pancreatic HCO$_3^-$ secretion regardless of their ability to conduct Cl$^-$. PS is associated with CFTR mutations that do not affect normal HCO$_3^-$ secretion whether or not they affect the ability of CFTR to conduct Cl$^-$. [271]. Mostly due to improved nutrition, CF patients who have PS have a better prognosis. These observations highlight the importance of CFTR’s role in HCO$_3^-$ secretion in the pathogenesis of pancreatic disease in CF.

**Gut**

The intestines of CF patients have difficulty breaking down and absorbing nutrients. This is mostly due to the lack of pancreatic enzymes in the gut, however, the intestinal contents also remain at a lower pH for a longer period of time further affecting digestion and nutrient absorption, partially due to the lack of pancreatic HCO$_3^-$ [542]. Most of the CFTR in the gut is expressed in intestinal crypt cells [58]. As with the rest of the organs affected in CF, the lack of CFTR has additional intestinal complications due to its role in maintaining the hydration of intestinal secretions [543-545]. Like the airway, intestinal mucus glands are plugged and dilated [546]. Mucus also accumulates in the salivary glands [547]. Meconium ileus (MI) and distal intestinal obstruction syndrome (DIOS) are the most common symptoms of CF involving the intestines. MI is an obstruction of the small bowel, due to impacted mucus mixed with undigested fecal material, occurring in 10-15% of CF infants [548]. Though MI used to be a major cause of death in CF patients, currently <10% of patients die from complications due to MI [549]. DIOS, often considered “late onset” MI, occurs in 15-20% of children and adults with CF due to malnutrition, thickened intestinal secretions and decreased intestinal motility [550]. Children with CF are also more prone to rectal prolapse.
CF patients are also at higher risk of developing gastroesophageal reflux and Crohn’s disease [551, 552]. There is not an increased risk of intestinal infections in the CF population however some studies have shown the increased presence of pro-inflammatory cytokines and monocytes in the intestines of CF patients possibly in response to enteric bacteria [553, 554]. CFTR in the gut has been shown to bind *Salmonella typhi*, mutants lacking this ability supporting the hypothesis that heterozygotes may be more able to survive severe intestinal infections [28, 29].

**Liver and Biliary Network**

CF patients can also develop liver and gall bladder pathology. Frank liver disease including multilobular obstructive biliary cirrhosis, portal fibrosis and portal hypertension occurs in only 1-2% of patients however it is the 2nd leading cause of death in the CF population [31]. More mild and more common liver disease includes hepatic steatosis and focal obstructive biliary cirrhosis [555]. Although few CF patients will develop liver cirrhosis, up to 30% have abnormal liver function mostly due to fat infiltration of the liver tissue. The peak age for presentation of liver disease is 16-20 years and it is more frequent in males [556]. Similar to the pancreas, lack of CFTR in biliary ducts leads to dehydration of bile fluid causing ducts to become plugged with secretions containing bile acids. The bile acids stuck in these ducts begin to destroy the ductal tissue and if the obstruction backs the secretions all the way up to the intrahepatic ducts, direct liver destruction ensues. This also results in reduced delivery of bile to the gut. Small gall bladders with reduced function are present in ~30% of CF patients [557]. Recurrent gall stones secondary to a reduction in bile acids occur in up to 20% of patients [558].
Genital Tract

The absence of CFTR in the genital tract also affects the viscosity of the mucus lining the epithelium of this organ. However, this has very different effects on men and women with CF. Women display dehydrated cervical mucus and occasionally mucus plugging of the cervical canal [559]. This can impair fertility, probably due to impaired sperm motility, however, the majority of women with CF are able to become pregnant and provided they have adequate nutrition and pulmonary function are able to carry a pregnancy full term [560, 561]. On the other hand, >95% of men with CF are infertile due to azoospermia secondary to the bilateral absence of the vas deferens (BAVD) or dilated/absent seminal vesicles [562]. The vas deferens is typically present in the fetus but likely becomes obstructed due to the thickened mucus present in the ducts and atrophies shortly after birth [563, 564]. Men with CF can also exhibit reduced fertility due to unilateral absence of the vas deferens, idiopathic epididymal obstruction or asthenospermia [358].

Sweat Glands

Unlike the previously mentioned organ systems, sweat glands do not undergo many pathologic changes in an individual with CF mainly due to its lack of mucus [565]. However, due to its dependence on CFTR activity for proper functionality, the sweat gland does provide one of the most diagnostic features of CF: the excretion of excessively salty sweat. Isotonic fluid is secreted by the sweat gland and is modified as it passes through the duct. CFTR is expressed in the epithelium of the sweat gland as well as the sweat ducts. It is the presence of CFTR in the sweat ducts that is required for the reclamation of Na+ and Cl− from the sweat as it passes through the duct. Unlike the situation in the respiratory tract, ENaC function in the sweat duct is dependent on the presence of CFTR thus in its absence both
Na\(^+\) and Cl\(^-\) fail to be reabsorbed from the sweat leading to elevated levels of salt (3-5 times normal levels) in the sweat excreted from CF patients [262, 566, 567]. Sweat volumes are normal in CF patients. As mentioned previously, a sweat Cl\(^-\) level of >60 mmol/L is diagnostic for CF but patients carrying the ΔF508 mutation frequently have levels exceeding 100 mmol/L. A minority of individuals with CF, carrying specific mutations, actually have normal concentrations of Cl\(^-\) in their sweat thus leading to delayed diagnosis [568]. Due to salt loss, CF patients are at greater risk of developing dehydration and heat shock. In addition to increased sweat Cl\(^-\), CF patients do not sweat in response to β adrenergic stimulation [569]. Heterozygotes for a CFTR mutation actually display reduced sweating in response to β adrenergic stimulation; this is one of the only ways to determine heterozygocity other than genotyping [570].

**Additional Complications in CF**

In addition to the organs which undergo the most substantial changes due to CF, other organs are also affected. Only mild, if any, renal abnormalities have been identified in CF patients including decreased ability to excrete salt, defects in the concentration/dilution of urine, altered filtration rate, increased proximal Na\(^+\) absorption and altered drug excretion [571-573]. Alterations in fatty acid metabolism have also been identified. Specifically, CF tissues exhibit increased phospholipid bound arachadonic acid and decreased phospholipid bound docosahexaenoic acid [574]. Also CF patients are at greater risk of osteoporosis secondary to poor nutrition, vitamin D deficiency, hypogonadism, chronic inflammation and intermittent corticosteroid use [575, 576].
Individuals with atypical CFTR mutations or those with a single mutated allele have been shown to be at risk for specific mono-organ disease related to the manifestations of CF. These individuals are said to have a CF-related syndrome [577]. Men can develop congenital BAVD (CBAVD) in the absence of other symptoms of CF indicating that CFTR function is extremely important to vas deferens function. Among men who exhibit CBAVD, 50-82% have a mutation in one of their CFTR alleles and ~10% have a mutation in both alleles [578]. Men with CBAVD or other CF-related syndromes are thought to have 10-50% CFTR function indicating that ~10% of CFTR function is enough to avoid the development of CF [579]. There is also an increase in the frequency of CFTR mutations among men with alternate forms of azoospermia [580]. There is also an increase in the CFTR mutation rate in individuals who develop recurrent or chronic idiopathic pancreatitis, chronic sinusitis or disseminated bronchiectasis and primary sclerosing cholangitis [365, 581-585].

Modifiers of CF Disease

The relationship between genotype and CF disease manifestation is complex. Most CF patients (85-90%) experience severe disease associated with a complete loss of CFTR function resulting in PI, increased sweat Cl⁻, pulmonary disease and BAVD. Most of these patients express class I-III mutations resulting in ≤1% of CFTR function [586]. The rest of the CF patients exhibit only mild or atypical disease with lower sweat Cl⁻ levels, PS and frequently, less lung disease probably due to the presence of residual (~5%) CFTR activity [541, 587]. Most of these patients express class VI and V mutations. Pancreatic involvement is strongly correlated with genotype therefore, CFTR mutations are classified as mild or severe based on whether they result in PS or PI [588, 589]. On the other hand, the severity of lung disease can vary highly, even among individuals who are homozygous for the same
CF mutation [540]. There is greater similarity in lung disease among siblings and even more among monozygotic twins compared to dizygotic twins [590]. It is thought, therefore, that CF phenotype, especially when it comes to lung disease progression and severity, can be influenced by environment and additional genetic factors. The contribution of modifying factors depends on the CF mutation and the organ system. The progression of CF lung disease involves multiple environmental and physiologic factors resulting in a greater potential for modulation. Pancreatic disease and sweat Cl⁻ are much more dependent on the basic CFTR defect and therefore are less likely to be modified by additional factors though modulation can occur [362, 541, 591, 592]. More rare CF complications including MI, DIOS and liver disease always occur in CF patients with PI but due to their infrequent occurrence are believed to be heavily influenced by additional environmental and genetic factors [541, 593-597]. The survival rate of CF patients is also highly variable though there is increased survival among those who are PS [586]. It is widely agreed that several modifiers probably contribute to the clinical course of CF caused by a given mutation. The identification of factors that modify CF disease will not only improve the understanding of disease pathogenesis, it will also aid in prognosis, improve the therapeutic effect of current treatments and aid in the development of future CF treatments.

Multiple environmental factors are thought to play a role in the development and severity of CF disease especially in the lung. The exposure of a CF patient to high levels of environmental pollution as well as household smoke are thought to result in a quicker decline in lung function [598, 599]. The timing of a patient's first airway infection is also thought to play a role in lung function decline. Nutritional status probably plays a role in all aspects of CF disease and can be impacted by both environmental and genetic factors [600,
Socioeconomic status has also been shown to play a role in the course of CF [602, 603]. A delay in diagnosis, the type of therapeutic interventions available and the patient’s adherence to the prescribed treatments probably all influence the development of CF related complications.

The best studied genetic modifiers of CF are polymorphisms in genes resulting in the modified expression of factors involved in inflammation and host defense. Polymorphisms in alpha1 antitrypsin resulting in deficiency have been suggested to correlate both with CF disease progression as well as milder forms of CF lung disease [604-607]. Polymorphisms in alpha1 antitrypsin resulting in increased expression have been associated with a better CF lung disease prognosis [608]. Still other studies have suggested there is no relationship between alpha1 antitrypsin expression and the severity of CF at all [609]. Contradictory results also exist with regards to a relationship between increased tumor necrosis factor alpha (TNFα) and transforming growth factor beta (TGFβ) expression and decreased lung function [610]. However, more recent studies indicate there is an association between TGFβ polymorphisms leading to increased expression and severe lung disease as well as liver disease in CF [611-613]. Recently a polymorphism in the SERPINA1 gene has also been linked to increased risk of CF liver disease [614]. Polymorphisms leading to decreased production of manose binding lectin correlate with more severe lung disease, an increase in inflammatory markers, liver disease and a survival disadvantage in CF patients [615-620]. Polymorphisms resulting in a decrease in the production of glutathione S transferase M1 and an increase in the production of glutathione S transferase M3 were more frequently present in patients with milder lung disease while altered activity of glutathione S transferase P1 was associated with liver disease [621, 622]. Studies have linked
polymorphisms in nitric oxide synthases leading to a decrease in nitric oxide to an increased risk of PA and *Aspergillus fumigatus* infection while those that cause an increase in nitric oxide production correlate with a reduced rate of pulmonary decline and a decreased risk of infection but only in females [623-625]. Increased risk of certain CF disease manifestations are also ascribed to carriers of certain human leukocyte antigen (HLA) alleles. Carriers of HLA-II DR4 and DR7 as well as those expressing auto antibodies to permeability increasing protein are at increased risk of early colonization with PA [626, 627]. One study showed that the presence of HLA DQ6, DQ7, DR15 or B7 was a risk factor for CF liver disease [628].

Polymorphisms in additional genes, as well as CFTR itself, may also affect the progression of CF. Polymorphisms in genes encoding other ion channels and proteins responsible for cellular repair, airway responsiveness and mucin production have all been linked to the progression of CF disease [629]. Polymorphisms in the β2 adrenergic receptor resulting in decreased expression correlate with reduced lung function and a faster decline of lung function in CF patients [630]. Polymorphisms in the angiotensin converting enzyme gene resulting in increased expression were associated with a quicker rate of pulmonary decline and an increased risk of liver disease [613]. A variant of the hemochromatosis gene is over represented in CF patients experiencing MI and a putative locus on chromosome 19 is also thought to be linked to the development of MI [631, 632]. Over 200 polymorphisms have been identified in CFTR [342]. CFTR polymorphisms do not cause CF but can alter CFTR production and function in a way that influences the CF phenotype of individuals who carry a CFTR mutation. For example, intron 8 of CFTR contains a stretch of thymines that can vary in length between 5, 7 and 9 [633]. Ten percent of the general population has 5 thymines in
this region resulting in organ-specific decreased CFTR production due to inefficient splicing of exon 9 [86, 634, 635]. This alone does not cause CF but if there is a CF causing mutation in the other CFTR allele, a CF phenotype loosely corresponding to that mutation will arise [636]. This variant is present in up to 50% of CBAVD cases [360, 578, 637].

Contradictory results regarding modifier genes may result from differences in the populations chosen for the study and in the study design including population size, clinical parameters and statistical analysis. Certain modifiers of CF may only operate in a certain gender, age group or at a certain disease stage. The effects a modifier has on a given phenotype may also depend on the CFTR mutation present and the treatment regime of the patient.

**Models to study CF**

Both *in vitro* and *in vivo* studies have led to our current understanding of CFTR function and CF pathology. Improving both *in vitro* and *in vivo* models will aid in further analysis of the pathogenesis of CF and developing new therapeutics to treat this disease.

Many types of cells have been used to examine the functions of CFTR, the effects of its absence and methods of correcting CFTR defects *in vitro*. A variety of immortalized cell lines have been used to examine the function of CFTR in a heterologous fashion. These include HeLa cells and Chinese hamster ovary cells due to their lack of CFTR expression. Cells from CF patients have also been transformed in order to study the effects of the absence of CFTR. These include airway cells (bronchial, serosal, nasal, submucosal) and cells of the pancreas. IB3-1, a CF bronchial cell line, and CFPAC-1, a CF pancreatic duct cell line, are two of the
most widely used [638]. Calu-3 cells are non-CF serosal cells widely used as a control for CFTR due to their high level of expression.

It is the culture of primary airway cells however, that has been the most instructive about the function of CFTR and the repercussions of its absence in the airway. These culture systems have also provided a suitable in vitro platform for testing potential CF treatments. Initially, the placement of bronchial and tracheal xenografts into mice with severe combined immune deficiency was the most widely used method of culturing primary CF and normal airway tissue [639, 640]. This culture method was replaced when it was discovered that primary airway cells could be grown and differentiate on porous supports. Airway cells collected from the lungs of CF patients undergoing lung transplantation and from donor lungs are first plated on plastic to obtain the basal cells capable of differentiation. To induce differentiation, these basal cells are placed on porous supports and once they become confluent the media on the apical side of the support is removed. The creation of an air-liquid interface stimulates the basal cells to differentiate creating a well differentiated culture of human airway epithelial cells (HAE) [641-643]. The development of a pseudostratified culture of HAE takes 1-2 months. These HAE cultures contain all of the pulmonary cell types present in the airway, secrete mucus and exhibit MCC. HAE cultures made from respiratory tissue from CF patients displays the characteristic differences between CF and non-CF airways including an imbalance in ion transport, reduced ASL, a dehydrated mucus layer and reduced MCC. Recently, a successful attempt was made to immortalize basal cells while maintaining their ability to differentiate [644]. Such an advance would improve the ease with which HAE cultures are produced. One deficiency of
these primary cultures is that they lack an immune component which is pivotal to the development of CF lung disease.

Though *in vitro* systems can be very useful, in order to observe the defects of the CF lung as a whole organ and draw conclusions about the efficacy of new treatments, *in vivo* models are needed. Multiple attempts have been made to generate an acceptable mouse model of CF. These include CFTR knockout mice and those with ΔF508 and G551D mutations. These mice were made on different backgrounds, with different CFTR mutations and thus have different CF phenotypes caused by residual CFTR function. All of these mice reliably develop intestinal obstruction although to varying degrees. The knockout mice generally develop severe obstruction leading to starvation and therefore do not live long. One CF mouse (CFTRtm1UNC) survives longer if put on a liquid diet [645]. This mouse has been modified to express CFTR in its intestinal tract, generating a model (CFTRtm1UNC-TgN(FAPCFTR)) that survives long enough to develop additional CF symptoms [646]. It was also back crossed to make a congenic mouse (B6-CFTRtm1UNC) [647]. None of the CF mice developed, however, have pancreatic dysfunction and therefore do not exhibit malabsorption. CF mice also do not develop serious liver disease. Only CFTRtm1Eur and CFTRtm1Cam male mice have reduced fertility while male B6-CFTRtm1UNC mice are completely infertile and females display severely reduced fertility.

The major problem with the mouse models of CF is that they do not recapitulate CF lung disease. These mice display abnormal ion transport in their nasal epithelium however only three mouse strains show any abnormalities in their lungs. CFTRtm1HGU and CFTRtm1G551D mice display reduced MCC, altered submucosal glands and abnormal pulmonary
inflammatory responses but their disease is very mild. The B6-CFTR<sup>tm1UNC</sup> mouse is the only one that develops spontaneous, progressive lung disease. Still, none of these mice are overly susceptible to common CF airway pathogens especially PA unless it is repeatedly administered or administered via embedded agar [648]. This vast difference in the development of lung disease could be due to the existence of compensatory Cl<sup>-</sup> channels in the murine airway and a lack of submucosal glands in the airways of the mouse. Mice also have a different distribution of CFTR expressing cells in their respiratory epithelium. It is therefore important to develop alternative animal models that better recapitulate human CF. Rabbit, ferret, pig and sheep models have been suggested due to their similarity to humans in respiratory architecture and CFTR sequence. Homozygous CFTR null and ΔF508 ferret and pig models have been generated and are currently being studied [649-652]. The homozygous null pig model is the furthest along and does display intestinal, pancreatic and pulmonary disease. Hopefully the generation of new, more analogous models will further the development of treatments for CF.

**Current CF Treatments**

The continually improving survival rate of CF patients is primarily due to the development of new therapeutic interventions [653]. Continually improving treatments may continue to do so allowing younger generations to enter adulthood in better health and thus live longer. Current treatments however, only treat the secondary pathologic events that precipitate from the loss of CFTR not the physiologic defect afforded by its absence. Early in the course of CF lung disease, the emphasis is on improving MCC and mucus clearance and limiting bacterial infection. Once chronic infection occurs the emphasis is on reducing bacterial load and exacerbations of disease. During end stage disease the emphasis is on preventing the
more serious outcomes of tissue damage such as severe hemoptysis and pneumothorax. The cost of these treatments can amount to more than $25,000/year but can allow a CF patient to maintain a reasonable quality of life until the end stages of their disease. As a CF patient ages and their disease advances, they require more frequent and more intense treatment. CF therapeutics need not only be effective, they must also be non-toxic and consistently effective with constant use. Therapeutic interventions include enzyme treatments to aid in food digestion, modalities to improve mucus clearance, antibiotics to treat infection and drugs to decrease inflammation.

One of the first treatments available for CF patients was that of pancreatic enzyme replacement therapy (PERT). The development of PERT allowed the survival of CF infants who would have otherwise died of malnutrition [654]. PERT consists of oral enteric coated capsules formulated to replace the pancreatic amylase, lipase and protease absent from the CF gut [655]. PERT helps to break down ingested material aiding in caloric intake and absorption of fat soluble vitamins [656]. CF patients are also required to take calorie dense and fat soluble vitamin supplements as well to ensure that they consume enough to handle the high metabolic demands of their disease. Because CF patients cannot maintain an intestinal pH that is sufficiently alkaline for PERT to function optimally, gastric acid secretion blockers are also used to improve the nutrition of patients since this plays such a large role in determining survival [657].

Since pulmonary deterioration is now the main cause of morbidity and mortality in the CF community, most additional treatments are aimed at improving lung function. Two main therapeutic techniques to accomplish this include mechanical clearance of mucus and
improving MCC. CF patients undergo daily physical therapy aimed at breaking up and clearing mucus from their airways. Airway clearance techniques include percussion, postural drainage and breathing techniques. Devices such as those imparting positive pressure and oscillation to the airways are also helpful [658]. These interventions do assist in the removal of secretions from the respiratory tract however their value is variable due to blockages that develop in the CF lung. Two treatments have therefore evolved to decrease mucus viscosity and thus improve mucus clearance. Inhaled recombinant human DNase is used by most if not all CF patients to improve mucus viscosity by digesting the high molecular weight neutrophilic DNA seen as a major contributing factor. Patients who use inhaled DNase have improved lung function and a reduction in the number of exacerbations [659-662]. More recently, inhaled hypertonic saline has begun to be used to reduce mucus viscosity. Hypertonic saline (3-12%) improves ASL hydration by drawing water into the airway lumen due to the change in ion concentration. The improved ASL hydration results in enhanced MCC, improvements in lung function and reductions in exacerbations [663-665]. The inhalation of powdered mannitol is currently being explored as an alternative to hypertonic saline [666, 667]. It is not thought, however, that these therapies can assist in the removal of the majority of adherent mucus plugs from the airway which may require stronger treatments that can disrupt the hydrophobic interactions that attach them, if they can be removed at all [668]. To this end inhaled N-acetylcysteine which breaks the disulfide bonds that exist between mucins has also been shown to improve MCC and lung function [669]. It is thought that these therapies are most beneficial if started before severe pathologic changes occur in the lung.
Next to improving nutrition and MCC the most important CF therapies involve preventing and eradicating pulmonary infections. CF patients receive intravenous, oral and inhaled antibiotics depending on their disease state. They require greater than normal doses for longer durations in order to counter the altered pharmacokinetics seen in the CF respiratory tract [670, 671]. CF patients most frequently receive antibiotics during exacerbations when they experience infection with a new pathogen or increased burden of a chronic pathogen. At this time patients undergo aggressive treatment with intravenous and oral antibiotics tailored to the sensitivity of the invading pathogen and depending on the severity of the exacerbation [672]. This usually involves more than one type of antibiotic given for 2-4 weeks resulting in a 75% eradication rate [673-675]. SA sometimes avoids eradication and PA becomes more difficult to eradicate as pulmonary disease progresses [676]. Once PA emerges, antibiotic treatments become even more aggressive in order to prevent chronic colonization. This involves 3 months of repeated rounds of treatment with alternating antibiotics which, at least early on, results in eradication [677-679]. These treatments usually involve oral ciprofloxacin and inhaled tobramycin in addition to antibiotics delivered intravenously. Once chronic PA colonization occurs, patients are kept on continuous, rotating antibiotics usually involving inhaled tobramycin [675, 680]. Twenty-eight day on/off cycles of inhaled tobramycin has been shown to reduce the bacterial burden and the number of exacerbations and improve the pulmonary function of those chronically infected with PA [681, 682]. Macrolide antibiotics, specifically azithromycin, have been instrumental in improving the quality of life for CF patients. Azithromycin not only interferes with PA virulence factor production and biofilms formation, it also alters cytokine production and neutrophil function making it a powerful treatment for CF airway disease [683-688]. Azithromycin has been shown to reduce
exacerbations, improve lung function and improve the nutritional status of CF patients [689-692]. The use of prophylactic antibiotics in the treatment of CF is controversial. Prophylactic treatment with cephalexin did reduce the number of exacerbations and decrease SA infection but it also reduced the time to PA acquisition [693-695]. For this reason the use of prophylactic antibiotics is not recommended for CF patients. It is also recommended that CF patients receive vaccines/prophylaxis for common respiratory viruses that are associated with disease exacerbations. While influenza vaccines and respiratory syncytial virus prophylaxis do not prevent virus infection they do reduce the symptoms [696].

Because inflammation plays such a large role in the progression of CF lung disease, anti-inflammatory drugs are also an important treatment modality. The repeated use of oral prednisone results in improved lung function and reduced exacerbations however it also causes serious side effects including glucose intolerance, cataracts and growth retardation. Therefore chronic use of oral steroids is not recommended for CF patients [697-700]. Inhaled steroids were not of any benefit [701-703]. High dose ibuprofen treatment is associated with a reduced rate of pulmonary decline in CF patients and is associated with less serious side effects [704]. Despite the side effects of chronic high dose ibuprofen, their use is recommended for CF patients. The frequent use of antibiotics to clear bacteria also has the secondary beneficial effect of reducing inflammation due to reduced stimulation.

Patients who are experiencing end stage pulmonary disease may be candidates for lung transplantation. The age and disease severity of a patient is very important when considering the option of transplantation. Patients who have an FEV1 of <30% the predicted
value and are *B. cepacia* free may be considered for transplantation [705, 706]. Lung transplantation has the potential to improve the quality of life and the survival of CF patients. Adults who undergo lung transplantation have a 50-70% 5-7 year survival rate however the rate is lower for children [707-709]. Inoculation of the transplanted lung with bacteria from the nasal cavity is a major concern during the immediate post-transplant period. Patients who are experiencing end stage liver disease but have limited pulmonary disease can be considered for liver transplantation [710].

The more infrequent disease manifestations of CF can also be treated. MI and DIOS are usually treated with enemas however, if necessary, surgery can be performed to remove the impaction. If severe, nasal polyps can be removed surgically. Liver disease can be treated with ursodeoxycholic acid [711]. CFRD is treated with insulin but early treatment with sulfonylurea may delay the need for insulin therapy [712].

Patients experience a reduced rate of disease progression when they attend specially designed CF centers [713]. These centers employ stringent protocols to limit their exposure to potentially devastating pathogens. Studies have indicated that centers which require more frequent visits, take more frequent pulmonary function measurements, take more frequent nasal cultures and prescribe antibiotics more frequently have patients with higher pulmonary function scores [714]. It is thought that more frequent testing results in earlier detection of pulmonary infection. Early detection increases the chance of eradication upon treatment.
**Proposed CF Treatments**

Further increases in the survival of CF patients will require the development of improved therapeutics attacking the basic ion transport defect. It is now known whether the structural and functional changes that occur with chronic disease would be reversible even with complete correction of the basic defect. These therapies should therefore be delivered early in life before the development of serious pathologic changes occurs. High throughput screening of small molecules has lead to the identification of many possible therapeutic compounds for CF. Some of these compounds may aid in the expression, maturation or function of mutant CFTR and thus target patients with a specific class of mutations. Other compounds affect ion channels other than CFTR.

Mutations in CFTR result in reduced production, maturation and function of the protein. Therapeutic compounds have been identified that can intervene in each of these steps leading to an increased presence of functional CFTR in the plasma membrane.

Aminoglycoside antibiotics can stimulate read through of the premature stop codons resulting from class I mutations [715-717]. Gentamicin improved CFTR expression and function when applied to the nasal mucosa of some CF patients [718, 719]. The results seemed to be patient and mutation specific. The high concentrations required however caused renal and ototoxicity. PTC124, a gentamicin derivative, also improved NPD in some patients but without the side effects [720, 721]. Multiple compounds have been able to increase the ability of ΔF508 CFTR to escape the ER without being degraded, these compounds have been termed “correctors”. Lowered temperatures (20-30°C), glycerol, trimethylamine N oxide and denatured water can increase expression of ΔF508 in the plasma membrane, however these treatments are not applicable in vivo [722-724]. Sodium
phenylbutyrate, quinazolines, osmolytes and multiple other compounds have also been shown to increase the amount of ΔF508 present in the membrane [725-728]. These compounds work through many different mechanisms, including acting as surrogate chaperones, altering the function of natural CFTR chaperones and increasing the stability of mutant channels at the membrane and may be applicable in vivo. Compounds that improve the function of mutant CFTR once it is in the plasma membrane could be helpful for patients with class III and IV mutations. These compounds are called “potentiators”. Alkylxanthines, sulfonamides, phenylglycines, dihydropyradines, flavonoids, pyrazoles and many other compounds can act as potentiators [727-735]. VX770 is a synthetic potentiator that, when given orally to CF patients, improves their lung function and sweat Cl− levels [736]. Because ΔF508 exhibits reduced function at the membrane, patients with this mutation may require treatment with both a corrector and a potentiator so identifying molecules that can act together or that function as both is important [737, 738]. Treatments that alter the efficiency of splicing have been suggested as therapeutic interventions for patients with class v mutations [739, 740].

It is difficult to determine the level of CFTR correction needed to avoid developing CF. It is thought that increasing the expression of functional CFTR to 5% of normal could drastically reduce disease severity because this is the amount present in many CF patients with PS [86, 635, 741-743]. This amount will not however, eliminate the symptoms of CF so any therapeutic for class I mutations should aim to induce the production of >5% of normal CFTR. When considering correction of ΔF508 CFTR, it is not only necessary to improve its maturation but also its function. It is thought that CFTR is expressed in 60-70% of airway epithelial cells. ΔF508 CFTR is only present at the membrane of 10-20% of epithelial cells of
homozygotes and ~40% of the epithelial cells in heterozygotes [350]. It is uncertain what degree of function is retained by ΔF508, studies have indicated that it is anywhere from 5% to 60% of non-mutant CFTR [355, 356, 744]. Therefore either the function of ΔF508 CFTR must be improved or it must be expressed in >60% of airway epithelial cells to avoid the symptoms of CF lung disease.

An alternate therapeutic strategy is to activate alternate Cl\textsuperscript- channels or inhibit Na\textsuperscript+ absorption. As mentioned previously, extracellular ATP and UTP can interact with purinergic receptors on the surface of airway epithelial cells and increase the concentration of intracellular Ca\textsuperscript{2+}. This not only activates CaCC, it also inhibits Na\textsuperscript+ absorption leading to reduced mucus viscosity, however, it has a relatively short period of activity [745-750]. Newly discovered/designed analogs provide more long lasting benefit [751, 752]. Denufosol is a UTP analog that can reduce ENaC function, increase MCC and improve lung function in patients with mild CF [753, 754]. Lancovutide and duramycin can also increase intracellular Ca\textsuperscript{2+}, stimulate Cl\textsuperscript- secretion and improve lung function [755, 756]. Activating CaCC could also facilitate HCO\textsubscript{3}\textsuperscript- secretion in pancreatic duct cells [757, 758]. Although inhibiting ENaC function with amiloride may improve MCC in the short term, it does not yield robust improvements in lung function mostly due to its short half life in the body [759-761]. Studies in the ENaC overexpressing mouse suggest that amiloride may be an effective preventative therapy if given before the development of pathologic changes in the lung [762]. Longer acting, more potent amiloride analogs are being developed [763-765].

Additional proposed therapies target infection and inflammation. Inhaled dextran, intravenous immunoglobulin and vaccination have all been suggested to delay and aid in
the clearance of PA [490, 766, 767]. Blocking PA quorum sensing has also been suggested to inhibit the transition of PA to its mucoid phenotype [768, 769]. Inhaled α1 antitrypsin has been shown to reduce the production of cytokines and neutrophil factors as well as the burden of PA but was not associated with improved lung function [770, 771]. Protease inhibitors can decrease cytokine production, restore some neutrophil function and reduce the burden of PA but these compounds have a short half life in the body and therefore need additional modification [772, 773]. Compounds that block specific cytokines or neutrophil factors are currently under investigation as is recombinant IL-10 [558]. The benefit of these compounds is unknown but some of them may have multiple or additive effects [774]. CFTR protein replacement therapy, where purified CFTR protein is delivered to the airways via liposomes, has been inefficient [775, 776].

**Gene Therapy**

Although improved treatments have lead to the increased survival of CF patients, this disease remains fatal. The ultimate therapy for CF patients is to restore functional CFTR to their diseased cells. This could be accomplished through gene therapy. Gene therapy is defined as the use of a gene transfer agent to deliver a therapeutic gene to target cells thereby normalizing their function. CF is a prime candidate for such a therapy because it is caused by defects in one gene, CFTR, which is only required in small quantities and the organ most affected by this disease, the lung, is easily accessible so the transfer vector would not need to be administered systemically. The feasibility of CF gene therapy was first demonstrated when the transfection of CFTR into primary airway epithelial cells corrected their Cl- transport defect [777]. It is not known for certain which cells in the respiratory tract are the most important to correct in order to avoid most of the CF lung pathology nor
is it known whether specific amounts of CFTR will be required to fix the defective functions in these cells. Some gene transfer vectors target airway cells non-specifically but most of the focus of CF gene therapy has been on delivering CFTR to the ciliated cells of the respiratory tract due to their major role in determining ASL height and MCC. These cells are terminally differentiated and are thought to turnover every 40-90 days. Because CF is a chronic disease, patients would need to be supplied with CFTR repeatedly, for their entire lives (~every 1-3 months). Alternatively, a gene therapy vector could target local lung progenitor cells to avoid the need for re-administration. It is also not known how many ciliated cells must be corrected in order to correct lung pathophysiology. An early experiment where immortalized CF bronchial cells were mixed with “corrected” cells at varying ratios suggested that insertion of normal CFTR into 6-10% of cells would result in Cl⁻ conductance indistinguishable from normal cultures [778]. Later experiments involving HAE cultures composed of CF and non-CF cells indicated that cultures containing 20% non-CF cells displayed Cl⁻ conductances that were 70% of those shown by non-CF cultures [779]. These studies suggested that a gene therapy vector will need to transduce at least 10%, but probably closer to 20%, of airway cells in order to correct the Cl⁻ transport defect in the CF lung. These studies did however not determine the amount of transduction required to correct Na⁺ hyperabsorption or the more pathologic features of the CF lung such as reduced ASL volume and decreased MCC. It has been suggested that correction of these defects may require transduction of >80% of airway cells [780, 781].

A CF gene therapy vector should specifically target the cells of the airway, efficiently deliver an appropriate amount of CFTR, provide sustained expression in progenitor cells or be able to be re-administered, exhibit very little toxicity, induce minimal inflammation and be easily
produced. Although the respiratory tract is accessible, it has evolved many physical and immunological barriers to withstand invasion. A CF gene therapy vector will need to penetrate and evade these barriers in order to successfully transduce airway cells. The vector must also be able to survive in and navigate through the harsh environment of the CF lung. Ultimately CF gene therapy would be performed on children before they develop severe lung pathology. Vector testing, however, must be performed on adults who have more severe lung disease even though it is not known to what extent these patients can be cured.

Viral and non-viral vectors have been proposed for the treatment of CF. The most studied virus vectors include adenovirus (Ad) and adeno-associated virus (AAV). The proposed non-viral vectors include different variations of cationic liposomes and DNA nanoparticles. These vectors have been tested in mouse models of the disease, rabbits, non-human primates as well as in human trials. They have been delivered by nasal instillation, intratracheally, intra-bronchially and by aerosol. Aerosol delivery via a commonly used nebulizer can efficiently deliver vectors to the entire lung [782]. However, by increasing the particle size delivered and delivering via an endobronchial spray, the vector is more efficiently delivered to the conducting airways and less is deposited in the alveoli [783]. Efficiency of CFTR delivery is determined by the presence of CFTR DNA, mRNA or protein or an alteration in Cl⁻ transport as measured by NPD. The usefulness of these surrogate endpoints in predicting the overall efficacy of a gene therapy vector is questionable however, because they do not evaluate the more clinically relevant features of CF lung disease such as Na⁺ hyperabsorption and decreased MCC. More clinically relevant endpoints such as FEV₁ may take longer than the length of the study to be altered. As gene
therapy studies progress the need for more informative surrogate endpoints that can be altered in the short period of a clinical trial must also be met.

**Adenovirus**

Ad was the first vector proposed for CF gene therapy. Ad is a double strand DNA virus in the *Adenoviridae* family. Its genome is a 36 kilobase linear strand of DNA containing early genes (E1-E4) encoding regulatory proteins and late genes (L1-L5) encoding structural proteins. Ad is non-enveloped and its genome is packaged into an icosahedral capsid. Ad is associated with respiratory illness, conjunctivitis and gastroenteritis in humans. It was chosen as a gene therapy vector because it infects non-dividing cells, it infects respiratory cells, it can generate high levels of gene expression and it can easily be produced to high titers. Over 50 serotypes of Ad exist but serotypes 2 and 5 were chosen for vector development because they were not associated with serious human disease. With the exception of group B, all adenoviruses bind the coxackievirus-adenovirus receptor (CAR) on target cells [784]. Integrins αvβ3/5 are also required for Ad infection [785]. First generation Ad gene therapy vectors lacked the E1 gene and sometimes also the E3 gene. Lack of the E1 gene rendered them replication deficient. These vectors used the human cytomegalovirus immediate early (CMV IE) promoter to produce high levels of CFTR. Unlike wild type viruses, replication deficient viruses do not spread so they must infect a large portion of cells upon the initial inoculation to function as efficient gene delivery vehicles. First generation Ad vectors were analyzed in CF xenografts (in SCID mice), rabbits, non-human primates and mouse models of CF. They exhibited high sustained expression of CFTR for 3-5 weeks in xenografts and 2-3 weeks in animal models with no signs of overt inflammation [786-789]. It was noticed however that if the vector reached the alveoli an inflammatory response ensued [790, 791].
Repeat administration of the vector also seemed well tolerated [792, 793]. When delivered to a CF mouse model, these vectors led to correction of Cl- transport [794]. These encouraging results led to the first human CF gene therapy trials in 1993. In the first trials Ad-CFTR was administered to the CF patients nasally. Some of these patients displayed some reduction in NPD even though CFTR mRNA could not be detected. This correction of Cl- transport was only transient (7-10 days) however. These patients also did not show any signs of inflammation or other adverse effects due to administration of the Ad vector [795, 796]. In an additional trial some inflammation was seen in patients receiving the highest doses [797]. Trials were also conducted involving the aerosol delivery of Ad-CFTR to the lungs of CF patients. In these trials again CFTR delivery was not efficient and was transient. One trial reported the development of inflammation in patients receiving the higher doses [798] while the other trial did not [799]. Two additional trials using first generation Ad vectors involved repeat administration of the vector. One trial involved nasal administration resulting in partial correction of Cl- transport in some patients but re-administration of the vector was blocked by the development of Ad neutralizing antibodies [800]. The other trial involved administration of the vector to the lungs via endobronchial spray. In this trial, patients receiving the highest doses were positive for CFTR after the first dose however this lasted for <30days. After the second dose, only the patients receiving the intermediate dose were positive for CFTR expression but again this lasted <30days. After the third dose no patients were positive for CFTR. All of the patients developed neutralizing antibodies to Ad and while there were no adverse reactions, patients receiving the highest doses did develop inflammation [801]. Additional studies in non-human primates suggested that the transient nature of the CFTR expression in these trials was due to a cellular immune response to Ad proteins that were still being produced in the absence of E1 and E3 [802,
Also the reduced efficacy upon readministration was due to the development of Ad neutralizing antibodies. This led to the development of second generation Ad vectors in which E2 and/or E4 was deleted. These vectors exhibited prolonged CFTR expression and induced less inflammation in animal models [804-806]. Two human trials were performed with these second generation vectors being delivered to the lungs of CF patients. In both cases CFTR expression was transient (<7 days) and some patients developed mild flu-like symptoms [807-809]. In one trial T cell responses and Ad neutralizing antibodies were present. It was concluded that the low efficiency of Ad delivery of CFTR and its transient nature was due to the host immune response.

First generation Ad vectors were found to stimulate an early, intense cytokine response similar to a regular viral infection probably due to the recognition of viral DNA [810-814]. There was also some suggestion that cytokines could be stimulated by the CMV IE promoter alone [815]. This innate response as well as the production of Ad proteins resulted in the development of a cellular and humoral adaptive response to the vector. CD8\(^+\) T cells were generated against the viral proteins being produced and led to the destruction of vector containing cells [816]. CD4\(^+\) T cells were generated and aided in the production of neutralizing antibodies [817]. These antibodies then prevented re-administration of the vector [792]. It was also determined that the vast majority of patients had preexisting antibodies to Ad from previous natural exposures, further decreasing in efficacy of Ad vectors [818]. Alveolar macrophages were also suggested to play a major role in the reduced efficiency of CFTR delivery by Ad vectors as well as the innate immune response to the vectors by internalizing virions before they reached their target cells [819, 820]. Ad vectors were also found to stimulate natural killer cells that also aided in the removal of
vector containing cells. This dose dependent inflammatory response led to inefficient delivery of CFTR to the airways of CF patients that decreased with repeat administration of the vector. Previous inflammation and PA infection as would be present in the CF lung also prevented the efficient transduction of Ad vectors and indeed the sputum of CF patients significantly inhibited the transduction of cultured cells [821, 822].

The location of Ad receptors further reduced the transduction efficiency of Ad vectors in the human airways. It was shown in HAE cells that Ad vectors do not infect the columnar ciliated epithelial cells very efficiently when delivered apically however they did when delivered basolaterally [823-825]. This was due to the fact that Ad receptors, CAR and \( \alpha_v\beta_3/\beta_5 \), are located on the basal surface of these cells and in the tight junctions between the cells but not on their apical surface [826]. It was suggested that the ability of Ad to target these basal cells may lead to more permanent correction of CF however, this was never demonstrated.

Treatments in addition to the delivery of the Ad vector have been shown to increase transduction and reduce vector induced inflammation. The use of transient immunosuppressants such as cyclophosphamide and antibodies to CTLA4 and CD40L, two T cell co-stimulatory molecules, improves Ad transduction efficiency, reduces the immune responses to Ad vectors and allows their re-administration in animal models [827-829]. Agents that cause the opening of tight junctions such as sodium caprate and lysophosphatidylcholine also improve Ad vector transduction [830, 831] However, immune suppression and tight junction opening would be undesirable for CF patients. Other
treatments such as mucolytics, steroids and neuraminidase have also improved the transduction of Ad vectors [832, 833].

Many alterations have been made to Ad vectors themselves in order to construct a vector that is more efficient at delivering CFTR to the human airway. Coating the virions with polyethylene glycol (PEG) improves transduction, reduces the immune response and alternating PEG formulations allows repeat administration [834, 835]. Encapsidating the virions in lipids protects them from antibodies and also allows re-administration [836, 837]. Precipitating the virions with calcium phosphate also improves transduction efficiency [838]. Alternating vectors derived from different Ad serotypes evades pre-existing immunity and allows repeat dosing [839, 840]. Retargeting Ad virions to cell surface molecules that are more plentiful in the surface of ciliated airway epithelial cells (purinergic receptors, urokinase plasminogen activator receptor, serpin enzyme complex receptor) via synthetic peptides of bi-specific antibodies also enhances transduction [841, 842]. The most significant development however, may be that of the “gutted” or helper-dependent (HD) Ad vector. This vector is devoid of all Ad genes, it contains only the inverse terminal repeat sequences and the packaging sequence [843]. In rabbits, HDAd exhibited a high transduction efficiency with minimal toxicity [844]. HDAd still induced cellular and humoral immune responses to its capsid proteins but these were much reduced compared to earlier vectors and did not prevent re-administration [845, 846] PEGylation of HDAd further reduced this immune response [847]. In a CF mouse model, HDAd treatment led to the expression of CFTR for 28 days and reduced inflammation and bacterial load [848]. Use of the cytokeratin promoter in place of the CMV IE promoter has been shown to improve the length of CFTR expression from HDAd vectors to up to 15 weeks [849]. The cytokeratin
promoter has an expression pattern similar to CFTR and results in much lower levels of CFTR expression. High quality HDAd vector has been difficult to produce, however, due to contamination with helper virus. In general, due to the many complications with Ad vectors, investigation into their use for CF gene therapy has diminished.

**Adeno-associated Virus**

AAV is a single strand DNA virus belonging to the *Dependovirus* genre of the *Parvovirus* family. There are 9 human AAV serotypes and more than 100 primate variants. AAV strains are thought to infect most mammals but are not associated with any human disease. AAV is non-enveloped with an icosahedral capsid. The AAV genome is ~5.2 kilobases and is composed of inverse terminal repeats, a Rep gene encoding four proteins essential for AAV replication, transcription and integration, and a Cap gene encoding the three proteins that compose the AAV capsid. AAV can infect a wide range of non-dividing cell types, in most cases specific serotypes target different organs more efficiently. AAV cannot replicate on its own, however, it requires co-infection with Ad or herpes simplex virus. In the absence of co-infection, AAV becomes latent existing as an episome or integrated into the host cell genome. Wild type AAV exhibits Rep-dependent, site specific integration into human chromosome 19. Recombinant AAV vectors are devoid of both Rep and Cap genes so they usually exist as episomes and rarely integrate [850, 851]. These AAV vectors are packaged in cells expressing Rep and Cap as well as Ad genes E1, E2a, E4 and VA either stably or by transient transfection. The packaging limit for AAV is 4.7 kilobases so the first AAV CF gene therapy vectors used the innate promoter capability of the inverse terminal repeat sequence to drive CFTR expression since there was no room in the genome to add an additional promoter. These vectors were based on AAV serotype 2 which binds heparan
sulfate, fibroblast growth factor receptor 1 and αvβ5 integrin [852-854]. AAV2 vectors expressing CFTR displayed very stable expression for 6 months in both rabbits and non-human primates [855, 856]. This expression was in the absence of inflammation, pathogenicity or toxicity. The ability to re-administer the vector, however, differed between the two models. Repeat administration was successful in non-human primates but not in rabbits [857, 858]. In both cases, there was AAV2 specific antibody production, however, there was no cellular immune response and no inflammation [859]. The first human trial involving the use of AAV vectors carrying CFTR to treat CF patients was in 1996. In the first few trials AAV vectors were delivered nasally. No inflammation developed in any of the patients treated in these trials and there were no adverse reactions indicating that these vectors were safe and well-tolerated [860]. In one trial patients were positive for CFTR DNA for 10 weeks, exhibited some correction of Cl⁻ transport and showed a reduction in IL-8 [861]. In another trial most patients were positive for CFTR for only 14 days but 1 patient was again positive for 40-70 days. These patients also exhibited partial correction of their Cl⁻ transport defect although it was transient [862]. The encouraging results from these trials led to a much larger trial in which patients did not develop improved Cl⁻ transport or a reduction in inflammatory markers [863]. They also led to the testing of this vector in the lungs of CF patients. In these trials patients receiving the highest doses were positive for CFTR for up to 30 days; however none exhibited a change in Cl⁻ transport. Some adverse reactions in the form of disease exacerbations were also noted as was the presence of AAV antibodies [864, 865]. Even with these questionable results, a trial testing the ability to re-administer this vector was undertaken. In this trial, even though all the patients were still positive for CFTR after three doses they only exhibited improved FEV₁ and reduced IL-8 after the first dose and all patients developed antibodies to AAV2 [866]. Due to the
encouraging results of this trial an additional larger trial was performed in which these results were not corroborated [867].

Even though AAV vectors seemed to be better tolerated than Ad vectors they still did not result in significantly better transduction efficiency, prolonged CFTR expression or the ability to be re-administered. This was due to many of the same reasons as the Ad vectors. Furthermore, as with Ad, most patients had preexisting antibodies to AAV2 [868]. This, in part, was responsible for the lack of transduction. Also patients developed AAV2 specific antibodies in response to vector administration which probably played a major role in preventing repeat administration. It has been suggested that the recognition of AAV DNA leads to a transient cytokine response, perhaps just enough to stimulate the production of AAV specific antibodies [869]. It has also been shown that AAV interacts with complement possibly leading to its phagocytosis by alveolar macrophages which could result in the induction of an innate and cell mediated immune response to the AAV2 capsid proteins [870]. Indeed, AAV vectors can stimulate the production of CD8+ T cells although to what extent they play a role in the eradication of vector containing cells is unknown [871] Similar to Ad, it was also shown that sputum from CF patients inhibited AAV2 transduction of cultured cells [872, 873]. However, in general, AAV vector transduction does not vastly alter cellular transcription [874]. Another reason for the poor transduction efficiency of AAV2 vectors could be the location of its receptors. As with Ad, AAV2 cannot efficiently transduce differentiated airway epithelium if it is applied apically [875, 876]. This is because the AAV2 receptors are located on the basal surface of these cells. It has also been shown that when AAV2 vectors do transduce cells at the apical surface they follow an alternative entry pathway that results in their sequestration in endosomes and eventual degradation [877].
Reduced CFTR expression from AAV vectors could be due to the weak promoter activity of the AAV inverted terminal repeats or diminished second strand synthesis due to the absence of a helper virus [878].

Most of the work to improve the transduction efficiency of AAV vectors has been done using alternate serotypes. AAV serotypes 1, 5, 6 and 9 can infect murine lung tissue much more efficiently than AAV2 [879]. AAV9 maintained stable gene expression for 9 months and could be re-administered however its transduction efficiency was highest in the more distal airways [880]. Both AAV5 and AAV6 can transduce HAE cultures much better than AAV2 and can maintain stable expression for 28 days [879], [881]. A single amino acid substitution in AAV6 improves its transduction efficiency even further [879]. Studies have shown that both AAV5 and AAV6 bind 2,3-linked sialic acid which is abundant on the apical surface of airway epithelial cells [881, 882]. Preexisting antibodies to these serotypes are less frequent in humans [868]. It is debated as to whether re-administration is possible with these serotypes [793, 883, 884]. AAV2 genomes can be packaged into the capsids of these other AAV serotypes creating “pseudotyped” vectors [885]. An AAV2/5 vector displayed improved transduction and expression in a non-human primate model with no inflammation [886]. Retargeting AAV vectors with the use of bi-specific antibodies and receptor targeting peptides has also been attempted [887, 888]

Directed evolution and rational design are two additional strategies taken to improve the potential of AAV as a gene therapy vector. Directed evolution involves the use of error-prone PCR and capsid fragment shuffling along with physical selection to obtain AAV capsids with desirable traits. One group employed this technique with AAV2 and AAV5,
generating an AAV hybrid that was much more efficient than either parental vector at transducing CF HAE cultures and correcting the Cl- transport defect [889]. Another group also used this technique but with AAV1, 6 and 9. This group obtained two hybrids: one containing sequences from AAV1 and 6, the other from all three serotypes. Again, the hybrids displayed improved transduction efficiency in CF HAE cultures and corrected the Cl- transport defect to 25-30% of that seen in non-CF HAE [890]. Rational design seeks to identify and modify neutralizing epitopes on the AAV capsid leading to the production of AAV vectors which can be re-administered [891, 892]. As with Ad, immune suppression has also been suggested as a mechanism to reduce the production of AAV neutralizing antibodies and indeed it is successful. However, again, it would be undesirable to suppress the immune system of CF patients in this manner [893, 894].

Improvements in the ability of AAV vectors to express CFTR have also been suggested. One of these is to shorten the coding sequence of CFTR to provide space for a better promoter in the vector. Removal of the first 117 or 264 amino acids or removal of a portion of the R domain made room for the inclusion of the chicken β-actin promoter [886, 895-897]. An AAV2/5 vector carrying a truncated CFTR gene reduced the inflammation due to PA in a mouse model of CF [885]. There is still some concern however as to whether these altered CFTR genes produce proteins that are capable of appropriate maturation and function. An alternative to altering the CFTR gene is to take advantage of trans-splicing or homologous recombination to deliver a promoter and the CFTR gene on two separate AAV vectors that will assemble in the nucleus. Both of these techniques involve the inclusion of specific sequences in the vectors that will either cause the joining of the vector DNA, in the case of homologous recombination, or the encoded mRNA, in the case of trans-splicing, in a
predetermined way [898-900]. This system was tested and was successful at normalizing 
Cl- transport in cultured IB3-1 cells [901]. Trans-splicing could also be used to minimize the 
amount of CFTR that needs to be carried by an AAV vector by inducing splicing between 
endogenously produced CFTR mRNA and a small portion of CFTR expressed by the vector. 
This technique was able to provide ~14% of the Cl- transport seen in non-CF HAE cultures 
[902]. The benefit of this technique is that CFTR would only be expressed in cells that 
normally express it; the downfall is that the specificity and frequency of the splicing is not 
well documented.

Additional suggestions for improving AAV vectors involve the intracellular processing of the 
vector. Proteosome inhibitors increase transduction efficiency by allowing more vectors to 
enter the nucleus [903]. Inhibition of DNA protein kinases increases the ability of AAV 
vectors to integrate thus improving their gene expression [904]. Co-expression of Rep from 
a second vector also increases the frequency of site specific integration [905]. To this end an 
Ad-AAV hybrid vector has been constructed that can integrate site specifically [906]. None 
of these suggestions, however, have drastically improved the efficacy of AAV vectors. Work 
continues to improve the potential for AAV as a gene therapy vector to treat CF.

**Lentivirus**

A retroviral vector was the first viral vector expressing CFTR to show that correction of the 
Cl-defect in cultured CF cells could be accomplished by delivery of a normal CFTR gene 
[907]. Lentivirus is a genre of viruses in the Orthoretroviridae subfamily of the Retroviridae 
family of single stranded RNA viruses that integrate DNA copies of their genomes into the 
genomes of their host cells. Lentiviruses are the only retroviruses that can infect non-
dividing cells [908]. The lentivirus genre includes human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV), feline immunodeficiency virus (FIV) and equine infectious anemia virus (EIAV). HIV, SIV and FIV based vectors have all been suggested for the treatment of CF. These vectors are pseudotyped with heterologous viral glycoproteins to more efficiently transduce the airway epithelium and contain cell specific promoters such as the cytokeratin promoter and the FOXJ1 promoter to limit gene expression to ciliated cells [909]. SIV pseudotyped with Sendai virus glycoproteins mediated efficient transduction of airway cells where gene expression persisted for over 15 months [910, 911]. FIV pseudotyped with baculovirus glycoprotein 64 was able to transduce murine nasal epithelia and persist for up to 50 weeks [912]. This vector was able to be re-administered with additive gene expression and very little immune response [913]. FIV-GP64 expressing CFTR was able to efficiently transduce and correct the Cl⁻ transport defect in CF HAE cultures for the life of the cultures (>3 months) [914]. HIV based vectors have been pseudotyped with glycoproteins from Ebola virus, baculovirus, and severe acute respiratory syndrome virus [915-917]. HIV pseudotyped with the vesicular stomatitis virus glycoprotein has shown encouraging efficiency of CFTR delivery and persistence in CF mouse models with little evidence of inflammation. In one study this led to Cl⁻ transport correction for 110 days [918]. In still another study partial correction was achieved for up to 12 months [919] However, these vectors did require opening of the tight junctions to achieve transduction which would probably not be acceptable for use in CF patients [920]. This long lived expression and correction suggests that these vectors are targeting airway progenitor cells and indeed, lentiviral vectors have been shown to transduce submucosal gland progenitor cells [921]. These lentiviral vectors also exhibit sustained expression because of their integration into the host cell genome. However, integration also leads to the
possibility of insertional mutagenesis which can lead to serious complications. Including specific DNA binding domains in the sequence of the vector has been shown to promote site specific integration therefore reducing the chance of insertional mutagenesis [922]. Also integrase defective vectors are being produced [923, 924]. No lentiviral vector has been tested in CF patients however, due to these safety concerns. However, because lentiviral vectors exhibit so many positive attributes (persistent expression, lack of immune stimulation, an ability to be re-administered, etc.) work continues to develop a safe, efficient lentiviral vector for the treatment of CF.

Simian Virus 40
Simian virus 40 (SV40) has also been tested as a vector for CF gene therapy. SV40 is a polyomavirus in the Polyomaviridae family of double strand DNA viruses. SV40 vectors have been instrumental in generating immortalized cells as well as other technical applications. The SV40 vector used for delivery of CFTR is a vector lacking all of the SV40 genes. This vector successfully delivered CFTR to immortalized CF airway cells and a CF mouse model in which it corrected the Cl- transport defect, reduced inflammation and inhibited pathology due to PA infection [925]. SV40, however, also has the potential to integrate into the host cell genome so safe integration will need to be assured before this vector could be tested in humans.

Natural Respiratory Viruses as Vectors
Any virus can be a potential vector for CF gene therapy provided that its pathogenic potential has been abolished and it can target the airway epithelium without inducing a harmful/protective immune response. Although some of the previously mentioned vectors
induce less of an immune response than others, none of them infect the apical ciliated cells of the airway with great efficiency. This, in part, led to the exploration of naturally airway-tropic viral vectors for the treatment of CF, particularly paramyxoviruses.

Sendai virus is in the Respirovirus genus of the Paramyxovirinae subfamily of the Paramyxoviridae family of enveloped, negative strand RNA viruses. It is the murine counterpart to human parainfluenza virus type 1 (PIV1) and has been shown to infect HAE cultures efficiently though it is not known to cause human disease [926]. Sendai virus was capable of expressing CFTR in a CF mouse model in which it corrected the Cl⁻ transport defect, albeit transiently (~7 days) [927]. However, this vector did induce inflammation and repeat administration was not effective [928]. A transmission incompetent Sendai virus vector lacking the Sendai virus fusion protein was also capable of delivering CFTR to CF mice which also corrected the Cl⁻ transport defect [927]. Use of this vector in humans is questionable though because of the presence of preexisting immunity to PIV1 which may also interfere with its transduction [929]. Also, when a virus that naturally infects one species, in this case mice, is used to infect another, in this case human, it induces a powerful innate immune response because viral mechanisms of immune evasion often do not operate efficiently in a foreign host [930]. Stimulation of innate immunity would also enhance the adaptive immune response resulting in inhibition of virus replication, destruction of vector containing cells and an inability to be re-administered.

PIV type 3 has also been suggested as a potential viral vector for CF gene therapy. PIV3 belongs to the same genus as Sendai virus and being a human respiratory virus also infects HAE cultures very efficiently [931]. PIV3 is a common respiratory virus in the human
population and usually results in common cold-like symptoms though it can result in more severe disease in infants and the immunocompromised. A PIV3 vector was shown to deliver CFTR to CF HAE cells very efficiently resulting in correction of both Cl− and Na+ transport as well as improved ASL height and MCC. PIV3 is the only vector to date that has shown correction of Na+ hyperabsorption, ASL dehydration or MCC in a clinically relevant model suggesting this can be accomplished. Correction of these defects required transduction of 25% of the culture by the vector [932]. PIV3 would certainly induce an immune response in humans so its antigenicity will need to be reduced in order for it to be a viable option for CF gene therapy.

Non-viral Vectors
Non-viral vectors include two distinct vector systems: cationic liposomes and DNA nanoparticles. In general, non-viral vectors express CFTR at a lower level than viral vectors and interact with target cells non-specifically, unless a targeting ligand is included in the complex, leading to less efficient transduction of the target cells. However, non-viral vectors are also less likely to induce an immune response that will prevent repeat administration of the vector. These vectors are easily synthesized, have no DNA packaging limit and are flexible in their composition and targeting.

Cationic liposomes are composed of cationic lipids complexed with plasmid DNA. The most commonly used cationic liposome vectors are composed of DC-cholesterol and dioleoylphosphatidylethanolamine (DC-chol/DOPE), N-{1-(2,3-dioleoyloxy)propyl}-N,N,N-trimethyl-ammoniummethylsulphate (DOTAP) or 1,2-dimyristoylsn-glycero-3-ethylphosphocholine, chloride salt (EDMPC). These vectors are 100-500 nanometers in
diameter. DC-chol/DOPE was shown to mediate the delivery of CFTR to the lungs of CF mice and partially correct the Cl\textsuperscript{\textendash}transport defect albeit transiently (~14 days) [794, 933]. This vector however did not induce inflammation and could be re-administered. Human trials in which DC-chol/DOPE delivered CFTR to the nasal epithelium of CF patients were moderately successful. CFTR expression and partial correction of the Cl\textsuperscript{\textendash}transport defect (~20% of normal) were observed for up to 15 days [934, 935]. DC-chol/DOPE could also be re-administered with very little immune stimulation [936]. Similar to DC-chol/DOPE, DOTAP could also efficiently deliver CFTR to the airway epithelium of CF mice and correct the Cl\textsuperscript{\textendash}transport defect but again only transiently, ~9 days [937, 938]. This vector could also be re-administered without any signs of inflammation. When tested in CF patients, DOTAP efficiently delivered CFTR to the nasal epithelium with little immune stimulation. Most patients were positive for CFTR and exhibited improved Cl\textsuperscript{\textendash}transport for up to 7 days however two patients were positive for expression and Cl\textsuperscript{\textendash}transport for 28 days [939]. When tested in CF mice and non-human primates, EDMPC was very efficient at delivering CFTR to the airways with no detectable inflammatory response [940, 941]. In humans, EDMPC successfully delivered CFTR to the nasal epithelium of CF patients, however, this did not result in a change in Cl\textsuperscript{\textendash}transport [942]. A fourth cationic liposome vector, GL67, was also tested in CF patients. Upon nasal administration, GL67 efficiently delivered CFTR inducing partial correction of the Cl\textsuperscript{\textendash}transport defect with no adverse reactions [943]. When administered to the lungs of CF patients, GL67 also mediated CFTR expression and partial correction of Cl\textsuperscript{\textendash}transport (~25% of normal), albeit transiently (up to 7 days). In one trial, a reduction in bacterial burden and inflammatory markers in the sputum was also observed. However, patients in both trials developed a flu-like illness [944, 945].
DNA nanoparticles are composed of a cationic polymer, usually poly-L-lysine (PLL) or polyethyleneimine (PEI), complexed with plasmid DNA. Substituting some of the side chains of PLL with PEG allows this vector (PEG substituted PLL-DNA) to be purified more easily [946]. It is thought that these particles bind to glycosaminoglycans on the surface of cells causing endocytosis of the particles [947, 948]. In the case of PEI-DNA particles, the PEI causes the endosomes containing the particles to burst liberating the particles so they can traffic to the nucleus [949]. PEI-DNA complexes can efficiently deliver CFTR to most cells of the proximal airways [950, 951]. They could also correct the Cl⁻ transport defect in CF mice however they did induce an inflammatory response [952, 953]. It has been suggested that this inflammatory response may be associated with the PEI-mediated rupture of endosomes [954]. PEG substituted PLL-DNA can also efficiently transduce the airway epithelium but with very little stimulation of an immune response [955, 956]. This vector could also be re-administered [957]. In a CF mouse model, PEG substituted PLL-DNA vector transduction partially corrected the Cl⁻ transport defect in all treated mice and fully corrected it in some mice with little inflammation [956]. When delivered to the nasal epithelium of CF patients, PEG substituted PLL-DNA efficiently delivered CFTR resulting in at least partial correction of the Cl⁻ transport defect lasting up to 15 days. Some inflammation was observed but no adverse reactions occurred [958].

Despite these promising results, neither cationic liposome vectors nor DNA nanoparticles have been efficacious in CF patients. As with Ad and AAV, sputum from CF patients has been shown to inhibit the transduction of these vectors [959]. It has been suggested that this is due to the presence of neutrophil proteases and elastase which degrade the vectors however the transduction of these vectors may also be inhibited by the thickened mucus in
the CF airway as well. Studies have also indicated that liposomes are somewhat inefficient at entering the airway epithelium apically [960, 961]. Nebulization can also damage liposomes. The low level of gene expression exhibited by these non-viral vectors may also reflect their lack of nuclear targeting and rather inefficient diffusion through the cytosol [962, 963]. The flu-like illness exhibited by patients receiving GL67 may result from an innate immune response to bacterial plasmid DNA mediated by Toll-like receptor 9 (TLR9) [964]. This response involves the production of cytokines and the attraction of neutrophils, macrophages and lymphocytes to the airways [965, 966]. DOTAP delivered DNA can also stimulate TLR9 whereas, PEG substituted PLL delivered DNA does not seem to [956, 967]. Additionally, certain combinations of lipids are more immunostimulatory than others and thus should be avoided.

Many advances in the field of non-viral vectors have been made to increase their efficacy for CF gene therapy. The use of mucolytic and anticholinergic drugs improves the transduction efficiency of non-viral vectors [968]. Altering the glycosylation pattern of DNA nanoparticles can also improve their transduction efficiency [969, 970]. Targeting strategies include the use of the serpin enzyme complex receptor, purinergic receptors and the polymeric immunoglobulin receptor [971]. This targeting reduces the non-specific uptake of these vectors by alveolar macrophages and therefore reduces immune stimulation.

Delivery of DNA nanoparticles via the serpin enzyme complex receptor to CF mice resulted in CFTR expression and Cl- transport correction for up to 12 days and this vector could be re-administered [971]. Use of polymeric immunoglobulin receptors can allow targeting to submucosal glands [972]. The ease of retargeting these vectors could allow the targeting of airway progenitor cells once they are definitively identified and could allow the targeting of
additional organs such as the liver and the pancreas. Indeed, non-viral vectors can be delivered intravenously [973, 974] Treatment with proteosome inhibitors as well as inclusion of a nuclear localization signal in the vectors improves their trafficking to the nucleus [975, 976]. As with viral vectors, the use of ciliated cell specific promoters has also been adopted by non-viral vectors as a means to ensure that CFTR will only be expressed in these cells upon delivery to the lungs [909, 977]. Reducing or eliminating bacterial CpG motifs largely inhibits the ability of these vectors to stimulate an immune response [978, 979]. PEGylation of liposome vectors also reduced their cytotoxicity [980]. Non-viral vectors are very appealing as gene therapy vectors for CF due to their lack of immune stimulation however their efficiency of delivery and their duration of gene expression need to improve in order to have real treatment potential.

The response to the vector will depend on its origin, purity, pseudotype characteristics, dose, route of administration and previous exposure. Future vectors will need improved production, targeting, transduction efficiency, persistence and reduced stimulation of the inflammatory response. Additional, longer studies are needed to determine if current vectors can improve chronic CF lung disease. Gene therapy studies in other diseases have shown that cellular immune responses can be directed toward the therapeutic gene resulting in loss of expression. T cell responses to CFTR have been noted in a CF mouse model however, this should only be a concern when treating CF patients who are homozygous for class I mutations and thus do not produce CFTR protein [981, 982].
Cell-mediated Therapy

In addition to gene therapy where a vector delivers CFTR to the cells of a patient, cell-based therapy where donor cells containing normal CFTR or patient cells in which a normal CFTR gene has been delivered *ex vivo* are administered to the patient has been suggested as a treatment for CF. Bone marrow transplantation has been suggested but results in very few newly engrafted cells in the airways [983]. Mesenchymal stem cells (MSCs) are capable of becoming airway cells [984]. When mixed with CF airway cells, “corrected” MSCs were capable of mediating a correction of Cl⁻ transport, however, when administered intravenously, very few MSCs engrafted into the airway [985, 986]. It has been suggested that extensive damage to the airway is a prerequisite for stem cell engraftment and it is unknown whether the amount of damage present in the CF respiratory tract is enough to facilitate sufficient engraftment to cause correction of the phenotypic defects in the CF lung.

An alternative is to use airway progenitor cells which locally repopulate injured airway tissue [987]. Though these cells have been difficult to identify, a population of Clara cells is capable of regenerating the cells of the respiratory tract when cultured alone in a similar manner as HAE cultures [988]. It has been suggested that patient-acquired progenitor cells could be “corrected” *ex vivo* with a lentiviral gene therapy vector then returned to the patient to repopulate the airways with “corrected” cells. However, this approach is yet to be tested. One problem had been the inability to amplify patient progenitor cells in culture while still maintaining their undifferentiated state. In addition to the infrequent engraftment, the risk of uncontrolled proliferation also plagues this type of therapy.

Continued advancements with the vectors discussed above and/or the development of new vectors will hopefully lead to a cure for this debilitating disease.
CHAPTER 2: RESPIRATORY SYNCYTIAL VIRUS

Classification

RSV is an enveloped, non-segmented, negative sense RNA virus belonging to the *Pneumovirus* genus of the *Pneumovirinae* subfamily of the *Paramyxoviridae* family of viruses in the order *Mononegavirales*. RSV was first isolated in 1956 from a laboratory chimpanzee exhibiting “cold-like” symptoms [989]. At this time it was termed chimpanzee coryza agent or CCA. Shortly after this first isolation however CCA was also recovered from infants hospitalized for acute respiratory disease and thereafter was termed respiratory syncytial virus or RSV [990, 991]. RSV received this moniker for its ability to cause cell-to-cell fusion or syncytia in non-polarized, cultured cells, a phenomenon that is not seen frequently in differentiated polarized cells, experimentally infected animals or infected humans [992-995]. Related RSV viruses have been isolated from various animal species including cows (bovine RSV), sheep (ovine RSV) and goats (carpine RSV). Human RSV and bovine RSV share ~73% identity [996]. Pneumovirus of mice (PVM), an indigenous rodent virus, is also classified with RSV and the two share ~52% identity [997]. The most closely related virus group (*Metapneumovirus*) includes human metapneumovirus which shares ~65% identity with RSV.

RSV has a single serotype with two antigenic subgroups, A and B. The two subgroups do not exist as variants but instead represent two divergent evolutionary paths which according to analysis of the attachment glycoprotein (G) diverged ~350 years ago [998]. The subgroups
are 25% related antigenically with the fusion protein (F) being the most related (~50%) and the G protein being the least related (1-7%) [999]. The prototypic strains of the A and B subgroups (A2 and B1) share 81% nucleotide homology. The genomic cis-acting signals (leader, trailer, gene start and gene end) are the most conserved and the non-coding regions are the most divergent. The ectodomains of the G protein and the small hydrophobic protein (SH) are the least conserved coding regions [1000]. Diversity within the subtypes is less prominent but follows the same pattern. RSV is relatively stable antigenically, observations indicate that escape mutations do not accumulate in the F protein over time though it is possible that this is the case because the F protein is less tolerant to such changes due to its strong structure/function relationship. Selective immune pressure may exert its effects on the G protein because it is much more tolerant to mutation. Compared with other viruses, however, the antigenic identity of G appears to change rather slowly, amino acid composition changes only ~0.25%/year [1001]. Sequencing data from natural RSV isolates indicates that nucleotide substitutions occur in G at a rate of $10^{-2.7}$/year implying that G is more diverse on the amino acid level than it is on the nucleotide level [1000]. The notable stability of the RSV genome in culture probably reflects a lack of selective pressure.

RSV can infect a wide variety of cell types from a number of different species in vitro. Almost all of what we know about the molecular mechanisms of RSV infection, replication/transcription, individual gene functions and virion formation come from the ability to grow RSV in cultured cells. RSV clinical isolates do not appear to require adaptation to grow efficiently in cultured cells. “Lab strains” of RSV maintain their virulence in chimpanzees and humans. Chimpanzees provide the most realistic animal model for RSV
disease but are rarely used due to cost and availability. RSV can infect mice, cotton rats, ferrets, guinea pigs and hamsters with varying degrees of efficiency [1002]. Mice do not become highly infected with RSV or show the overt signs of respiratory disease seen in humans but due to their abundance of immunologic and other reagents much of what we know about RSV infection and disease comes from experimental infection of the mouse. Of all mouse strains, the BALB/c mouse is the most permissive to RSV infection.

**Genome Organization**

The non-segmented, negative sense RNA genome of RSV is 15,222 nucleotides in length. It contains 10 genes (NS1-NS2-N-P-M-SH-G-F-M2-L) encoding 11 proteins (NS1-NS2-N-P-M-SH-G-F-M2-1/M2-2-L). The additional protein is due to the translation of two open reading frames (ORFs) in the M2 gene. The genome is flanked by a 44 nucleotide leader (Le) sequence at the 3’ end and a 155 nucleotide trailer (Tr) sequence at the 5’ end [1003, 1004]. The Le serves as a polymerase binding site and the promoter for genome replication and gene transcription [1005, 1006]. Each gene is flanked by a highly conserved 10 nucleotide gene start (GS) sequence, responsible for initiating transcription, and a less conserved 12-13 nucleotide gene end (GE) sequence that terminates transcription of the gene and generates a poly A tail for the mRNA [1007-1010]. Each pair of genes is separated by an intergenic region of varying sequence and length (1-58 nucleotides). The lengths of the RSV intergenic regions are conserved among RSV strains but the sequences are variable [1011]. RSV does not follow the “rule of six”, a requirement that its genome length be divisible by six, like most of the Paramyxoviruses. The RSV genome exists in virions and infected cells as an α-helical, RNAse resistant nucleocapsid.
The polymerase complex is composed of the large (L) protein and the phosphoprotein (P), which can associate with the nucleocapsid (N) protein and the accessory protein encoded by the first ORF of the M2 gene (M2-1). The accessory protein encoded by the second ORF of the M2 gene (M2-2) also plays a role in polymerase activity and so will be discussed here as well.

The L gene is a 6578 nucleotide gene encoding a 250 kiloDalton (kDa) protein that functions as the RSV polymerase and is responsible for RSV replication and transcription. The L protein has six domains containing polymerase motifs that are highly conserved among Paramyxoviruses [1012]. Due to their high level of amino acid conservation the functions of these domains are believed to be conserved. There is a putative nucleotide binding domain in the central region (domain II) that is also believed to play a role in mRNA capping [1013, 1014]. The nucleotide polymeration site is located in domain III, guanyltransferase activity is attributed to domain V and methyltransferase activity is attributed to domain VI [1015, 1016]. Domains I and IV are still without assigned functions.

The P gene is 914 nucleotides encoding a 33 kDa protein that is required to form stable polymerase complexes. It has been shown that the P protein is required for efficient binding of the L protein to the nucleocapsid and that the C-terminal portion of P is what interacts with both L and the nucleocapsid. The P protein must be phosphorylated to function as a polymerase cofactor. Multiple phosphorylation sites have been identified, however, only phosphorylation at serine 232 has been shown to be important for RNA elongation [1017].
The N gene is 1203 nucleotides encoding a 45 kDa protein responsible for encapsidating the RSV genome. N is an RNA binding protein which binds to both the RSV genome and antigenome to form the RNAse resistant α-helical nucleocapsids, an activity requiring the N-terminal portion of the N protein [1018]. N forms a stable complex with the P protein such that P acts as a chaperone for soluble N protein. When N is expressed in the absence of P it forms aggregates and is incapable of encapsidating the RSV genome, P maintains N in a soluble state so that it is available for genome encapsidation [1019].

The M2 gene, so named because when first discovered it was thought to produce a second matrix protein, is a 961 nucleotide gene encoding a 22 kDa protein from its first ORF (M2-1) and an 11 kDa protein from its second, overlapping ORF (M2-2) [1020]. The M2-1 and M2-2 proteins are believed to be produced by a ribosomal termination-reinitiation mechanism; after translating the M2-1 protein, the ribosome can reinitiate translation at any one of three AUGs located upstream of the M2-1 stop codon to generate the M2-2 protein [1021]. Termination-reinitiation is a very inefficient process which probably accounts for, at least in part, the low levels of M2-2 present in RSV infected cells. The M2-1 protein serves as a polymerase anti-termination factor that is essential for RSV transcription as it keeps the polymerase complex bound to the genome while it is transcribing [1022, 1023]. In the absence of M2-1, transcription terminates prematurely and nonspecifically. The activity of M2-1 is dependent on its N-terminal cysteine-histidine zinc finger motif which is required for its ability to bind RNA [1024]. It also appears that an interaction with P is required for M2-1 function [1025]. M2-1 is present in infected cells in phosphorylated and unphosphorylated species but the significance of this is unknown [1024]. The M2-2 accessory protein has been shown to down-regulate transcription and up-regulate
replication and is thought to be a “switch” between transcription and replication [1026]. As the abundance of M2-2 increases during RSV infection, genome replication begins to be favored over transcription. It has been suggested that the accumulation of M2-2 signals that enough transcription has taken place and now it is time to replicate the genome in preparation for virion formation.

The Structural Proteins

RSV expresses three membrane glycoproteins: the fusion protein (F), the attachment glycoprotein (G) and the small hydrophobic protein (SH). RSV also expresses a matrix protein (M) that lines the inner surface of the virion membrane.

The F gene is 1903 nucleotides in length and encodes a 70 kDa protein that is responsible for the virus-cell fusion that initiates viral infection. The F protein is a type I transmembrane glycoprotein, with its transmembrane domain located near its C-terminus while its N-terminus is extracellular. The signal sequence guiding the F protein to the endoplasmic reticulum (ER) is located at the N-terminus and is cleaved following entry into the ER. F is initially produced as a precursor protein called F₀. F₀ is cleaved in the trans Golgi network by a furin-like protease yielding the disulfide linked F₁ (amino acids 137-574) and F₂ (amino acids 1-130) subunits [1027, 1028]. The F protein is cleaved twice, in the trans Golgi, yielding a 27 amino acid fragment termed pep27. Other paramyxovirus F proteins are not cleaved at a second position and the purpose of this second cleavage and the fragment it releases is yet to be determined. The F protein is N-glycosylated in the ER and forms trimers as its functional unit. The placement of cysteine, glycine and proline residues is conserved among Paramyxovirus F proteins suggesting that they share a similar structure [1029]. F
not only directs the fusion of the viral envelope with the host cell plasma membrane, it is also responsible for the cell-to-cell fusion or syncytium formation seen in cultured cells. The RSV F protein can mediate this fusion independent of any other viral proteins, supporting virus replication in cultured cells in the absence of the attachment protein [1030, 1031]. This characteristic is unique to the members of the *Pneumoviridae*. The F protein is highly conserved among RSV strains and subtypes and is responsible for most of the antibody cross-neutralization.

The G gene is 923 nucleotides in length and encodes a ~90 kDa protein responsible for the initial attachment of the RSV virion to the host cell. The G protein is produced in both membrane bound and soluble forms [1032]. The membrane bound form of G is a type II transmembrane glycoprotein, with its transmembrane anchor located at its N-terminus leaving its C-terminus to be oriented extracellularly. The signal sequence guiding G to the ER is also located at its N-terminus and is not cleaved following entry into the ER. The secreted form of G is translated from a second start codon (AUG) located in the transmembrane domain. Once soluble G (sG) arrives at the plasma membrane the rest of the transmembrane domain is proteolytically cleaved releasing sG from the membrane. The sG protein is believed to account for ~20% of the total G protein produced by an RSV infected cell [1033]. The RSV G protein is N-glycosylated and heavily O-glycosylated. N-glycosylation of G occurs cotranslationally at 4-5 sites located throughout the protein. O-glycosylation occurs in the Golgi. The G protein contains ~70 potential O-glycosylation sites of which approximately 25 are believed to be used routinely [1034]. Due to its high degree of O-glycosylation the G protein is said to resemble a family of cellular glycoproteins called mucins. The O-glycosylation pattern of G can vary from molecule to molecule which could
provide some shielding from immune recognition. The G protein sequence is not well conserved among RSV strains except for a central conserved domain (amino acids 164-198) containing overlapping cysteine rich (amino acids 173-186) and heparin binding (amino acids 184-198) domains. The four cysteines in the cysteine rich region are spaced in such a way that they form a “cysteine noose” believed to be important for the attachment function of G, however, data to the contrary also exists [1035, 1036]. The central conserved region is also required for the inhibition of nuclear factor-kappa B (NFκB) exhibited by the G protein [1037]. The G protein is not absolutely required for virion assembly or RSV infection of cultured cells however it greatly enhances viral growth and infectivity. Viruses lacking the G gene grow to lower titers in cell culture and are attenuated in vivo [1031, 1038-1040]. It is believed that the G protein forms trimers or tetramers [1041].

The SH gene is a 410 nucleotide gene encoding proteins varying from 5-60 kDa. Like G, SH is a type II transmembrane glycoprotein whose membrane anchor and signal sequence are located at the N-terminus. SH, however, is produced in four distinct forms: SH\(_o\), SH\(_p\), SH\(_t\) and SH\(_i\) [1042, 1043]. SH\(_o\) is the most abundant form consisting of a full length, non-glycosylated SH protein (~7.5 kDa). SH\(_p\) has a single N-glycosylated residue (13-15 kDa). SH\(_p\) has a single N-linked side chain containing polylactosaminoglycan (21-60 kDa). SH\(_t\) is a truncated, non-glycosylated form of SH that is translated from a second, in-frame AUG in the SH ORF (~5 kDa). SH\(_o\) and SH\(_p\) are the predominant forms of SH on the surface of RSV virions but the significance of the multiple forms of SH is not known. The precise function of SH is also not known as ΔSH viruses are completely viable in cultured cells and are only slightly attenuated in the upper respiratory tracts of mice and chimps [1044, 1045]. Studies have indicated that SH forms pentamers which may act as cation specific membrane pores,
however this “viroporin” function has not yet been found to play a role during RSV infection [1046-1048]. Additional studies have indicated that SH may play a role in the inhibition of tumor necrosis factor α (TNFα) signaling resulting in the inhibition of apoptosis in RSV infected cells [1049].

The M gene is 958 nucleotides in length and encodes a 25 kDa protein required for RSV virion formation and budding. The M protein associates with both the plasma membrane of infected cells and the RSV nucleocapsid and is believed to be responsible for attaching the nucleocapsid to the plasma membrane beneath where a virion is about to form [1050-1052]. The M protein is unglycosylated but contains a hydrophobic domain in its C-terminus that probably mediates its interaction with the host cell membrane/virion envelope. The M protein of Sendai virus has been shown to self associate to form two dimensional paracrystalline structures beneath the host cell plasma membrane that not only interact with the nucleocapsid but also interact with the cytoplasmic tails of the Sendai virus glycoproteins [1053, 1054]. While no specific interaction between the M protein of RSV and the RSV glycoproteins has been identified, M is positioned so that such an interaction could occur and it is commonly accepted that M plays a central role in RSV virion organization and formation.

The Non-structural Proteins

The first two genes in the RSV genome are the non-structural proteins 1 (NS1) and 2 (NS2). The NS1 gene is 532 nucleotides in length and encodes a 13.8 kDa protein involved in blocking the interferon α/β response in RSV infected cells. The NS2 gene is 503 nucleotides in length and encodes a 14.5 kDa protein that is also involved in blocking the interferon
(IFN) α/β response in RSV infected cells. The exact mechanisms that NS1 and NS2 employ to interfere with IFN α/β induction and signaling will be discussed in more detail in a later section. Deletion of NS1 or NS2 separately or in combination results in a virus that is attenuated both in vitro and in vivo, most likely because of their roles in defeating the IFN system [1045, 1055-1060]. These viruses are also attenuated in Vero cells, however, which lack a competent IFN α/β pathway, indicating that NS1 and NS2 may play important yet undiscovered roles in RSV infection [1056]. In an RSV minireplicon system, NS1 was shown to strongly inhibit RSV replication and transcription but neither the mechanism nor the importance of this function are known [1061].

**RSV infection**

**Attachment and Entry**

RSV has been shown to infect the luminal ciliated epithelial cells of the respiratory tract through experiments using the well differentiated human airway epithelial cell model (HAE) [992]. This mirrors the infection pattern seen in infected patients and in animal models with the inclusion of type I pneumocytes in severe lower respiratory tract disease [994, 995].

Initial RSV attachment is believed to occur through the G protein [1062]. The G protein has been shown to bind to N-sulfated, iduronic acid containing glycosaminoglycans (GAGs), specifically heparan sulfate and chondroitin B sulfate, which are present on the cell surface of most cells [1063, 1064]. The heparin binding domain of the G protein, which contains multiple basic amino acids, is probably responsible for this attachment. GAGs may not be the G protein receptor on respiratory cells however, since heparan sulfate cannot be
detected on the apical surface of the ciliated cells infected by RSV [931]. Other potential G protein receptors (annexin II, L-selectin, fractilkine receptor) are being studied [1065, 1066]. The G protein is not absolutely required for attachment of RSV in vitro; the F protein can mediate attachment albeit less efficiently, presumably through binding GAGs [1031]. In fact, a B1 strain virus that was grown in Vero cells kept at successively colder temperatures developed a spontaneous deletion of the G gene, as well as SH [1030]. In vivo, however, ΔG viruses are highly attenuated indicating that the auxiliary attachment function of F is not efficient during natural infection [1038].

RSV enters a cell through membrane fusion mediated by the F protein and F is the only glycoprotein absolutely necessary for entry. The F receptor is not known but several candidates (RhoA and ICAM-1, for example) are being studied [1067-1069]. Cleavage of the F protein in the trans Golgi, yields the disulfide linked F1 and F2 subunits and exposes the hydrophobic “fusion peptide” at the N-terminus of the F1 subunit. The F protein exists on the surface of RSV virions in this metastable form, waiting to be triggered by contact with its receptor. Once triggered, the F protein undergoes a dramatic conformational change resulting in insertion of its fusion peptide into the target cell membrane. The F1 segment then folds back on itself bringing together the hydrophobic, heptad repeat regions, HRA and HRB, located adjacent to the fusion peptide and the transmembrane domain, respectively. The HRA and HRB associate to form an anti-parallel “six helix bundle” bringing the virion and cellular membranes together and initiating membrane fusion [1070].

Once the virion and cellular membranes have fused, the RSV nucleocapsid and virion-incorporated proteins are spilled into the cytoplasm of the newly infected cell and are
immediately ready for transcription. The exact process by which the nucleocapsid detaches from the virion envelope and more specifically the M protein to become free in the cytoplasm is not well understood.

Transcription and Replication

RSV transcribes and replicates its genome in the cytoplasm of infected cells with no nuclear involvement then uses the cellular machinery to translate its proteins. Polymerase complexes bound to RSV nucleocapsids form dense cytoplasmic inclusion bodies in infected cells [1071]. Experiments performed in a cell-free system have shown that cellular actin and profilin were required for efficient RSV transcription and replication potentially by forming the structure on which these processes occur [1072-1077].

Early in infection transcription is favored over replication until enough N protein has been produced to encapsidated the newly created antigenome and genome. The polymerase complex required for transcription consists of the N, P, L and M2-1 proteins and is packaged in the virion so transcription can begin immediately upon the arrival of the nucleocapsid in the cytoplasm of the infected cell. There is no evidence that transcription or replication occurs within the virion. It has been shown, in a cell free system, that the P and L proteins are sufficient for promoter recognition, RNA synthesis, 5’ capping and 3’ polyadenylation [1078]. For efficient transcription of all RSV genes, though, the M2-1 “anti-termination factor” is also required. The promoter for transcription is located at the 3’ end of the genome in the Le region (nucleotides 1-11), this is the only promoter in the RSV genome. This region is also believed to function as the polymerase binding site. It is not known for certain how the polymerase gets around the nucleocapsid protein that encapsidates the
genome to reach its binding site on the genome but it is assumed that either the polymerase temporarily displaces the N protein from the genome or it can reach the nucleotide bases between the whorls of N protein due to the flexibility of the nucleocapsid. The first 15 nucleotides of the Le plus a GS are required to initiate transcription; the efficiency of transcription is greatly increased though with the inclusion of nucleotides 36-44 of the Le, a uridine rich region located directly before the NS1 GS [1006, 1079]. It is not known for certain how the polymerase gets from the promoter region at the beginning of the Le to the NS1 GS where it begins transcription. One suggestion is that if the Le is transcribed but not capped, because there is no GS preceding it, transcription would terminate after ~50 nucleotides allowing the polymerase to restart transcription at the NS1 GS [1080]. Transcription begins at the first nucleotide of the GS though it is not known how the polymerase recognizes this as the location to begin transcription. The GS of the first nine RSV genes is CCCCGUUUA U/C, the GS of the L gene is CCCUGUUUUA but this difference in sequence has no obvious effect on transcription efficiency [1007]. Each mRNA is capped and methylated at the 5’ end with an m7G(5’)ppp(5’)Gp cap and capping of the mRNA seems to be required for efficient transcription of the rest of the gene [1013].

After transcribing a gene the polymerase encounters a GE. The GE is a semi-conserved sequence (UCAA/UG x3-4 UUUU) that directs both polyadenylation of the mRNA and the release of the mRNA from the template/polymerase [1007]. It is believed that polyadenylation occurs as a result of polymerase “slippage” on the tract of uridines at the end of the GE with each “slip” resulting in the addition of one adenosine to the 3’ end of the mRNA [1081]. After release of the nascent mRNA, the polymerase complex scans along the genome for the next GS at which it reinitiates transcription. The GEs vary in their ability to
terminate transcription leading to “read-through” mRNAs. Some “read-through” mRNAs are common (NS1/NS2, NS2/N) due to frequent inefficient termination, while others are very infrequent (SH/G) due to very faithful termination [1007, 1082-1084]. Studies have shown that termination efficiency is, in part, due to the number of uridines at the end of the GE [1085]. In most cases, only the upstream ORF will be translated from a “read-through” mRNA leading to some degree of “transcriptional attenuation”. The presence of M2-1 at high concentrations has also been shown to increase “read-through” at the gene junctions [1023, 1086, 1087]. The same gene junctions that are more prone to GE mediated “read-through” are also more susceptible to M2-1 mediated “read-through”. In this case, a role of M2-1 may be to deliver more polymerase to the distal regions of the genome. In any case, read-through accounts for only 10% of the total mRNA [997].

The first nine genes of RSV are non-overlapping however an overlap of 68 nucleotides exists between the M2 and L genes meaning that the GE of the M2 gene is in L and the GS of the L gene is located in M2. This unique arrangement requires that, when the polymerase is finished transcribing M2, it must scan backward to find the L GS indicating that the polymerase is capable of scanning for a GS in both the forward and reverse directions [1088]. It has been determined experimentally that ~90% of the transcripts that begin at the L GS terminate at the M2 GE making the complete transcription of L somewhat dependent on “read-through” at the M2 GE [1089]. This arrangement also suggests that polymerase complexes that terminate at the M2 GE have the potential to recycle back to the L GS to retry L transcription.
Genes are transcribed sequentially from the 3’ end of the genome in the start-stop fashion described above. Genes closer to the 3’ promoter are transcribed more frequently than downstream genes because a portion of polymerase disassociates from the genome template at each gene junction resulting in a polar transcription gradient. It is not well understood what causes this dissociation but, as with read-through, the potential for dissociation varies among gene junctions. Studies have indicated, however, that dissociation is independent of intergenic region length [1090, 1091]. Due to the conservation of GS sequences it is unlikely that they would play a role, so as with read-through, the GE is implicated in polymerase dissociation at the gene junctions. Studies have indicated however, that some sequences in the intergenic regions may affect polymerase activity at the GE [1084, 1092]. The higher concentrations of M2-1 that induce “read-through” at gene junctions can also alleviate some of the afore mentioned transcriptional gradient by allowing more polymerase molecules to reach the downstream genes.

Once enough N protein has been produced to encapsidate the RSV genome and antigenome, replication of the RSV genome can take place. It has been shown experimentally that the only proteins required for RSV genome replication are the N, P and L proteins [1093]. As mentioned previously, P and L are sufficient for promoter recognition and RNA synthesis but in the case of replication, concurrent encapsidation of the newly synthesized RNA is required for efficient antigenome and genome replication. As with transcription, the first 11 nucleotides of the Le serve as the replication promoter and the polymerase binding site. The first 15 nucleotides of the Le are required to initiate replication but the next 17 nucleotides are necessary for efficient encapsidation and replication of the entire genome. Replication of the RSV genome occurs in two steps: first,
complete complementary (+) sense antigenome copies are synthesized from the (-) sense genome template, second, the (+) sense antigenomes are used as a template for the synthesis of more (-) sense genome. Both the genome and antigenome are encapsidated by N protein as they are generated. It is assumed that a conformational change in N upon its binding to RNA is responsible for its release from the P protein allowing encapsidation to occur [1080]. Encapsidation serves to protect the genome and antigenome from degradation and reduce the formation of double strand RNA (dsRNA).

The 26 nucleotides at the 3' end of the genome in the Le and the 3’ end of the antigenome in the trailer complement (TrC) are highly conserved, indicating the presence of the genomic and antigenomic promoters, respectively, but their sequences differ thereafter [1079]. The antigenome is 10-20 fold less abundant than the genome in infected cells indicating that the antigenomic promoter directs a higher level of replication than the genomic promoter [1094]. Saturation mutagenesis has been used to determine that the differences in sequence of the Le and TrC dictate that the Le can promote efficient replication as well as transcription while the TrC is optimized for promoting only replication [1005, 1079, 1095]. The first 36 nucleotides of the TrC are sufficient to initiate replication and encapsidation but nucleotides 36-155 greatly enhance this process [1079, 1080]. RSV does not participate in homologous or non-homologous recombination. Only one recombination event has ever been recorded and that occurred under extreme recombination pressure in a laboratory setting [1096].

The factors that dictate whether the polymerase complex is going to participate in transcription or replication upon binding to the genome are not well understood. One
possibility is that the initial interaction of the polymerase with the 3’ end of the Le will
determine what process that polymerase complex will participate in. It is also possible that
the polymerase complex exists in two forms: a transcriptase consisting of L, P and M2-1 and
a replicase consisting of L, P and N [1080]. Binding of the transcriptase to the genome
template dictates that transcription will occur, while binding of the replicase to the genome
will mean that the antigeneome will be produced. It has been suggested that N and M2-1
share a binding site on the P protein which would allow only one to be a component of any
given polymerase complex supporting the transcriptase/replicase hypothesis [1080]. To
date however, two forms of the RSV polymerase complex have not been isolated. A third
possibility is that the potential for replication versus transcription is determined by the
concentrations of specific RSV proteins. The M2-2 and N protein are believed to play major
roles in the switch from initial RSV transcription to genome replication. RSV lacking the M2-2
gene exhibits increased transcription and decreased replication [1026, 1097, 1098].
Increased levels of N have been shown to cause increased levels of replication but do not
affect the ratio of replication to transcription suggesting that M2-2 and N may work
cooperatively to dictate the activity of the polymerase [1019]. Early in infection when
transcription is needed to produce enough N protein to participate in replication, the
amount of M2-2 is also low allowing transcription to be the favored process. During
infection while the levels of N are increasing so are the levels of M2-2. M2-2 may act to
dampen transcription in favor of replication which is then possible due to the higher levels
of N. During replication the GS and GE signals flanking each RSV gene must also be ignored
in order to replicate the entire genome. It is unknown how RSV accomplishes this but in
Sendai virus it is believed that concurrent encapsidation of the newly synthesized RNA
increases processivity of the polymerase making it easier to ignore these signals [1099, 1100].

**Budding**

Once the RSV proteins have been produced and the genome has undergone sufficient amplification, RSV begins to form infectious virions. RSV buds into irregular spherical virions ~100-350 nm in diameter or filamentous particles 60-200 nm in diameter and up to 10 μm in length. The envelope of the RSV virion consists of a lipid bilayer derived from the host cell’s plasma membrane. In polarized cells, budding occurs from the apical surface [992, 1101]. Movement of the glycoproteins to the apical surface involves the cellular apical recycling endosome pathway [1102]. Once at the cell surface, the RSV glycoproteins collect in lipid rafts [1103-1105]. Lipid rafts where RSV glycoproteins collect tend to have high concentrations of sphingolipids, cholesterol, caveolin-1 and ganglioside GM-1. The significance of the association of the RSV glycoproteins with lipid rafts, however, is yet to be determined [1106, 1107]. The M protein accumulates below the lipid rafts containing the viral glycoproteins, bringing the nucleocapsid to be packaged into the envelope. RSV budding is believed to require both M and F but it is unknown as to whether it also requires the RSV nucleocapsid which has been shown to associate independently with lipid rafts [1051].

The genome and antigenome tend to exist in virions in equal proportions implying that there is no discrimination in binding to M or packaging into virions [1094]. It is known that the NS1, NS2, SH, G, M2-1 and M2-2 proteins are not absolutely required for budding [1040]. G and M2-2, however, have been shown to enhance virion formation. Actin is
believed to play a major role in virion formation and release as actin is packaged into the RSV virion and disruption of actin filaments leads to reduced virion formation [1076, 1077]. Microtubules are also believed to play a role in RSV virion formation and release and RhoA has been shown to be required for filamentous virion formation as well as cell-to-cell fusion [1067]. RSV may produce defective interfering particles but this possibility has not been thoroughly studied [1108]. It has been found that a large portion of the RSV virions produced in vitro appear to be empty and non-infectious [1109].

**RSV Disease**

**Epidemiology**

RSV is the most common cause of hospitalization due to lower respiratory tract disease in infants and children in the world. In a 13 year surveillance study of infants in Washington D.C., RSV was present in ~23% of all hospitalizations for respiratory tract disease and ~40% of all hospitalizations for lower respiratory tract disease (43% of all bronchiolitis cases and 25% of all pneumonia cases) [1110, 1111].

The WHO estimates that there are 64 million RSV infections annually worldwide. Most individuals become infected with RSV during their first years of life with the peak age for infection occurring between 2-4 months [1111, 1112] In a two year surveillance study, ~69% of at risk infants were infected during their first RSV season and ~83% were infected during their second RSV season with all the children being infected at least once by the age of 24 months [1113]. A child’s environment can also affect the chance of RSV infection; while ~50% of susceptible infants will become infected with RSV during an outbreak, infection can be as high 98% for children in day care settings [1114]. Healthy infants usually
develop only upper respiratory symptoms with RSV infection, however in a portion of those infected RSV disseminates below the larynx resulting in lower respiratory infection (LRI). Of those that develop LRI, ~0.5% (1-20 per 1000 infants) under 1 year of age, will require hospitalization; this can be as high as 25% for high risk infants [1113]. Risk factors for LRI include: prematurity, low birth weight, chronic cardiac/pulmonary disease, immunodeficiency, family history of allergic disease, low socioeconomic status and exposure to smoking. Infants with cystic fibrosis are at increased risk of developing severe disease and being hospitalized due to RSV infection but their need for hospitalization appears to decline with age as it does for healthy children [1115-1117]. It has also been found that native populations including Native Americans, native Alaskans and the Maori of New Zealand have increased frequencies of severe disease [1118-1120]. Death due to RSV-induced disease is uncommon in developed countries. Estimates from the 1970s put the risk of death at 0.5-2.5% for hospitalized individuals with RSV in the United States while more recent estimates put the risk at <0.3% with the vast majority of deaths due to severe RSV-induced disease occurring in those with underlying congenital cardiac/pulmonary disease or immunodeficiency [1121-1123]. More recent studies have estimated that ~100 children die annually of complications due to RSV infection in the U.S. However, RSV may be responsible for >17000 deaths/year in the elderly population [1124]. This study underlines the growing evidence that RSV may be an important pathogen in the ever increasing elderly population. In another study 3-7% of healthy elderly and 4-10% of high risk elderly, those with underlying cardiac/respiratory disease or those who are immunocompromised, developed serious RSV-associated disease again indicating the importance of this pathogen in this population [1125].
RSV outbreaks show distinct seasonality in temperate climates. Outbreaks can occur in late fall through early spring with the peak months being February and March [1110, 1126, 1127]. It has been noticed, though, if a large outbreak occurs early in a season there may be a smaller late season outbreak [1128]. Outbreaks are hardly ever seen in the summer months and it is not known how RSV can seemingly disappear for the summer only to reappear in the fall. There is typically a 1-2 year alternating pattern of subtype dominance but both A and B subtypes can co-circulate during any given RSV season.

RSV is unique in that it can reinfect individuals throughout their lifetimes, even with the same or a very similar strain of virus indicating that antigenic drift is not required for host susceptibility [1113, 1114]. Reinfection with an alternate subtype is favored but is not a requirement for reinfection. A study done with healthy health care providers who had just had a recent natural RSV infection demonstrated that individuals could be reinfected with a lab strain of RSV 2, 4, 8, 14, 20 and 26 months after their initial infection. At each challenge time point ≥25% of the study participants became reinfected. Within the 26 months, 73% had ≥2 infections and 47% had ≥3 infections [1129]. Reinfection can result in symptomatic or asymptomatic disease but as a general rule symptoms do tend to be reduced during reinfection. Elderly and immunocompromised patients, however, can often develop severe lower respiratory tract disease upon reinfection with RSV.

**Clinical Manifestation**

As mentioned previously, most RSV infections result in mild self-limiting upper respiratory disease. In the infant population these symptoms may include: rhinorrhea, sneezing, low grade fever, cough, decreased appetite and wheezing. Episodes of apnea may also be
present in infants born prematurely or those <6 weeks of age but these are seldom of any concern and RSV infection is not believed to be associated with sudden infant death syndrome [1130]. The incubation time or the time from initial infection to the development of symptoms is 4-5 days with the symptoms generally resolving within 7-12 days [1131]. In severe cases coughing and wheezing may lead to dyspnea or tachypnea indicating lower respiratory tract involvement. Lower respiratory tract symptoms usually begin within a few days of the initial upper respiratory tract symptoms. LRI will develop in 25-40% of primary RSV infections most frequently causing bronchiolitis or pneumonia [1113]. Bronchiolitis and pneumonia occur most frequently in infants 6 weeks to 9 months of age with the peak chance occurring between 2-7 months [1111, 1112]. Bronchiolitis is the obstruction of bronchioles and alveoli due to the necrosis and proliferation of the bronchiolar epithelium, loss of ciliated epithelial cells, peribronchiolar infiltration of leukocytes, lymphocytes, macrophages and plasma cells, edema of the submucosal and adventitial tissues and the increased production of mucus [995]. Increased airway resistance and “air trapping” are also characteristic of bronchiolitis. Pneumonia is the filling of alveolar spaces with fluid because of alveolar wall thickening due to mononuclear cell infiltration [995]. Of those experiencing bronchiolitis or pneumonia, 1-3% will require hospitalization [1094]. Dehydration, respiratory distress and hypoxia are indications that hospitalization may be needed [1132]. Infants and children with underlying cardiac/respiratory disease may experience a more rapid progression of symptoms.

Abnormalities in pulmonary function have been known to occur and persist after resolution of RSV-induced bronchiolitis and pneumonia. These abnormalities include: decreased pulmonary function, increased frequency of coughing and wheezing, asthma and bronchitis
These abnormalities can occur in up to 50% of patients recovering from RSV-induced bronchiolitis and pneumonia, but tend to resolve by the time the child is 8-10 years old [1136]. One study indicated, however, that it is the development of bronchiolitis, not the RSV infection, which predisposed the children to later pulmonary abnormalities [1137]. Also, it has been suggested that it is the host response to infection, rather than the infection itself that predisposes a child to later allergic disease [1138]. It is unclear at this time, however, whether past bronchiolitis or pneumonia predisposes a child to abnormal pulmonary function later in life, or whether having a propensity for reduced pulmonary function predisposes a child to develop bronchiolitis or pneumonia upon infection with RSV. Studies have indicated, though, that there is a strong correlation between having a prior reduction in pulmonary function and developing RSV-induced bronchiolitis with increased wheezing post-recovery [1134, 1139]. Other studies have shown that if a parent developed bronchiolitis with post-recovery pulmonary function abnormalities after RSV infection, this increases the child’s risk of developing the same disease [1140]. This “genetic predisposition” suggests that a child’s genetic background plays a role in the risk of developing severe disease in response to RSV infection as well as the potential for lingering pulmonary function abnormalities after recovery. It is most likely that the risk of developing abnormalities in pulmonary function following RSV-induced bronchiolitis or pneumonia depends on many factors including: the age at which the child was first infected, the presence of an anatomical/functional respiratory abnormality, a tendency toward hyper-reactivity or other aberrant immune responses, the frequency of recurrent respiratory infections, RSV persistence, genetic background and the child’s exposure to cigarette smoke.
Otitis media is another complication associated with RSV infection. Otitis media is a type of ear infection usually caused by *Streptococcus pneumonia*, *Haemophilus influenzae* (typeable and non-typable) and *Moraxella catarrhalis*. RSV proteins and nucleic acid have both been found in the middle ear along with the colonizing bacteria suggesting that RSV may play a role in this disease [1141, 1142]. It has been shown that bacterial superinfection after infection with RSV is unusual so prior presence of the bacteria or simultaneous infection with RSV and the bacteria is more likely with otitis media occurring if RSV travels to the middle ear [1143, 1144].

Healthy adults that develop symptomatic RSV infection can present with: rhinorrhea, cough, pharyngitis, fever, headache, fatigue or bronchitis. Infection is usually limited to the upper airways however and usually lasts ~5 days. As mentioned before, adults with underlying cardiac/pulmonary disease or immunodeficiency, as well as the elderly, can develop severe lower respiratory tract disease upon infection with RSV usually leading to pneumonia.

**Pathogenesis**

RSV is spread by large droplet respiratory secretions or fomite contamination with such secretions. Once an individual has come into contact with RSV, initial virus replication probably takes place in the nasopharynx. As mentioned previously, RSV infects the apical ciliated cells of the respiratory epithelium as shown by infections of pulmonary explants and well differentiated HAE cultures. Infection can be patchy *in vivo* and groups of infected cells can be found in the bronchial, bronchiolar and even alveolar epithelium [994, 995]. Spread is believed to occur primarily by aspiration of pulmonary fluid deeper into the lung. Nasal washes from infected infants have suggested that in the upper airway RSV virions are
produced at $10^5$ plaque forming units per milliliter [1145]. Bronchiolar-alveolar lavage samples taken from intubated patients indicate a similar production of virions in the lower respiratory tract. Infection is believed to be limited to the lungs as only a few cases of RSV viremia have been reported [1146]. RSV proteins and RNA have been detected, however, in circulating mononuclear leukocytes [1147, 1148].

Infection alone does not cause much gross pathology as determined by experiments in HAE cultures [992]. Cell to cell fusion does not occur frequently in vivo, though it is a hallmark of giant cell pneumonia seen in patients with extreme T cell deficiency [1149]. RSV does cause sloughing of the infected ciliated cells which can result in decreased movement of the bronchociliary apparatus leading to the accumulation of cell debris in the airway [1150]. RSV has also been shown to increase the volume of airway surface liquid in vivo through the inhibition of ENaCs, possibly providing a mechanism for spread [1151]. RSV may also contribute to airway remodeling through the activation of matrix metalloproteinases [1152, 1153].

It is the accumulation of cell debris and the increase in airway surface liquid plus the infiltration of immune cells, such as monocytes, macrophages, granulocytes and lymphocytes, occurring due to RSV infection that results in clinical RSV disease. Infants have smaller diameter airways so they are easier to obstruct which is one reason why infants are more susceptible to severe RSV-mediated bronchiolitis. The exact mechanism of RSV-induced cell death is unknown. Cells could be undergoing apoptosis stimulated by the perturbation of the plasma membrane, the immune response to the expression of foreign antigen or a general stress response to infection. The F protein alone has been implicated in
mediating p53-dependent apoptosis of RSV infected cells [1154]. However, other reports suggest that RSV can inhibit apoptosis [1049, 1155-1158]. Infected cells could also be killed directly by immune cells; natural killer (NK) cells and cytotoxic T lymphocytes (CTLs) can both destroy RSV infected cells via perforin/granzyme mediated lysis, leading to tissue damage and increased debris in the airway. Also, the binding of neutrophils to infected cells has been shown to augment epithelial cell damage and detachment [1159]. Indeed, the contributions of direct RSV-mediated cytopathology and immune mediated cellular damage to RSV-induced disease are not fully understood.

RSV can cause serious disease in immunocompromised patients indicating that a fully functioning adaptive immune response is not required for disease. However, disease pathogenesis in these patients is different than that seen in cases of severe RSV-mediated disease in individuals with a fully functional immune system; they tend to develop alveolitis rather than bronchiolitis, indicating that there may still be a role for the immune response in mediating severe RSV-induced bronchiolitis [1160]. Studies have indicated, though, that there is a strong correlation between the level of RSV replication and disease severity indicating that RSV-mediated cytopathology does play a role in the pathogenesis of RSV-associated disease [1145, 1161]. However, reducing RSV replication in an already infected individual does not markedly decrease disease pathology [1162-1165]. Also, there is broad disease heterogeneity even when the infecting strain of RSV is the same or very similar indicating that host factors are definitely involved in RSV-associated disease pathogenesis. In the end, RSV-associated disease is probably dependent on multiple viral activities as well as the host responses to those activities. In addition to disease induced by RSV alone, RSV infected cells have been shown to bind *Pseudomonas aeruginosa*, nontypable *Haemophilus*
influenza, and Streptococcus pneumonia more readily indicating that RSV infection may increase the chance of developing an infection with one of these bacterial pathogens [519, 1166].

During the course of a synchronous RSV infection of cultured cells, RSV mRNA and protein can be detected at 4-6 hours post infection (hpi) but reach their peak at 12-16 hpi and 18-20 hpi, respectively. The release of RSV virions can be detected as early as 8-10 hpi but peaks at ~24 hpi and continues until the infected cell dies between 30-48 hpi [1094]. RSV infection of HAE cultures peaks 2-3 days post infection then the number of infected cells decreases rapidly to ~25% of the originally infected cells and remains steady for up to 3 months [992]. In the mouse, RSV replication peaks at 4 days post infection then the virus is rapidly cleared. Only scattered cells become infected in the mouse and while mice do not show overt signs of disease they do exhibit weight loss, ruffled fur and some changes in pulmonary function upon RSV infection. Even though there are few signs of RSV disease in the mouse, histopathological changes in the airways can be seen [1002, 1167]. RSV is not believed to cause a persistent or latent infection however recent studies have indicated that mice can remain positive for RSV RNA and protein up to 100 days post infection and cultured cells can remain infected for >3 years [1168-1170]. Some have suggested that RSV infection of immune cells or pulmonary nerve cells may play a role in RSV persistence/latency [1171, 1172]. In addition, studies in the mouse have suggested that persistence of RSV infection may play a role in inducing airway hyperreactivity [1173, 1174].
RSV infection has little effect on global cellular protein synthesis. RSV infection does however alter the expression of >1300 genes including, but not limited to: chemokines, cytokines, antiviral factors, growth factors, cell surface receptors/markers, signaling molecules, transcription factors and cytoskeletal components [1175, 1176]. Most of the alterations are increases in expression but some decreases have been noted. RSV infection can also cause diminished cellular DNA and RNA synthesis [989]. As a whole, the alterations seem to represent a typical response to cellular injury.

**Immune Responses to RSV**

**Innate Immune Response**

The innate immune system is responsible for initial identification and defense against invading pathogens. The innate immune response also helps to determine the magnitude and quality of the adaptive immune response. The first line of the innate immune system is its pathogen recognition receptors which recognize pathogen associated molecular patterns. Of the pattern recognition receptors, the Toll-like receptors (TLRs) and the cytosolic receptor, retinoic acid inducible gene I or RIG-I, are the most important in the recognition of RSV infection. Activation of the innate immune response through the TLRs and RIG-I results in the production of multiple cytokines and chemokines that contribute to the migration of innate immune cells and the activation of the adaptive immune system in response to RSV.

RSV proteins as well as its replication and transcription are responsible for the activation of the host’s pathogen recognition receptors. Studies have shown that the F protein interacts directly with the TLR-4 and CD14 receptor complex resulting in activation of the NFκB
signaling pathway in epithelial cells [1177, 1178]. The recognition of RSV infection by RIG-I also results in the activation of NFκB as does the activation of the phosphatidylinositol 3-kinase pathway in infected cells [1155, 1179]. NFκB is a transcription factor that, once activated, translocates to the nucleus causing the production of cytokines, specifically RANTES and interleukin-8 (IL-8) [1180]. Additional studies have shown that the M2-1 protein and the NS2 protein of RSV can also activate NFκB while other studies suggest that the G protein may inhibit its nuclear translocation [1037, 1060, 1181]. The stimulation of other cytokines and chemokines in response to RSV infection is dependent on the activation of the TLR-3 pathway. TLR-3 is located in the endosome of some immune cells and expression on the surface of epithelial cells can be induced by RSV infection [1182]. The exact mechanism by which RSV stimulates TLR-3 in epithelial cells is unknown but in immune cells, specifically dendritic cells (DCs), it is believed that endocytosis of RSV antigens causes TLR-3 activation. Pulmonary DCs are believed to play an important role in eliminating RSV through their secretion of cytokines and their presentation of antigen [1183].

In addition to IL-8 and RANTES, RSV stimulates the production of IL-1β, 2, 4, 6 and 10, IFNγ, TNFα and macrophage inflammatory protein 1α/β (MIP-1α/β) [1184-1186]. Infants requiring hospitalization for severe RSV-induced bronchiolitis display increased levels of IL-8, RANTES, TNFα and MIP-1α [1186-1188]. Also, certain polymorphisms in genes encoding IL-4, 8 and 10 as well as TNFα, the IL-4 receptor, TLR4 and CD14 are overrepresented in infants that develop serious RSV-associated disease and post-RSV reactive airway disease suggesting their importance in disease pathogenesis [1189-1192]. TNFα, for instance, has been shown to play a direct role in inhibiting RSV replication,
however, it has also been shown to contribute to RSV-induced pathology in the mouse model [1193, 1194]. One of the major roles of cytokines and chemokines in the innate immune response is to attract and activate leukocytes (monocytes, macrophages, natural killer cells, neutrophils, basophils and eosinophils) as well as lymphocytes (CD4+ and CD8+ T cells and B cells). Neutrophils are the predominant innate immune cell present during RSV infection and these are attracted by IL-8 [1195]. RANTES and MIP-1 α/β attract and activate eosinophils, among other leukocytes, which are believed to play a role in severe RSV-mediated disease [1196-1198]. MIP-1 α also induces the migration and activation of NK cells [1199]. According to mouse studies, neutrophils arrive first in the airways of RSV infected mice and are present on days 1-6 of infection. NK cells arrive around day 3 of infection. The presence of monocytes peaks between days 3-11. Lymphocytes enter the airways between days 4-8 of infection and reach peak numbers around day 11 [1200, 1201].

Leukocytes perpetuate the immune response by secreting additional immunoreactive molecules and some, such as NK cells, have the ability to directly kill RSV infected cells. Other innate immunoreactive molecules thought to play a role in the immune response to RSV include cysteinyi leukotrienes substance P and surfactant protein A. RSV infection stimulates the expression of substance P, a neuropeptide that has been shown to stimulate the production of cytokines as well as attract immune cells such as macrophages, NK cells and T lymphocytes [1202, 1203]. Surfactant protein A is a C-type lectin that specifically binds to the RSV F protein and is believed to enhance phagocytosis of RSV virions [1204, 1205]. Cysteinyi leukotrienes have been found in the nasalpharyngeal and tracheobronchial
secretions of infants with RSV and are believed to play a role in airway obstruction [1206-1208].

IFNs are another major group of antiviral signaling molecules that are activated during an innate immune response. Type I IFNs (IFN $\alpha/\beta$) are expressed after TLR stimulation leads to the phosphorylation and nuclear translocation of interferon regulatory factor 3 and 7 (IRF3, IRF7). IRF3 and IRF7 form the transcription factor complex that is responsible for the induction of IFN $\alpha_4/\beta$ transcription. IFN $\alpha_4/\beta$ is secreted and can then signal through its own pathway consisting of the Janus kinases (JAK) and signal transducers and activators of transcription proteins 1 and 2 (STAT1, STAT2) to induce transcription of all IFN $\alpha/\beta$ genes, thereby amplifying the production of IFN. IFN also stimulates transcription of antiviral genes. RSV, however, stimulates the production of much less IFN $\alpha/\beta$ than most other viral pathogens. Furthermore, RSV is much less sensitive to the antiviral effects mediated by IFN $\alpha/\beta$ [1209-1213]. This is probably due to the activities of the RSV NS1/2 proteins.

NS1, along with NS2, inhibits the phosphorylation and nuclear localization of IRF3 by reducing the level of the kinase (IKKe) that is required for IRF3 phosphorylation [1060, 1214]. Lack of nuclear IRF3 leads to lack of IFN $\alpha/\beta$ production following RSV infection. Deletion of either NS1 or NS2 causes RSV to become a much better stimulator of IFN $\alpha/\beta$ production [1059]. NS2, along with NS1, is also responsible for the proteosome-mediated degradation of STAT2 [1057, 1215, 1216]. Lack of STAT2 leads to decreased expression of interferon stimulated genes in response to IFN $\alpha/\beta$ or $\lambda$, a type III IFN which also signals through STAT2. Studies in STAT1 -/- mice have shown, however, that a complete lack of IFN $\alpha/\beta$ signaling results in increased RSV replication and increased lung pathology following
RSV infection indicating that, although it is not produced in significant amounts, IFN $\alpha/\beta$ must play some role in the immune response to RSV [1217]. In addition, interferon stimulated genes such as Mx protein A, protein kinase R and 2', 5' oligoadenylate synthetase are expressed in RSV infected cells indicating that IFN $\alpha/\beta$ signaling is occurring even if substantial amounts of IFN $\alpha/\beta$ cannot be detected. The ability of NS1/2 to disrupt the IFN signaling pathway is also thought to be a determining factor in RSV host range [1218].

**Adaptive Immune Response**

The adaptive immune response to RSV consists of cell-mediated immunity and antibody-mediated immunity. Cell-mediated immunity is more responsible for the eradication of RSV while antibody-mediated immunity is more responsible for preventing reinfection. As mentioned previously, the effectiveness of the adaptive immune response highly depends on an appropriate innate immune response.

Cellular immunity is mediated by CD4$^+$ and CD8$^+$ T cells. The RSV F and G proteins are able to induce epitope specific CD4$^+$ cells. Multiple epitopes are recognized on the F protein however only the central conserved region of G is recognized [1219, 1220]. The NS2, N, M, SH, F, and M2-1 proteins can stimulate the production of RSV specific memory CD8$^+$ cytotoxic T lymphocytes (CTLs) with N, F and M2-1 expressing the dominant epitopes [1221-1224]. There are no CD8$^+$ T cell epitopes on the G protein. Immunodepletion studies conducted in mice have determined that both CD4$^+$ and CD8$^+$ T cells contribute to the eradication of RSV and the limiting of viral shedding but CD8$^+$ CTLs are required for complete clearance [1225]. These same studies also determined that both CD4$^+$ and CD8$^+$ T cells contribute to the pathology seen during RSV infection indicating that although cell-
mediated immunity is required to eliminate RSV from the host it may also play a role in the
development of RSV-mediated disease. Similar studies have also determined that neither
CD4+ nor CD8+ T cells play a significant role in preventing reinfection because their effects
are so short lived [1226]. It would appear then that although the host mounts CD4+ and
CD8+ T cell responses to RSV they are not fully functional. Studies have shown that although
RSV specific CTLs exist in the respiratory tract, they have impaired cytokine secretion and
cytotoxicity possibly due to a direct effect of RSV, specifically the F protein, on their
activation or because RSV is a poor inducer of IFN α/β which is required to establish T cell
functionality [1227-1231]. This lack of effector function is specific to the RSV-specific T cells
in the lung as RSV-specific T cells in secondary lymphoid tissue maintain their effector
functions [1232]. The CD4+ and CD8+ T cells that are present during RSV infection also
appear to die out following resolution rather than developing into fully functioning memory
cells [1232]. This could again be due to direct suppression by RSV or a result of the lack of
IFN α/β production. Additional studies have also shown that reinfection with RSV does not
boost virus specific CTL responses [1233]. The presence of RSV specific T cells in the
respiratory tract during infection but their inability to effectively clear the virus could result
in prolonged inflammation and enhanced pulmonary injury.

Once stimulated with antigen, CD4+ T cells are able to differentiate into type 1 or type 2 T
helper cells, Th1 and Th2 respectively, depending on the context of antigen stimulation and
the cytokine environment. A Th1 response seems to be better at eradicating RSV while a
Th2 response seems to be more pathogenic to the host and somewhat less efficient at RSV
elimination. Th1 cells secrete IFN γ which enhances the antigen presentation of professional
antigen presenting cells and the antiviral effects of CD4+ and CD8+ T cells leading to rapid
and efficient eradication of the invading pathogen. On the other hand, Th2 cells secrete IL-4, 5 and 13 which play a role in generating an environment of hyperresponsiveness. IL-5 and 13 stimulate the migration and activation of eosinophils which are believed to play a role in the development of severe RSV-mediated disease as well as post-RSV reactive airway disease [1234-1236]. Eosinophils also stimulate the production of histamine and leukotrienes which play a role in prolonged wheezing and reactive airway disease following RSV-mediated bronchiolitis [1207, 1237]. For these reasons, it has been suggested that a Th2 biased response may play a role in RSV infection and disease pathogenesis. During an infection the production of Th1 cells is enhanced by the secretion of IFN-γ from NK cells and CTLs which also act to inhibit the production of Th2 cells [1201, 1238, 1239]. Infants, however, express reduced levels of IFN-γ during severe RSV infection [1240-1242]. Infants also display Th2 biased responses to pathogens partly because they have reduced CD8+ T cell responses due to their immunologic immaturity and also because their Th1 cells are directly inhibited as part of the mechanism used to prevent immune rejection of the fetus during pregnancy [1243-1245]. It has been shown that infants hospitalized for RSV-mediated disease exhibit a Th2 biased response to RSV while older children and adults exhibit a Th1 biased recall response [1246-1248]. Asthmatics may also be predisposed to developing a Th2 biased response leading to more severe RSV mediated disease and prolonged respiratory deficits [1249]. RSV itself is also believed to promote the generation of a Th2 biased immune response. The development of a robust Th1 response is dependent on the production of IFN-α/β, IL-12 and IFN-γ [1217, 1239, 1250]. The fact that RSV is a poor inducer of IFN-α/β has already been discussed. RSV also appears to inhibit the production of IL-12 from DCs and macrophages which then leads to the reduced production of IFN-γ from the T cells stimulated by these antigen presenting cells [1251-1255].
The RSV G protein is thought to directly stimulate the production of Th2 cells as shown by experiments where it alone was expressed by a vaccinia virus vector [1256]. When the F protein was expressed by the same vector it stimulated a Th1 response [1257]. This may be compounded by the fact that G is produced in a soluble form and does not stimulate the production of CTLs. Soluble antigens have been shown to be inefficient at stimulating CD8+ T cell responses and indeed sG stimulates an enhanced Th2 response [1258]. A shift to a Th2 biased response was also seen when the F protein was expressed alone in a soluble form [1259]. In the presence of a “normal” immune response any stimulation toward a Th2 bias exhibited by the G protein is probably overcome by the responses generated toward the other RSV proteins. This is supported by experiments in which the G protein was fused to a CD8+ T cell epitope from the M2-1 protein then expressed by a vaccinia virus vector. In this case, there was suppression of the G-specific Th2 response [1238]. This is also supported by data from the mouse model of RSV disease where the initial response is a mixed Th1/Th2 response but early production of IFN γ, presumably by infiltrating CD8+ T cells, stimulates a shift toward a Th1 response [1239, 1250, 1260]. Indeed, lack of IFN γ signaling in mice skews the Th1/Th2 cytokine balance in response to RSV infection toward a Th2 response [1250, 1261]. In an infant however, where the immune system is already Th2 biased, the additional Th2 stimulation by the G protein probably ensures that the overall CD4+ response will be Th2 biased. It has been suggested that the immune response to a child’s first RSV infection may affect his or her responses to subsequent infections, a phenomenon termed immunological imprinting. This may especially be the case if the infection took place during a critical time in the development of the lung or the immune system or if the child is in some way genetically susceptible to aberrant immune responses. It may also mediate long term changes to either the structure of the lung or the quality of
future immune responses [1262-1266]. Experiments performed in the mouse have shown that if primary RSV infection occurs at an early age this leads to increased disease severity upon reinfection in adulthood [1262, 1267].

The only RSV proteins that stimulate the production of neutralizing antibodies are F and G [1226, 1268, 1269]. The antibodies generated against F and G are sufficient to neutralize RSV infectivity and confer some resistance to reinfection. Many of the neutralizing antibodies generated against F inhibit its ability to induce fusion and are conformation dependent as they do not recognize the denatured form of the F protein. The four major antigenic sites, all of which are located in F1, induce the production of cross-neutralizing antibodies that recognize both A and B subtypes of RSV [1270-1272]. Some G neutralizing antibodies recognize conserved epitopes located in the central conserved region of G and are thus cross-neutralizing. Other G specific antibodies are subtype specific but most G neutralizing antibodies are strain specific due to the heterogeneity of the G protein. The antibodies that recognize the conserved and subtype specific epitopes react with G regardless of its glycosylation pattern. However, most of the strain specific antibodies react with the C-terminus of G and are dependent on glycosylation [1273]. Individual G neutralizing antibodies are not efficient at neutralizing RSV infectivity however combinations of them are indicating that G may be an inducer of poorly neutralizing antibodies [1274]. Also most G specific antibodies are not conformation dependent and thus recognize G in its native and a denatured state [1273, 1275]. Vaccine trials have determined that newborn infants primarily generate an antibody response to G [1276]. Infants have been shown to generate a blunted antibody response to both F and G however, most likely due to the presence of maternal serum antibodies [1277-1279]. Maternal antibodies are
present in an infant up to four months after birth and while these infants can still become infected with RSV, probably because their own immune systems are not completely functional, some studies have indicated that they are less susceptible to severe RSV disease when they were carried for the full term of pregnancy and have no underlying conditions [1280].

Even though RSV neutralizing antibodies are not required for elimination of the virus once infection has taken place, they are required to restrict the degree of infection upon re-exposure to RSV [1281]. The major antibody isotypes stimulated by F and G are nasal secretory IgA and serum IgG. Secretory IgA antibodies appear in the respiratory tract 2-5 days after RSV infection and are associated with a decrease in virus shedding [1282]. The presence of secretory IgA also confers resistance to reinfection [1283-1287]. RSV specific secretory IgA is very short lived however, because even though RSV-specific plasma cells are present at the site of infection, they fail to expand and eventually die out [1288]. Multiple RSV infections can boost the levels of nasal secretory IgA, however, symptomatic upper respiratory infection can still occur even with a short interval between infections [1114, 1283, 1284, 1289]. RSV specific serum IgG peaks at 20-30 days post infection and it can remain high for up to a year [1287]. Serum IgG, however, is relatively inefficient at entering the respiratory tract though it can gain access to the lower airways more readily than the upper airways [1290]. In healthy adults, most RSV infection of the lower airway can be prevented by RSV-specific serum IgG antibodies. Indeed, the challenge study done with healthy health care providers indicated that increased serum antibody levels correlated with less frequent reinfection and less symptomatic disease upon reinfection. This suggests that although protection from RSV infection is incomplete there is still enough
protection to shield the host from the more severe forms of RSV disease. Decreases in the magnitude of immune responses seen in the elderly are therefore probably a major factor in the susceptibility of this population to more serious forms of RSV-mediated disease.

There are multiple factors that contribute to the lack of a long lived immune response to RSV allowing for frequent reinfection. In addition to the immune evasion strategies already discussed, one possibility is that RSV is so infectious and the disease course is so rapid that the host is unable to properly lay the groundwork for a successful memory response. Another suggestion is that the tropism of RSV for the superficial epithelium of the respiratory tract and its low degree of invasiveness cause only minimal immune stimulation. Then there are the unusual antigenic properties of the G protein. G displays modest antigenic diversity and is believed to stimulate poorly neutralizing antibodies. G has been shown to inhibit the activities of NFκB, MIP-1 α/β and MIP-2 [1037, 1291]. G also contains a CX3C motif in its central conserved domain that has some similarity to the CX3C domain of the chemokine, fractalkine [1066]. The CX3C motif of G was shown to interact with the fractalkine receptor and possibly alter the trafficking and function of immune cells expressing the receptor such as monocytes, macrophages, NK cells, CTLs and Th1 CD4+ T cells [1066, 1292]. Th2 CD4+ T cells express only low levels of the fractalkine receptor and therefore may not be as responsive to G-mediated inhibition. It has been suggested that inhibition of the more effective virus clearing T cells may result in prolonged virus replication and thus enhanced pathology [1292].

Both soluble and membrane bound G have been shown to interfere with TLR responses to additional pathogen associated molecular patterns [1037]. In addition, sG has also been
shown to interfere with the activities of NFκB, IL-8 and RANTES [1293]. The primarily function of sG, however, is believed to be that of an antibody decoy which will minimize the effects of G neutralizing antibodies on RSV infectivity [1294]. RSV has also been shown to infect immune cells, such as monocytes and macrophages, and interfere with their trafficking, activation, maturation and function directly [1148]. RSV infection of plasmacytoid DCs has been shown to decrease their secretion of IFNα, while infection of myeloid DCs reduces their ability to activate CD4+ T cells [1295, 1296]. Even the ability of RSV to inhibit apoptosis may play a role in its immune evasion. Fewer RSV infected cells undergoing apoptosis means that there will be less free antigen to be phagocytosed by professional antigen presenting cells and presented to T cells. In addition, a recent study showed that, upon reinfection, RSV can infect cells and replicate without producing virions further limiting the ability of the immune response to identify and fight off this pathogen [1297].

Vaccines

There is no commercially available vaccine for RSV yet it has been and remains one of the pathogens deemed most important for vaccine development. Some of the reasons why no vaccine is currently available revolve around a failed vaccine trial in the late 1960s [1298-1300]. For these trials, the Bernett strain of RSV was grown in Vervet monkey kidney cells, formalin inactivated, concentrated by centrifugation then again by alum precipitation. Children 2 months to 9 years of age were given 1-3 inoculations of this inactivated vaccine (FI-RSV) intramuscularly. The vaccine was successful at boosting the RSV specific serum antibody levels however it did not protect the children from RSV infection. As a matter of fact, children <24 months of age that received the vaccine developed an enhanced disease
upon natural infection with ~80% of them requiring hospitalization. Children receiving the control vaccines did not develop enhanced disease or require more frequent hospitalization. Two children aged 14 and 16 months died after developing enhanced disease yet no fatalities occurred in the control group [1299]. The enhanced diseased was marked by high levels of serum antibody, increased RSV replication, increased lymphocyte proliferation, the increased presence of eosinophils and increased lung blockage [1301].

Later investigation into the cause of the enhanced disease seen after FI-RSV inoculation determined that the vaccine induced a weaker adaptive immune response than did natural RSV infection. The FI-RSV vaccine did not stimulate the production of secretory IgA antibodies because it was not delivered intranasally. In addition, the serum antibodies that were generated against F either did not recognize the true F neutralizing epitopes because they were altered due to the formalin inactivation process or they reacted with F at a low avidity meaning that they bound to F but did not effectively neutralize the virus [1302, 1303]. There is some suggestion that the non-neutralizing antibodies formed immune complexes with RSV virions thus causing even further tissue damage [1304]. The production of low avidity F antibodies has been linked to the fact that the FI-RSV vaccine did not replicate and therefore did not stimulate the proper TLRs [1302]. Non-replicating vaccines have also been shown to be inefficient at inducing CD8+ CTLs and indeed although the FI-RSV vaccine stimulated the production of CD4+ T cells, no RSV specific CD8+ T cells were found in the mouse model of enhanced disease [1305, 1306]. The mouse model also showed that there was a Th2 biased recall response to natural RSV infection following FI-RSV inoculation which probably would have been amplified in infants [1305]. In the mouse, depletion of CD4+ T cells, IL-4 or IL-10 eliminated the development of enhanced disease
The lack of an efficient recall response upon natural RSV infection probably led to increased RSV replication, compared with normal reinfection, as well as increased immune mediated damage in the infants who received the Fl-RSV vaccine leading to the development of enhanced disease. Older children who received the vaccine did not develop enhanced disease possibly because they had already developed an effective immune response to RSV based on their earlier exposures.

Most of the recent attempts at creating an effective vaccine for RSV have centered on live attenuated vaccine candidates. A live attenuated vaccine can be administered intranasally and will replicate which should allow it to stimulate an immune response similar to that of natural RSV infection. The main populations in need of a vaccine are infants and the elderly. The problem is developing a candidate that is attenuated enough not to cause disease, especially in the infant population, but that can still elicit a protective immune response even in the presence of neutralizing antibodies, either maternal or those from earlier contact with the virus, and an immature/deteriorating immune system. There is some concern though that a vaccine that would be attenuated enough for infants would be too attenuated for the elderly suggesting that there may be a need for two different vaccines.

The live attenuated vaccine candidates have been developed from the A2 strain of RSV that was “cold-passaged”, or grown in cultured cells (Vero) kept at increasingly lower temperatures, for 52 passages (cpRSV). cpRSV contained five point mutations: one in N, two in F and two in L [1309, 1310]. cpRSV, however, was still insufficiently attenuated for use in infants so it underwent chemical mutagenesis and biological selection for additional “temperature sensitive” mutations that restricted its replication further under lower
respiratory tract physiological conditions (cptsRSV). Six of the most promising cptsRSV candidates were sequenced and found to contain one additional point mutation each: five were located in L but the most attenuating mutation was found in the GS of M2 [1311-1315]. cptsRSV 248/404 was created by combining two temperature sensitive mutations in L with the M2 GS mutation and was the first recombinant vaccine candidate to be tested in humans. cptsRSV 248/404 was tested in 1-2 month old infants and was found to exhibit moderate immunogenicity that was protective against a second dose of the vaccine but it still induced mild congestion in the infants suggesting that more attenuation was needed [1276]. The deletion of genes from the cptsRSV candidates was suggested to additionally attenuate or alter the immune response to the vaccine while also increasing safety because they would be less likely to revert than simple point mutations. The deletion of NS1 and NS2 was suggested to increase the IFN response to the vaccine while the deletion of M2-2 was suggested to increase the amount of protein/antigen production. These deletions resulted in attenuated vaccine candidates that were somewhat more immunogenic [1045, 1055]. SH was also deleted but this did not result in further attenuation or increased immunogenicity in the infant [1045, 1316]. The most recent vaccine candidate tested in infants was cptsRSV 248/404/1030/ΔSH which contains three temperature sensitive mutations in the L gene, the M2 GS mutation and the deletion of the SH gene. cptsRSV 248/404/1030/ΔSH was found to be sufficiently attenuated in the infant and immunogenic enough to protect against a second dose of the vaccine [1316].

Other potential modes of vaccination have also been suggested for RSV such as: purified protein vaccines, protein subunit vaccines, synthetic peptide vaccines, and DNA vaccines. The problem with many of these though is that they are non-replicating vaccines that could
potentially prime for enhanced disease in the RSV-naïve infant. They could, however, be safe for individuals who have already been exposed to RSV and thus be used to boost the already existing immune response such as in the elderly. This is supported by experiments in the mouse where a purified F protein vaccine only primed for enhanced disease if the animal had not been previously exposed to RSV [1317]. The purified F protein vaccine was well tolerated and moderately immunogenic in previously RSV-exposed children, adults and elderly [1318-1320]. The purified F protein vaccine did not however provide protection from RSV infection when it was administered to children 1-12 years of age with cystic fibrosis even though it did induce a robust immune response [1321]. Other viruses have also been suggested as vaccine vectors for RSV proteins. Adenovirus, vesicular stomatitis virus, Newcastle disease virus, vaccinia virus and Venezuelan equine encephalitis virus are all under investigation [1322-1325]. Parainfluenza virus 3 expressing the RSV F protein was examined and determined to be protective in African green monkeys [1326]. Bovine RSV has also been suggested as a potential vaccine candidate for RSV however it was deemed over-attenuated and insufficiently immunogenic [996]. Efforts are underway to create an RSV-bovine RSV chimera which could take advantage of the attenuated phenotype of bovine RSV [1327]. It has also been suggested that an RSV subtype chimeric vaccine, created by putting the F and G genes of a B subtype virus into the backbone of an attenuated A subtype vaccine candidate, could elicit a protective immune response to both subtypes of RSV with a single vaccine [1328].

**Treatments and Preventatives**

Just as there is no current vaccine for RSV there are also no good treatments for those who become infected with RSV or develop severe RSV-mediated disease. Once hospitalized,
patients infected with RSV get mostly supportive care including mechanical removal of nasal/bronchial secretions, administration of humidified oxygen and respiratory assistance if needed. Anti-inflammatories, such as corticosteroids, are given to help diminish the immune response even though they have not been shown to be very effective at limiting the severity of RSV-mediated disease [1329]. Bronchodilators are given if the child is wheezing and seem to help slightly but since there is very little smooth muscle involvement in the cause of wheezing during an RSV infection it is understandable why bronchodilators do not provide more relief [1330]. An antagonist to cysteiny1 leukotrienes has been shown to help with episodes of wheezing following severe RSV disease [1331]. Ribavirin, a nucleoside analog, was shown to decrease clinical illness and virus shedding in hospitalized patients however it has no effect on the duration of hospitalization, the need for supportive care or the rate of mortality [1332-1334]. The administration of RSV specific neutralizing antibodies during infection is effective at reducing RSV replication and disease severity in animal models however when given to patients it did reduce RSV replication and virus shedding but it did not improve disease pathogenesis [1162-1165, 1335-1337]. It is possible that decreasing RSV replication in a child already experiencing severe disease will not significantly improve the symptoms because most of the damage has been done. Racemic epinephrine has been shown to relieve some respiratory symptoms but again has no effect on the length of hospitalization [1338]. siRNAs directed toward the NS1, N and F proteins have been shown to improve the symptoms of RSV infection in preclinical studies but further testing is needed before they will become an approved treatment [1339-1342]. Small molecule inhibitors of the F protein that inhibit fusion are also being studied as a treatment modality [1343-1347]. In mice, the anti-oxidant, butylated hydroxyanisole, has been shown to alleviate the symptoms of RSV infection, presumably by limiting the
production of cytokines and chemokines in response to infection [1348]. The inhibition of TNFα has also been shown to limit the symptoms of RSV infection, in the mouse, without affecting viral clearance indicating that it may be a potential treatment [1349].

Although an effective RSV vaccine is unavailable and there are no effective treatments for those infected, advances have been made in the area of preventing RSV infection via passive immunophylaxis. In 1993 RSV-IVIG, or RespiGam as it was known commercially, was shown to reduce the rate and duration of hospitalization for infants infected with RSV [1164, 1350]. RSV-IVIG was fractionated human IgG antibodies from donors that was prescreened for high neutralizing activity. It was administered to high risk infants, those who were born prematurely or have chronic cardiac/respiratory disorders, by intravenous injection monthly during their first RSV season. In 1999 palivizumab, or Synagis as it is known commercially, was created by combining the complementarity determining regions of a highly neutralizing F antibody from the mouse with a human IgG1 backbone [1351]. In the mouse, palivizumab treatment reduced viral load, progression of infection to the lower respiratory tract, overt symptoms, and the occurrence of post-infection lung function abnormalities [1352]. In humans, palivizumab was shown to be 50-100 fold more effective at preventing RSV disease than RSV-IVIG on a weight basis and was shown to reduce hospitalization due to RSV infection by ~55% [1353]. Palivizumab is effective at neutralizing almost all available RSV strains and was also shown to prevent recurrent wheezing post RSV infection [1094, 1354]. Palivizumab is given via monthly intramuscular injections to high risk infants during their first two RSV seasons and is sometimes used to protect newborns during hospital outbreaks of RSV. However, studies have shown that palivizumab is ineffective at treating established RSV infections and a recent study showed
that palivizumab was ineffective as a treatment for children with cystic fibrosis experiencing their first infection with RSV [696, 1355]. Since most of the severe RSV-mediated disease occurs in the infant and infection at an early age is thought to play a role in later pulmonary function and immune response abnormalities, delaying the age at which a child develops RSV disease may provide an effective therapeutic strategy. Unfortunately, palivizumab escape mutants have been identified [1356-1358]. An even more potent derivative of palivizumab, motavizumab, is now being tested [1359].

**The RSV Reverse Genetics System**

Understanding the pathogenicity of RSV, its infection, replication and spread, as well as developing preventative and treatments for RSV disease was made much easier by the creation of a reverse genetics system for growing RSV *in vitro*. A complete infectious recombinant RSV virus can be recovered from a cDNA copy of the RSV genome in the form of a plasmid. The plasmid contains the entire RSV genome sequence preceded by a T7 promoter. To launch recombinant virus, the RSV cDNA plasmid is transfected into cultured cells along with plasmids expressing the N, P, L and M2-1 proteins, also preceded by T7 promoters [1360]. A source of T7 polymerase is also provided by either a modified vaccinia virus or another plasmid. Upon transfection into cultured cells the T7 polymerase serves to transcribe the RSV cDNA and “support” plasmids resulting in the production of N, P, L and M2-1 proteins and RSV antigenome. The antigenome is encapsidated by the N protein, then replicated and transcribed by the P, L and M2-1 proteins, thereby launching the recombinant RSV virus. HeLa, HEp2, Vero and A549 cells are most commonly used to produce recombinant RSV. Once RSV transcription and replication take place the virus will behave similarly to “wild-type” RSV with respect to virion formation and infection.
Recombinant virions being produced will contain the genomic sequence present in the originally transfected RSV cDNA plasmid. A typical yield for recombinant RSV is 10-100 particle forming units (pfu) per infected cell. Virions formed by this system are mostly filamentous and ~90% will remain cell-associated [1101]. Recombinant RSV can rapidly lose its infectivity during storage or concentration; addition of buffers, sucrose and magnesium sulfate can help to retain RSV infectivity [1361, 1362].

The reverse genetic system allows RSV genes to be mutated, sequences deleted and foreign genes added to the RSV genome, which will then be packaged into infectious virions. Foreign genes have been shown to be stable in the recombinant RSV genome with no deletions of the unnecessary gene occurring [1363]. A cDNA copy of the RSV genome has been engineered to contain the gene for the green fluorescent protein (GFP) from *Renilla reniformis* (Stratagene) thus creating recombinant GFP-expressing RSV or rgRSV. Once RSV is replicating within a cell, GFP is expressed like any other RSV protein, thus acting as a marker for infected cells capable of producing RSV virions. The RSV reverse genetics system has also made it much easier to create live, attenuated vaccine candidates containing multiple, known attenuating mutations as mentioned previously.

**The RSV Replicon**

Our laboratory has developed an RSV replicon in which the three RSV glycoprotein genes, F, G and SH, have been removed. This replicon is launched in the same manner as recombinant RSV. Once established, the replicon is able to replicate and produce viral proteins, but it is unable to produce progeny virions because of the absence of the glycoproteins. The RSV replicon is launched in the BHK-SR19 T7 cell line, a baby hamster kidney cell line that
constitutively expresses the T7 polymerase from a Sindbis virus replicon which means that there is no requirement for an exogenous source of T7 polymerase as there is for the launch of recombinant RSV [1364]. Once the plasmid containing the cDNA copy of the RSV replicon and the support plasmids are transcribed in the BHK-SR19 T7 cells, RSV replicon replication and transcription proceeds in the same manner as the whole virus described above. The replicon, however, will replicate and transcribe its genes but will not form virions. In order to “mobilize” replicon virions, the RSV F and G proteins must be supplied in trans. This is done by transfecting replicon containing cells with plasmids expressing the F and G proteins. After RSV F and G are produced in the replicon containing cells, infectious RSV replicon virions containing the replicon genome will be formed and released. These replicon virions can go on to infect other cells but once in these new cells the replicon will again be trapped, producing all of its encoded proteins but unable to form virions due to the lack of the glycoprotein genes. The replicon is thus said to form “one step” virions or OSV. In place of the glycoprotein genes the RSV replicon contains a blasticidin resistance gene for use in selecting and cloning the cells that contain the replicon. The replicon genome also contains the GFP gene, like its parent RSV genome, so that cells containing the replicon can be easily visualized. The replicon has been studied extensively in our laboratory and has been shown to be stable and non-cytotoxic in a variety of cultured cell types. Similar transmission incompetent viruses have also been made with vesicular stomatitis virus and equine arteritis virus [1365, 1366]. Sendai virus replicons lacking the fusion, hemagglutinin-neuraminidase and matrix genes have also been constructed as have packaging cell lines for each of them [1327, 1367-1369]. Sendai replicons lacking the fusion and matrix genes as well as all three were shown to exhibit reduced cytotoxicity and immune stimulation upon infection.
CHAPTER 3: WHOLE VIRUS VECTORS

Introduction
RSV has many features that suggest it might be successful as a gene therapy vector for the delivery of CFTR to the airways of CF patients. RSV specifically infects the ciliated cells of the respiratory tract; the cells to which most of the gene therapy vectors for CF are being targeted due to their major role in determining the hydration of the airway mucosal surface. RSV can infect the lungs of CF patients, so it appears that it can make its way through the sticky and mucus-rich environment of the CF lung unlike many of the currently studied vectors. RSV has the ability to re-infect, implying that multiple sequential administrations of an RSV-based vector would be possible. RSV also has a reverse genetics system that allows the addition and deletion, as well as mutation, of genes contained in its genome.

Here, we tested the utility of RSV as a CFTR gene transfer vector. The CFTR gene was inserted into four different sites in the RSV genome to obtain a range of expression levels. The vector was then evaluated for the ability to deliver CFTR to HAE cultures. The RSV vectors were able to deliver a sufficient amount of functional CFTR to CF HAE cultures to fully correct the Cl⁻ transport bioelectric defect and enable the cells to maintain their proper ASL height.
Materials and Methods

Viruses and Cells

Recombinant, green fluorescent protein-expressing rgRSV was used as the vector backbone in this study. All viruses were rescued and grown in HeLa cells in DMEM supplemented with 10% fetal bovine serum (FBS) in a humidified incubator supplied with 5% CO₂.

Recombinant Virus Construction and Rescue

The full-length RSV cDNA construct, RW30, was used as the backbone for CFTR gene insertion. This was an updated version of the original RSV cDNA [1360]. This cDNA contained a 5’ hammerhead ribozyme sequence responsible for leaving the exact, native 5’ terminus [1370]; the Renilla recombinant green fluorescent protein (RrFP), a PCR product from pFBRrFP (Stratagene) without mammalian optimization, inserted as the first viral gene; unique restriction sites inserted in the intergenic regions flanking each of the membrane associated protein genes; a BsiWI restriction site inserted following the L gene and a 3’ hepatitis D virus antigenomic ribozyme responsible for generating the exact 3’ native terminus [1371]. These insertions and mutations were produced by PCR mutagenesis in small plasmids and moved into the full-length cDNA by standard subcloning techniques [1372].

The human CFTR cDNA, received from Dr. John Riordan, University of North Carolina-Chapel Hill, was first flanked by RSV gene start (GS) and gene end (GE) sequences as well as unique restriction sites to enable the insertion of this gene unit into RW30. A series of plasmids were created containing the GS and GE sequences from the RSV F gene, internally flanked by SacII and Apal restriction sites and externally flanked by PvuI and Xhol, BssHII,
Xhol or BsiWI restriction sites. The CFTR gene was inserted into these plasmids using SacII and ApaI. The resulting CFTR gene units were inserted at the BssHII, Xhol or BsiWI restriction sites in RW30, creating rgRSV-CFTR6, rgRSV-CFTR10 and rgRSV-CFTR12, respectively (Fig. 1). rgRSV-CFTR2 was created by first inserting the CFTR gene unit into a partial rgRSV plasmid using PvuI and Xhol. The CFTR gene unit and a partial genome segment containing the NS1, NS2, N, and P genes were then cloned into the complete rgRSV plasmid using the KpnI and BssHII sites. The names of the resulting plasmids include a number that describes the position occupied by the CFTR gene relative to the 3’ end of the genome.

All viruses were rescued in HeLa cells using the reverse genetics system described previously [1360, 1373]. Briefly, a plasmid containing a full length cDNA of the virus genome (1.2 µg) along with support plasmids expressing the RSV N, P, L and M2-1 genes (0.8 µg) were transfected into 6 well plates of 40% confluent HeLa cells, using the TransIT HeLa Monster transfection kit (Mirrus) according to the manufacturer’s protocol. Initial replication of the plasmids was driven by a T7 polymerase supplied by co-infection of the cells with vaccinia virus MVA-T7. The existence and clustering of GFP containing cells indicated the presence of virus replication and spread. Virus was harvested by scraping the cells from the plate, vigorously resuspending them and then pelleting them by centrifugation (2000 x g for 5 min.). The virus-containing supernatant was passaged onto successively larger plates to obtain higher titered virus stocks. All viruses were amplified and titered in HeLa cells. Stocks were titered by counting the number of green cells at 24 hours in duplicate wells of a 96 well plate infected with serially diluted virus.
**Western Blot**

HeLa cells at 60% confluency in 100 mm dishes were inoculated with rgRSV, rgRSV-CFTR2, rgRSV-CFTR6, rgRSV-CFTR10 and rgRSV-CFTR12 at an MOI of 0.5, or mock inoculated. Cells were lysed at 72 hr post inoculation using 500 μl lysis buffer [1% Triton X-100, 150 mM NaCl, 50 mM Tris and an EDTA-free protease inhibitor cocktail (Roche)]. Laemmli sample buffer containing 5% β-mercaptoethanol was added to equal volumes of lysate. Samples were incubated at 37°C for 30 min then electrophoresed on a 10% SDS-PAGE gel [1374]. Samples were transferred from the gel to an Immobilon-P membrane (Millipore) which was blocked overnight at 4°C with 5% dry milk in phosphate-buffered saline plus 0.2% Tween-20 (PBST). The membrane was probed with CFTR monoclonal antibody #596 (Dr. John Riordan, University of North Carolina-Chapel Hill) diluted 1:1000. A goat anti-mouse peroxidase-labeled secondary antibody (Chemicon) was used at a dilution of 1:10,000. The blot was developed with LumiLight chemiluminescent substrate (Roche). Lysate from Calu3 cells, prepared in the same manner, was used as a positive control for CFTR.

**Growth Curve**

Duplicate wells of 40% confluent HeLa cells in 12-well plates were inoculated with rgRSV, rgRSV-CFTR10 or rgRSV-CFTR12 at an MOI of 0.1. At indicated time points, virus was harvested from duplicate wells. The virus-containing supernatant was transferred to a new tube and frozen on dry ice. Samples were titered on HeLa cells once all of them were collected. Growth curves was constructed based on the viral titers at each time point.
Generation of CF HAE

Primary human tracheobronchial epithelial cells from CF patients (ΔF508 homozygous) and non-CF donors were obtained from tracheobronchial tissues by the UNC CF Center Tissue Culture Core under UNC IRB-approved protocols. Cells were plated at a density of 250,000 cells per well on permeable type IV collagen-coated Millicells (0.4 μm pore size, 12 mm diameter, Millipore). Cultures were allowed to differentiate at an air-liquid interface for 4-6 weeks to form well-differentiated cultures with morphological characteristics that resemble those of the human cartilaginous pseudostratified ciliated epithelium in vivo, as previously described [643, 1375].

Inoculation of CF HAE with rgRSV-CFTR Viruses

Prior to viral inoculation, the apical surfaces of HAE were rinsed 3 times with PBS to remove accumulated apical secretions. The apical surfaces of quadruplicate cultures were then inoculated with 200 μl of rgRSV, rgRSV-CFTR10 or rgRSV-CFTR12 (2 × 10^5 pfu, 2 × 10^5 pfu and 1 × 10^6 pfu, respectively) or mock inoculated, and incubated for 2 hr at 37°C. After removal of inoculum, HAE were returned to a humidified incubator until assays were performed.

Quantification of Infection of CF HAE Cultures

CF HAE inoculated with rgRSV, rgRSV-CFTR10, or rgRSV-CFTR12 (2 × 10^5 pfu, 2 × 10^5 pfu and 1 × 10^6 pfu, respectively) were photographed with a Leica DMIRB Inverted Fluorescent Microscope 24 hr post inoculation and en face percentages of GFP-positive cells were quantified with ImageJ software (NIH).
Quantitation of CFTR and RSV N mRNA Following CF HAE Inoculation

Total RNA was isolated from quadruplicate cultures of CF HAE 48 hr post inoculation with rgRSV, rgRSV-CFTR10, rgRSV-CFTR12 (2 × 10^5 pfu, 2 × 10^5 pfu and 1 × 10^6 pfu, respectively) or mock inoculation using acid phenol-guanidine thiocyanate followed by DNase digestion and further purification using the Qiagen RNeasy Mini Kit. The cDNA was synthesized with oligo(dT) and SuperScript II reverse transcriptase (Invitrogen). Quantitative PCR was performed using a Roche LightCycler with the Roche FastStart DNA Master SYBR Green I Kit according to the manufacturer’s protocols. Levels of CFTR mRNA were normalized to the level of GAPDH measured using the LightCycler Software version 4.0. Levels of RSV N mRNA in cultures inoculated with rgRSV-CFTR10 and rgRSV-CFTR12 were normalized to the level in rgRSV inoculated cultures which was set to 1.

Immunofluorescent Staining

To immunolocalize CFTR in CF HAE inoculated with rgRSV, rgRSV-CFTR10, rgRSV-CFTR12 or mock inoculated, cells were gently scraped from the Millicells with a pipette tip then resuspended in PBS. Cells were then pelleted onto glass slides with Cytospin and air-dried. Cells were fixed with 4% paraformaldehyde then CFTR was detected with the human CFTR monoclonal antibody #596 and Alexafluor568 conjugated goat anti-mouse secondary antibody. Cell nuclei were counter-stained with Hoechst 33342 (Invitrogen). Images were taken with a Leica SP2 Laser Scanning Confocal Microscope, and processed with Adobe Photoshop CS2.
**Ion Transport Measurements**

CF HAE inoculated with rgRSV, rgRSV-CFTR10, or rgRSV-CFTR12 (2 × 10⁵ pfu, 2 × 10⁵ pfu and 1 × 10⁶ pfu, respectively) or mock inoculated were mounted in Ussing chambers for measurement of transepithelial resistance (Rt) and transepithelial potential difference (PD) under open circuit conditions using a Physiologic Instruments Voltage clamp (San Diego, CA) 48 hr post-inoculation. The electrical potential difference across the tissue was continually recorded and a constant current pulse (2-10 μA) was applied across the tissue at one-minute intervals to calculate tissue resistance. From these measurements, the equivalent short-circuit current (I_{sc}) was calculated. Additional details of Ussing chamber techniques have been published previously [1376]. Ussing chamber experiments were performed in bilateral Krebs Bicarbonate Ringer solution gassed with 95% O₂, 5% CO₂. Drugs [amiloride (10⁻⁴ M), forskolin (10⁻⁵ M), and CFTR172 (10⁻⁵ M)] were added sequentially to the indicated final concentration from concentrated stock solutions (Sigma-Aldrich) to lumenal and/or basal surfaces. CFTR172 (10⁻⁵ M), a potent small molecule inhibitor of CFTR, was synthesized according to appropriate standards and used as previously described [1377]. Untreated non-CF HAE cultures were tested in parallel as controls.

**Air Surface Liquid Measurements**

At 48 hr post inoculation of the CF HAE cultures with rgRSV, rgRSV-CFTR10, or rgRSV-CFTR12 (2 × 10⁵ pfu, 2 × 10⁵ pfu and 1 × 10⁶ pfu, respectively) or mock inoculation, 25 µl of PBS containing 0.2% Texas Red-dextran (Invitrogen) was added to the apical surface giving the cultures an initial ASL height of 20-30 µm. Images of the ASL were acquired using laser scanning confocal microscopy (Zeiss Model 510) 48 hr post-inoculation. ASL height was
determined by averaging the heights obtained from the XZ scans of four culture regions. Additional details concerning ASL measurement have been published previously [1378].

**Results**

**Construction and rescue of recombinant rgRSV vectors expressing CFTR**

A CFTR gene unit containing the 4.5 kb CFTR gene flanked by a set of RSV transcription signals was inserted into the full length recombinant GFP-expressing RSV (rgRSV) cDNA in four different positions creating the plasmid templates for rgRSV-CFTR2, rgRSV-CFTR6, rgRSV-CFTR10 and rgRSV-CFTR12, which were numbered according to the CFTR gene position in the viral genome (Fig. 1A and B). Virus was rescued from multiple clones of each rgRSV-CFTR cDNA by transfection into HeLa cells. Similar to rgRSV, rgRSV-CFTR10 and rgRSV-CFTR12 virus began to spread by 4 days post-transfection, as visualized by fluorescent microscopy. The rgRSV-CFTR2 and rgRSV-CFTR6 viruses required an additional 2-4 days to spread and were more cytotoxic (i.e. they caused more cell death and the cells in the culture were visibly less healthy) during this period and subsequent early virus passages compared to the other viruses, including the parental rgRSV. This increase in cytopathology could account for the extra time required to rescue these viruses. By the third passage, however, these viruses caused similar cytopathology to the rgRSV-CFTR10 and rgRSV-CFTR12 viruses. Large stocks were grown of all viruses and each was titered.
Figure 1. rgRSV-CFTR Constructs. The CFTR gene, flanked by the RSV GS and GE was inserted into the rgRSV cDNA at 4 positions. (A) Sequences flanking the CFTR gene unit in the four constructs. (B) Schematic of CFTR location within rgRSV-CFTR genomes. Constructs are named for the position of the CFTR gene relative to the viral promoter at the 3’ end of the genome.
**rgRSV Can Express Full-length, Mature CFTR**

To test whether these viruses expressed CFTR, we inoculated HeLa cells at an MOI of 0.5, harvested cell lysates at 72 hr post-inoculation (pi), and assayed for CFTR by western blot. As a positive control, we used lysate from the human lung cell line, Calu3, which endogenously expresses a high level of mature CFTR. HeLa cells inoculated with rgRSV-CFTR10 and rgRSV-CFTR12 expressed mature CFTR protein, band C (Fig. 2, lanes 3 and 4), with more CFTR being produced by rgRSV-CFTR10 than by rgRSV-CFTR12. The immunoreactive lower band detected in HeLa cells inoculated with rgRSV-CFTR10 and rgRSV-CFTR12 likely represents CFTR in its immature, under-glycosylated state. In contrast, the rgRSV-CFTR2 and rgRSV-CFTR6 viruses failed to produce full-length, mature CFTR protein. Four clones of each of these viruses were tested and none were capable of expressing full-length, mature CFTR. Though occasionally what may be considered truncated forms of CFTR were present. Representative clones of rgRSV-CFTR2 and rgRSV-CFTR6 are shown in Figure 2 (lanes 1 and 2). No CFTR immunoreactivity was detectable in mock inoculated HeLa cells or in HeLa cells inoculated with rgRSV alone. These data indicate that mature CFTR can be expressed from the rgRSV genome but that the location of the CFTR gene in the genome may impact its expression. In addition, the difference in CFTR expression level between rgRSV-CFTR10 and rgRSV-CFTR12 is consistent with the polarity of RSV expression.

The observations that rgRSV-CFTR2 and rgRSV-CFTR6 produced little to no full-length CFTR are likely related to the increased CPE of these vectors during rescue. The rgRSV-CFTR2 and rgRSV-CFTR6 viruses would have been expected to express substantially higher levels of CFTR than rgRSV-CFTR10 and rgRSV-CFTR12 because they bear the CFTR gene.
unit in positions closer to the RSV promoter. It is conceivable that these high levels of CFTR were cytotoxic during the rescue and early passages of these viruses. The loss of cytotoxicity upon serial passage might be explained by the selection of adventitious mutations that block expression of a functional CFTR protein. This may explain why multiple clones of the rescued rgRSV-CFTR2 and rgRSV-CFTR6 viruses lost both their cytopathic phenotype and their ability to express complete CFTR. Due to their lack of CFTR expression, rgRSV-CFTR2 and rgRSV-CFTR6 viruses were not further evaluated in this study.

Figure 2. CFTR Expression from rgRSV. A western blot to detect CFTR protein in HeLa cells infected with rgRSV-CFTR vectors. Band C (~190 kDa) represents full-length, mature CFTR. Band B (~150 kDa) represents immature CFTR. Endogenous CFTR from Calu3 cells was used as a positive control (Lane 5). Lysates from mock-infected HeLa cells and rgRSV inoculated HeLa cells were negative controls (Lane 6 & 7). This is a representative blot from four experiments.
Growth Kinetics of rgRSV-CFTR Viruses

We compared the growth kinetics of rgRSV-CFTR10 and rgRSV-CFTR12 to rgRSV to determine whether the 4.5 Kb CFTR gene insertion or the position at which it was inserted affected virus replication. Cells were infected with each virus at an MOI of 0.1 and the virus yield was determined over 4 days. The shapes of all the curves were similar, though it seemed that rgRSV-CFTR10 and rgRSV-CFTR12 had a lower beginning infectivity (Fig. 3). By 72 hr the yields for all viruses were similar, indicating that the insertion of CFTR may have delayed virus replication somewhat but had little effect on the final virus yield. Because the growth kinetics of rgRSV-CFTR10 were similar to those of rgRSV-CFTR12, we concluded that the insertion of the CFTR gene, not its location in the genome, was responsible for the delay in virus yield. We hypothesize that the 23% increase in the rgRSV genome length that results from the insertion of CFTR is causing a delay in virus replication, most likely due to the additional time needed for the RSV polymerase to traverse the longer genome, leading to a delay in virus yield.
Figure 3. Growth Analysis of rgRSV-CFTR Vectors. Growth curves for rgRSV-CFTR10 and rgRSV-CFTR12 compared to parental rgRSV. Duplicate wells of HeLa cells were inoculated with 0.1 MOI of either rgRSV, rgRSV-CFTR10 or rgRSV-CFTR12. Virus was harvested at the indicated times and titrated.
**rgRSV Can Deliver CFTR to CF HAE Cultures.**

To determine whether RSV can deliver CFTR to well differentiated airway cells, quadruplicate HAE cultures derived from airway tissue of CF patients with a ΔF508 homozygous genotype (CF HAE) were inoculated with 200 µl of rgRSV (2 × 10⁵ pfu), rgRSV-CFTR10 (2 × 10⁵ pfu) or rgRSV-CFTR12 (1 × 10⁶ pfu), or mock inoculated. These inocula resulted in multiplicities of infection of ~0.2 for rgRSV and rgRSV-CFTR10 and ~1.0 for rgRSV-CFTR12 for ciliated cells in the cultures. At 24 hr pi, GFP-positive cells were visible in all infected cultures (Fig. 4A). The transduction efficiency for rgRSV, rgRSV-CFTR10 and rgRSV-CFTR12 was 14%, 10% and 35%, respectively (Fig. 4B).

To quantify CFTR expression levels, we measured CFTR mRNA 48 hr pi from the quadruplicate infected CF HAE cultures described above and compared it to endogenous CFTR mRNA from mock infected cultures. Since the amount of CFTR mRNA is the same in ΔF508 CF tissues and non-CF tissues, the endogenous levels of CFTR mRNA in these CF HAE cultures would be representative of that in non-CF cultures. Our data showed that the endogenous CFTR mRNA levels in these cultures were not altered by rgRSV infection. However, CF HAE inoculated with rgRSV-CFTR10 and rgRSV-CFTR12 had a 94-fold and a 64-fold higher level of CFTR mRNA, respectively, compared to levels in CF HAE inoculated with rgRSV or mock-infected (Fig. 5A). The relative levels of CFTR mRNA expression by these two vectors correlated more closely with the genome position of the CFTR gene and the amount of CFTR protein produced by each virus in HeLa cells (Fig. 2), than with the proportion of CF HAE cells transduced by each virus (Fig. 4B).
To correlate the amount of CFTR mRNA generated by the vectors to virus replication, we also quantified the relative levels of N protein mRNA in these cultures (Fig. 5B). RSV N protein mRNA levels correlated closely with infection efficiency (Fig. 4A), indicating a similar amount of N protein mRNA production and, therefore, similar levels of replication for each virus. This data suggests that the level of replication of these viruses does not account for the difference in levels of CFTR mRNA expression. By comparing the amount of CFTR mRNA with the N mRNA being expressed by each virus, the rgRSV-CFTR10 vector produced approximately 4-fold more CFTR mRNA per infected cell than the rgRSV-CFTR12 vector.

To determine whether the CFTR protein produced by rgRSV-CFTR10 and rgRSV-CFTR12 was correctly localized at the apical surface of the ciliated cells of the CF HAE, we immunoprobed CF HAE cells 48 hr pi for CFTR protein. These studies confirmed that CFTR protein was produced in ciliated cells by both vectors and was detected at the apical surface (Fig. 6), thus recapitulating the localization of endogenous CFTR in non-CF ciliated HAE cells as previously reported [373, 1379].
Figure 4. **rgRSV-CFTR Infection of CF HAE.** Quadruplicate CF HAE cultures were inoculated with $2.0 \times 10^5$ pfu, $2.0 \times 10^5$ pfu and $1.0 \times 10^6$ pfu of rgRSV, rgRSV-CFTR10 or rgRSV-CFTR12, respectively. (A) At 24 hr post inoculation GFP expression was detectable in cultures infected with rgRSV, rgRSV-CFTR10 and rgRSV-CFTR12, indicating infection (representative images are shown). (B) The percentage of GFP positive cells at 24 hr pi were quantified and results displayed as mean ± stdev. Results depicted are representative of two separate experiments each including quadruplicate cultures for each experimental condition.
Figure 5. Expression of CFTR mRNA in rgRSV-CFTR Infected CF HAE. CF HAE cultures were infected with rgRSV, rgRSV-CFTR10, or rgRSV-CFTR12 (2 × 10^5 pfu, 2 × 10^5 pfu and 1 × 10^6 pfu, respectively) or mock-infected. Total mRNA was collected from infected and mock infected cultures 48 hr post-inoculation (n=4). (A) The amount of CFTR mRNA present in infected cultures was compared to that in untreated CF HAE cultures. (B) The amount of viral N protein mRNA present in cultures infected with the rgRSV-CFTR vectors was compared to that in rgRSV infected cultures. All results are shown as mean ± stdev. “*” indicates a statistically significant difference (p value < 0.05) as determined by an unpaired student t-test. Results depicted are representative of two separate experiments each including quadruplicate cultures for each experimental condition.
Figure 6. CFTR Localization in CF HAE Infected with rgRSV-CFTR Vectors. At 48 hr post inoculation, CF HAE cultures that were mock infected or infected with rgRSV, rgRSV-CFTR10 or rgRSV-CFTR12 as indicated were sectioned and stained for CFTR with MAb #596 followed by a goat anti-mouse Ig- AlexaFluor568 conjugated secondary antibody (red). Nuclei were stained with Hoechst 33342 (blue). Representative images are shown.
**CFTR Delivered to CF HAE by rgRSV-CFTR Viruses is Functional**

To determine whether the CFTR produced by the RSV vectors was functional as a Cl⁻ channel, CF HAE cultures were inoculated with rgRSV, rgRSV-CFTR10, and rgRSV-CFTR12 (2 × 10⁵ pfu, 2 × 10⁵ pfu and 1 × 10⁶ pfu, respectively) or were mock-inoculated, and assessed for ion channel activity in Ussing chambers. Two days after virus inoculations, cultures were mounted in Ussing chambers and ion transport was assessed using previously established methods [1376]. Briefly, electric current across the tissue (called the equivalent short-circuit current, or I_{sc}) in response to applied electric pulses was measured while a series of drugs was applied in the following order: amiloride, a potent inhibitor of Na⁺ ion channels (specifically ENaCs), was used to prime the cells for maximal CFTR activity; forskolin, an activator of adenylate cyclase, was used to maximally stimulate CFTR channel activity; CFTR172, a potent inhibitor of CFTR, was added to confirm the specificity of any forskolin induced change in I_{sc}; and UTP, a stimulator of Ca²⁺-activated Cl⁻ channels was added to activate Cl⁻ channels other than CFTR as a positive control. Representative traces from Ussing chamber experiments are shown in Fig. 7A. CF HAE infected with rgRSV-CFTR12 exhibited a lower I_{sc} before treatment with amiloride and therefore a reduced ΔI_{sc} after amiloride treatment, suggesting that the Na⁺ channel hyperactivity exhibited by ENaCs in these cells was reduced somewhat by this treatment (Fig. 7A and B). However, due to the lack of other factors involved in the control of ENaC function, this trend will need to be tested further to determine if this decrease in ENaC hyperactivity is significant. CF HAE inoculated with rgRSV-CFTR10 and rgRSV-CFTR12 did however show increases in I_{sc} after forskolin treatment (Fig. 7A and C). These increases returned to baseline following treatment with CFTR172 (Fig. 7A and D), indicating that they can be attributed to the presence of functional CFTR channels provided by the RSV-CFTR viruses. When the
magnitude of the forskolin-induced changes in $I_{sc}$ produced by rgRSV-CFTR10, rgRSV-CFTR12 and rgRSV infected cells was quantified and compared in parallel experiments to that produced in non-CF HAE, infection with rgRSV-CFTR12 resulted in a 25.84 ± 10.76 μA/cm² increase, which is not statistically different than that seen in non-CF HAE cultures (16.34 ± 4.26 μA/cm²) (Fig. 7C). Although infection with rgRSV-CFTR10 also resulted in increased forskolin-activated ion channel activity (6.12 ± 1.23 μA/cm²), this increase was not as high as that in non-CF HAE or CF HAE infected with rgRSV-CFTR12 (p-values of 0.04 and 0.006, respectively). This suggested that full CFTR Cl⁻ transport correction occurred in response to rgRSV-CFTR12 but not rgRSV-CFTR10 (which had been administered at a lower dose and infected fewer cells, as shown in Fig. 4). If the forskolin-induced change in $I_{sc}$ is adjusted for the number of rgRSV-CFTR containing cells in the CF HAE cultures, the “CFTR function per cell” is very similar for rgRSV-CFTR10 and rgRSV-CFTR12-containing cells even though there is more CFTR mRNA in rgRSV-CFTR10-containing cells (Fig. 8).

Treatments to measure ASL were performed on CF HAE cultures 48 hr after cultures were inoculated with rgRSV, rgRSV-CFTR10, and rgRSV-CFTR12 (2 × 10⁵ pfu, 2 × 10⁵ pfu and 1 × 10⁶ pfu, respectively) or were mock-inoculated, in order to determine if the RSV-supplied CFTR could improve the maintenance of ASL height. Texas Red-dextran labeled PBS was applied to each culture and the height of this overlay was measured 48 h later. In CF HAE that had been mock-infected, over half of the fluid was absorbed (Fig. 9). Also, in cultures infected with rgRSV-CFTR10, most of the ASL had been absorbed. In contrast, cultures infected with rgRSV-CFTR12 were able to maintain their ASL height, indicating that the CFTR supplied by the rgRSV-CFTR12 vector was functional as an ion channel and also performed its downstream role of equilibrating liquid absorption from the ASL.
Figure 7. Ion Transport Properties of rgRSV-CFTR Infected CF HAE. CF HAE cultures were inoculated with rgRSV, rgRSV-CFTR10, or rgRSV-CFTR12 (2 × 10⁵ pfu, 2 × 10⁵ pfu and 1 × 10⁶ pfu, respectively) or mock-infected and measured for ion transport properties in Ussing chambers at 48 hr pi. (A) Representative traces of the short circuit current (I_SC) responses (y-axis) in infected cultures in response to sequential treatment with amiloride (Amil) to inhibit ENaCs, forskolin (Fskl) to activate CFTR activity, CFTR172 to inhibit CFTR activity, and UTP to stimulate Ca²⁺-activated Cl⁻ channels as a positive control. The maximum changes in I_SC (∆I_SC) in response to individual treatments are shown in B-D. (B) ∆I_SC of CF HAE after amiloride treatment. (C) Forskolin-induced ∆I_SC of CF HAE, indicating the presence of functional CFTR. (D) ∆I_SC of CF HAE following treatment with CFTR172. The magnitude of forskolin-activated changes in CF HAE infected with the RSV-CFTR viruses was comparable to that of non-CF HAE (n=7). All results are shown as mean ± stdev. "ns" indicates a difference that is not statistically significant (p value > 0.05). "*" indicates a difference that is statistically significant (p value < 0.05), as determined by an unpaired student t-test. Results depicted are representative of two separate experiments each including quadruplicate cultures for each experimental condition.
Figure 8. CFTR Function per Cell Containing an rgRSV-CFTR Vector. The average change in I_{SC} after the addition of forskolin to CF HAE cultures infected with rgRSV, rgRSV-CFTR10, rgRSV-CFTR12 (2 × 10^5 pfu, 2 × 10^5 pfu and 1 × 10^6 pfu, respectively) was divided by the average number of infected cells in the culture to obtain an estimate of forskolin-induced ΔI_{SC} contributed by each cell containing an rgRSV-CFTR vector.
Figure 9. ASL Measurements of CF HAE Infection with rgRSV-CFTR Vectors. ASL height was measured 48 h pi after infection with rgRSV, rgRSV-CFTR10, rgRSV-CFTR12 (2 × 10^5 pfu, 2 × 10^5 pfu and 1 × 10^6 pfu, respectively) or mock-infection. Each bar represents three measurements on each of four cultures. All results are shown as mean ± stdev. “ns” indicates a difference that is not statistically significant (p value > 0.05). “*” indicates a statistically significant difference (p value < 0.05) as determined by an unpaired student t-test.
Discussion

While advances are continuing to be made in the fields of AAV, lentivirus and non-viral vectors, their transduction efficiencies for the ciliated cells of the human airway are still quite low. Also, delivery of CFTR by these vectors has yet to show improvement of CF pulmonary defects other than correction of the Cl⁻ transport defect. We have chosen to take a different approach to vector construction by exploiting a human respiratory virus, RSV, which has a natural tropism for the ciliated cells of the airway lumen.

To test our hypothesis that RSV could deliver sufficient CFTR to ciliated cells to correct the CF bioelectric defect, we inserted the CFTR gene into four different positions in the RSV genome to produce different amounts of CFTR. All four viruses were rescued, but CFTR was only expressed from the two expected to produce the lowest amounts of CFTR, suggesting that higher levels of CFTR were cytotoxic. Over-expression of CFTR in fibroblasts has previously been shown to slow cellular proliferation and possibly cause an unfolded protein stress response resulting in cell death [1380]. Overexpression of a protein by RSV is not, by itself, cytotoxic since other transgenes, such as GFP, can be expressed at a high level from the first genome position without toxicity. The viruses capable of expressing CFTR, rgRSV-CFTR10 and rgRSV-CFTR12, were passaged four times in HeLa cells without losing CFTR expression, indicating that they are genetically stable despite the inclusion of a large gene that is not necessary for virus replication. CFTR expression from these vectors should continue to be monitored with further passage, however, to ensure the stability of the CFTR gene in the RSV genome.
Our data show that ~3.5X more ciliated cells in CF HAE were transduced with rgRSV-CFTR12 than with rgRSV-CFTR10, correlating roughly with the viral inoculum (Fig. 4) and with the amounts of RSV N protein mRNA produced (Fig. 5B). However, cultures infected with rgRSV-CFTR10 produced ~5.3X more CFTR mRNA than cultures infected with rgRSV-CFTR12 (Fig. 5A). From the N and CFTR mRNA measurements, we can extrapolate that the CFTR gene inserted into the tenth position of the RSV genome is transcribed ~4 times more efficiently than from the twelfth position. This result illustrates the ability to control the amount of transgene expression simply by changing the gene position in the RSV genome.

The level of N mRNA was used to normalize gene expression because the N protein is expressed at a specific level in RSV infected cells. The N gene is also located in the fourth position of the RSV genome, upstream of the CFTR genes inserted into the tenth and twelfth positions. N mRNA expression should therefore not be affected by the CFTR gene insertions allowing it to be comparable between rgRSV, rgRSV-CFTR10 and rgRSV-CFTR12.

Both rgRSV-CFTR10 and rgRSV-CFTR12 produced functional CFTR, as measured by Cl− transport in Ussing chambers, but the magnitude of forskolin-mediated CFTR activation correlated closely with the number of infected cells rather than with the levels of total CFTR mRNA being produced. When the amount of forskolin induced change in I_sc was normalized for the number of cells in the culture containing an rgRSV-CFTR vector, the amounts were very similar for rgRSV-CFTR10 and rgRSV-CFTR12 even though there was more CFTR mRNA in rgRSV-CFTR10-containing cells (Fig. 8). This result suggests that there is a limit to the CFTR activity per cell and that this limit had been reached by both vectors. One possible explanation for this limit on CFTR function is that there are a limited number of CFTR membrane sites and, once these are occupied, no additional CFTR molecules are allowed
into the membrane and no further effects on intracellular ion balance are possible. So even though rgRSV-CFTR10 produced more CFTR per cell (Fig. 2A and 5A), rgRSV-CFTR12 caused greater overall CFTR function (Fig. 7C) because it was delivered to more cells in these particular experiments (Fig. 4). These results are consistent with previous findings indicating that as long as CFTR is expressed above endogenous levels, it is more effective to deliver CFTR to more cells than to express a larger amount of CFTR in fewer cells [779, 932, 1381].

At the MOI of infection used in these experiments, rgRSV-CFTR10 infected 10% of the CF HAE apical cells (Fig. 4) and exhibited a cAMP-activated CFTR activity that was 37% of that in non-CF HAE (Fig. 7C). rgRSV-CFTR12 infecting 35% of CF HAE generated a CFTR response that was statistically equivalent to non-CF cells, though. In both cases, overexpression of CFTR in individual infected cells led to greater CFTR function per cell than in non-CF cells. With rgRSV-CFTR12, given its higher input MOI, we were able to target more than the suggested number of cells needed to reverse the chloride ion transport defect in the CF lung [778]. Also, the degree of CFTR function provided by rgRSV-CFTR12 exceeds the amount thought to be needed to reverse many of the other defects in the CF lung such as sodium hyperabsorption and ASL dehydration [779, 932]. Indeed our results suggest that RSV expression of CFTR in CF HAE is having some effect on ENaC function (Fig. 7B) and confirm that the ASL is maintained at a higher volume in cultures infected with rgRSV-CFTR12 (Fig. 9). In addition to correcting the ASL dehydration phenotype of CF HAE, rgRSV-CFTR12 also infected a percentage of cells that exceeds the amount (35% vs. 25%) speculated as being required for the correction of other defects (i.e. ciliary beat frequency and mucociliary transport efficiency) as shown in the PIVCFTR vector study [932].
RSV infection does elicit a muted adaptive host immune response that allows repeated infections throughout life without the need for significant antigenic change [1129, 1233, 1288], suggesting that an RSV-based gene therapy vector might be successfully re-administered. RSV suppresses the innate immune response in human cells via its two unique non-structural proteins, NS1 and NS2. The reduced interferon response to RSV infection may play a role in dampening the adaptive immune response to RSV [1229, 1230].

In one study, 7 of 15 individuals could be infected three or more times over a 26 month period as detected by shed virus or a rise in RSV antibody titer. Furthermore, infection can occur without virus shedding suggesting that infection may have occurred even more frequently. Recent in vivo data have shown that while previous exposure to RSV does not prevent re-infection, it instead causes the challenge infection to become abortive: RSV enters cells and replicates but fails to generate infectious progeny virions [1297]. This situation might be ideal for a gene transfer vector, reducing the likelihood of cell to cell spread, exposure of the vector to the immune system and potential disease symptoms. Repeat infections with RSV are usually limited to the upper respiratory tract, suggesting that the level of pre-existing antibody is not high enough to be protective, but that the memory immune response is successful at preventing the spread of RSV from the upper to the lower respiratory tract. However, a vector delivered by aerosol could be distributed throughout the lung simultaneously enabling vector infection before a memory response could be mounted.

In addition, through efforts to develop RSV vaccines, mutations and gene deletions that attenuate the virus have been identified [1309, 1311-1314], some of which could be incorporated into RSV-CFTR vectors to reduce or eliminate pathogenesis. The observation
that CFTR expression from RSV is robust, and possibly too robust from the second and sixth positions, indicates that substantial reductions in overall RSV gene expression could be accommodated while still maintaining adequate levels of CFTR expression to correct ciliated cell defects. Attenuation could also reduce the immunogenicity of the infecting virus, by reducing the production of viral proteins as has been found in some clinical trials with live-attenuated vaccine candidates, in turn facilitating re-infection. Further studies in applicable animal models will need to be performed to determine the immune response to this vector as well as its persistence and its ability to infect upon re-administration. Newly developed models of CF lung disease in the pig and ferret may provide more appropriate models for testing an RSV-based vector due to their ability to be infected with RSV and their similarities in lung physiology to humans [650-652, 1002, 1382, 1383].
CHAPTER 4: RSV REPLICON VECTORS

Introduction

In Chapter 2 we showed that RSV is capable of expressing CFTR, the gene in which mutations cause CF. We found that rgRSV expressing CFTR is capable of correcting the physiologic defects seen in primary CF airway epithelia. Unfortunately, most rgRSV infected cells die within seven days of becoming infected, limiting the persistence of CFTR expression. Also, RSV weakly activates the immune system, resulting in the attraction of leukocytes and cytotoxic T lymphocytes to the airways which can also kill infected cells.

In order to generate an improved RSV-based vector for CF gene therapy our laboratory created an RSV replicon by removing the three glycoprotein genes from the rgRSV genome (Malykhina, O., Yednak, M.A., Collins, P.L., Olivo, P. and Peeples, M.E. manuscript in preparation). Once in cells, the replicon replicates and produces its proteins but is unable to spread due to the lack of its glycoproteins. To move the replicon from cells containing it, the RSV F and G proteins can be provided in trans, producing “one-step-virus” (OSV). OSV is capable of delivering the replicon to fresh cells where it again produces its encoded proteins, but cannot spread. This vector has been shown to be non-cytotoxic in several immortalized cell lines.

Here, we tested the ability of the RSV replicon to express and deliver CFTR. The CFTR gene was inserted into four different sites in the RSV replicon genome to obtain a range of expression levels. These vectors were then evaluated for their ability to express CFTR and to deliver it to fresh cells as OSV. All four replicons were capable of producing the
CFTR protein and once mobilized were able to deliver it to fresh cells. When CFTR-expressing replicon OSV were used to infect primary human airway epithelial cultures, replicon containing cells remained within the cultures for 12-20 days, indicating that replicon containing cells do not die as quickly as those infected with complete RSV. The titer of the CFTR-expressing replicon OSV was not high enough however, to infect enough primary airway cells to test the functionality of the replicon expressed CFTR. Future studies will need to improve the mobilization efficiency of the replicon OSV in order to generate high enough titered stocks to complete the functional testing of replicon-expressed CFTR in primary HAE cultures from CF patients. Otherwise, the RSV replicon is a promising second generation RSV-based vector for CF gene therapy.

**Materials and Methods**

**Viruses and Cells**

Recombinant, green fluorescent protein-expressing rgRSV was used as the vector backbone in this study. All replicons were rescued in BHK SR19-T7 cells grown in MEM supplemented with 10% fetal bovine serum (FBS). Replicons were transferred to HeLa cells grown in DMEM supplemented with 10% FBS. All cells were grown in a humidified incubator supplied with 5% CO₂.

**rgRSV Replicon Vector Construction**

The RSV replicon was created by removing the three glycoproteins from the full-length RSV cDNA construct, RW30, by digestion with PvuI and XhoI. In place of the glycoproteins, a blasticidin resistance gene, flanked by the GS and GS from the RSV F protein, was inserted again using the PvuI and XhoI enzyme sites to create the MP355 construct.
The plasmids containing the CFTR gene unit, the CFTR gene flanked by the RSV F GS and GE sequences, externally flanked by PvuI and XhoI, BssHII, XhoI or BsiWI restriction sites used to insert the CFTR gene into RW30 in Chapter 2 were also used to insert the CFTR gene into MP355. The CFTR gene units were inserted at the BssHII, XhoI or BsiWI restriction sites in MP355, creating rgRSVrep-CFTR6, rgRSVrep-CFTR8 and rgRSVrep-CFTR10, respectively (Fig. 9). rgRSVrep-CFTR2 was created by first inserting the CFTR gene unit into a partial rgRSV plasmid using PvuI and XhoI. The CFTR gene unit and a partial genome segment containing the NS1, NS2, N, and P genes were then cloned into the MP355 plasmid using the KpnI and BssHII sites. The number included in the names of the resulting plasmids indicates the gene position occupied by the CFTR gene relative to the 3’ end of the genome.

**rgRSVrep-CFTR Vector Rescue and Mobilization**

All replicons were rescued in the BHK SR19-T7 cell line, a baby hamster kidney cell line that constitutively expresses T7 polymerase from a Sindbis virus replicon [1364]. A plasmid containing a full length cDNA of the replicon vector genome (1.2 µg) was transfected into 40% confluent BHK SR19-T7 cells in 6 well plates along with a mixture of support plasmids (0.8 µg) expressing the RSV N, P, L and M2-1 genes. This was done using the TransIT-LT1 transfection kit (Mirrus) according to the manufacturer’s protocol. Transcription of the plasmids was driven by the T7 polymerase. The presence of GFP containing cells indicated functioning replicons. Replicon containing cells were then selected with 10 µg/ml blasticidin and cloned.
OSV was mobilized by transfecting replicon containing BHK SR19-T7 cells in 150 mm dishes with plasmids expressing the RSV F (MP340) and G (MP341) proteins under the control of a CMV promoter in pcDNA3.1 (Invitrogen) using the TransIT-LT1 transfection kit (Mirrus). After 48 hr, OSV was harvested by scraping the cells from the plate, vigorously pipetting and vortexing them and then pelleting out the cells by centrifugation (2000 x g for 5 min.). The OSV-containing supernatant was used to infect additional cells or snap frozen on dry ice and stored at -80°C for later use. OSV stocks were titrated by counting the number of green cells at 24 hours in duplicate wells of a 96 well plate of HeLa cells infected with serially diluted virus stocks.

**Western Blot**

Replicon-containing BHK SR19-T7 cells or HeLa cells in confluent 100 mm dishes were lysed using 500 μl lysis buffer [1% Triton X-100, 150 mM NaCl, 50 mM Tris and an EDTA-free protease inhibitor cocktail (Roche)]. Laemmli sample buffer containing 5% β-mercaptoethanol was added to equal volumes of lysate. Samples were incubated at 37°C for 30 min then electrophoresed on a 10% SDS-PAGE gel [1374]. Samples were transferred from the gel to an Immobilon-P membrane (Millipore) which was blocked overnight at 4°C with 5% dry milk in phosphate-buffered saline plus 0.2% Tween-20 (PBST). The membrane was probed with CFTR monoclonal antibody #596 (Dr. John Riordan, University of North Carolina-Chapel Hill) diluted 1:1000. A goat anti-mouse peroxidase-labeled secondary antibody (Chemicon) was used at a dilution of 1:10,000. The blot was developed with LumiLight chemiluminescent substrate (Roche). Lysate from Calu3 cells, prepared in the same manner, was used as a positive control for CFTR.
**Generation of CF HAE**

Primary human tracheobronchial epithelial cells from non-CF donors were obtained from tracheobronchial tissues by the UNC CF Center Tissue Culture Core under UNC IRB-approved protocols. Cells were plated at a density of 250,000 cells per well on permeable type IV collagen-coated Millicells (0.4 μm pore size, 12 mm diameter, Millipore). Cultures were allowed to differentiate at an air-liquid interface for 4-6 weeks to form well-differentiated cultures with morphological characteristics that resemble those of the human cartilaginous pseudostratified ciliated epithelium in vivo, as previously described [643].

**Inoculation of CF HAE with rgRSVrep-CFTR Vectors**

Prior to viral inoculation, the apical surfaces of HAE were rinsed 3 times with PBS to remove accumulated apical secretions. The apical surfaces of duplicate cultures were then inoculated with 200 μl of rgRSVrep-CFTR2, rgRSVrep-CFTR6, rgRSVrep-CFTR8 or rgRSVrep-CFTR10 (5 × 10^2 pfu, 9 × 10^2 pfu, 1 × 10^3 pfu and 1 × 10^3 pfu, respectively) and incubated for 2 hr at 37°C after which the inoculums was removed and the cultures were returned to a humidified incubator.

**Longevity of rgRSVrep-CFTR in CF HAE cultures**

CF HAE inoculated with rgRSVrep-CFTR2, rgRSVrep-CFTR6, rgRSVrep-CFTR8 or rgRSVrep-CFTR10 (5 × 10^2 pfu, 9 × 10^2 pfu, 1 × 10^3 pfu and 1 × 10^3 pfu, respectively) were photographed with a Leica DMIRB Inverted Fluorescent Microscope every 24-48 hr for 21 days in the same locations to determine the longevity of rgRSVrep-CFTR containing cells.
Results

Construction and Rescue of rgRSV Replicon Vectors Expressing CFTR

A CFTR gene unit containing the 4.5 kb CFTR gene flanked by a set of RSV transcription signals was inserted into the recombinant GFP-expressing RSV replicon (rgRSVrep) cDNA in four different positions creating the plasmid templates for rgRSVrep-CFTR2, rgRSVrep-CFTR6, rgRSVrep-CFTR8 and rgRSVrep-CFTR10, which were numbered according to the CFTR gene position within the viral genome (Fig. 10). rgRSVrep-CFTR vectors were rescued in BHK SR19-T7 cells.

After GFP expressing cells were detectable, indicating that rgRSVrep-CFTR was replicating, the BHK SR19-T7 cells were treated with 10 µg/ml blasticidin to eliminate cells which did not contain a replicon. Twelve clones of BHK SR19 T7 cells containing each of the rgRSVrep-CFTR vectors were isolated and expanded. None of the clones exhibited any cytotoxic effects due to the presence of any of the rgRSVrep-CFTR vectors.
Figure 10. **rgRSVrep-CFTR Constructs.** The CFTR gene, flanked by the RSV GS and GE was inserted into the rgRSVrep cDNA at 4 positions. Constructs are named for the position of the CFTR gene relative to the viral promoter at the 3’ end of the genome.
rgRSVrep Vectors Can Express Full-length, Mature CFTR

To test whether these vectors expressed CFTR, we harvested cell lysates from replicon containing BHK SR19-T7 cells and assayed for CFTR by western blot. As a positive control, we used lysate from the human lung cell line, Calu3, which endogenously expresses a high level of mature CFTR. BHK SR19-T7 cells containing each of the rgRSVrep-CFTR vectors expressed mature CFTR protein (Fig. 11). The expression level of CFTR in one representative clone of each is shown in Figure 10. No CFTR was detected in BHK SR19-T7 cells without a replicon or in BHK SR19-T7 cells containing rgRSVrep. The gradient of transcription exhibited by RSV is illustrated by the gradient of CFTR expression based on its position. These data indicate that mature CFTR can be expressed from the rgRSVrep genome and that the location of the CFTR gene determines its expression level.

Figure 11. CFTR Expression in rgRSV-CFTRrep-containing BHK SR19-T7 Cells. Lysates from BHK SR19-T7 cells containing rgRSVrep-CFTR vectors were run on an SDS-PAGE gel and probed for CFTR. Endogenous CFTR in Calu3 cells was used as a positive control. Lysates from rgRSVrep-containing BHK SR19-T7 cells were negative controls.
rgRSVrep Vectors Are Capable of Delivering CFTR to Fresh Cells.

rGPSVrep-CFTR vectors were mobilized from BHK SR19-T7 cells as infectious OSV by transfecting these cells with plasmids expressing RSV F and G. OSV was then used to infect HeLa cells. Once GFP-expressing cells appeared, indicating rgRSVrep-CFTR replication, HeLa cells were treated with 10 µg/ml blasticidin to eliminate cells which did not contain a replicon. Lysates were harvested from confluent 100 mm dishes of rgRSVrep-CFTR-containing HeLa cells and assayed for CFTR expression by western blot. HeLa cells containing all the rgRSVrep-CFTR vectors expressed mature CFTR protein that co-migrated with the CFTR produced by Calu3 cells (Fig. 12). Again, no CFTR immunoreactivity was detectable in HeLa cells alone or in HeLa cells containing rgRSVrep. The gradient of CFTR expression can also be seen in these cells. These data indicate that CFTR can be delivered to fresh cells via rgRSVrep-CFTR OSV vectors.

![Western blot of CFTR expression](image)

**Figure 12.** rgRSVrep-CFTR OSV Are Capable of CFTR Delivery. HeLa cells infected with rgRSVrep-CFTR OSV and selected by blasticidin produce CFTR, as detected by western blot. Endogenous CFTR from Calu3 cells was used as a positive control. Lysates from rgRSVrep-containing BHK SR19-T7 cells were negative controls.
rgRSVrep-CFTR Vectors Persist in HAE Cultures

To determine whether rgRSVrep-CFTR vectors were less cytotoxic than rgRSV in CF HAE cultures, duplicate wells of non-CF HAE cultures were inoculated with 200 µl of rgRSVrep-CFTR2 (5 × 10^2 pfu), rgRSVrep-CFTR6 (9 × 10^2 pfu), rgRSVrep-CFTR8 (1 × 10^3 pfu) or rgRSVrep-CFTR10 (1 × 10^3 pfu). Starting at 24 hr post inoculation photographs of designated areas of the cultures exhibiting green rgRSVrep-CFTR vector-containing cells were taken every 24-48 hr for 21 days. Individual cells were identified and the longevity of rgRSVrep-CFTR containing cells was determined by counting the number of days an individual rgRSVrep-CFTR cell remained in the culture. Overall, rgRSVrep-CFTR containing cells remained in the cultures longer than rgRSV infected cells. rgRSVrep-CFTR2 containing cells survived for ~18 days, rgRSVrep-CFTR6 containing cells for ~12 days, rgRSVrep-CFTR8 containing cells for ~13 days and rgRSVrep-CFTR10 for ~20 days (Fig. 13).
Figure 13. Longevity of rgRSVrep-CFTR-containing HAE Cells. Duplicate cultures of normal (non-CF) HAE were inoculated with $5 \times 10^2$ pfu, $9 \times 10^2$ pfu, $1 \times 10^3$ pfu and $1 \times 10^3$ pfu of rgRSVrep-CFTR2, rgRSVrep-CFTR6, rgRSV-CFTR8 or rgRSV-CFTR10, respectively. Every 24-48 HR for 21 days, photographs were taken of the cultures in the same locations. Individual vector-containing cells were followed to determine the length of their survival.
Discussion

The ciliated cells of the respiratory tract are terminally differentiated, with a 40-90 day lifespan. Any CF gene therapy vector targeting these cells will need to be re-administered. We showed in Chapter 2 that rgRSV is capable of delivering CFTR to the ciliated cells in well differentiated CF HAE cultures and that this RSV-delivered CFTR is capable of correcting the physiologic defects in these cells. However, rgRSV infection of ciliated cells in HAE cultures causes them to undergo more rapid elimination from the culture resulting in elimination of most of the rgRSV infected cells by seven days post inoculation. Induction of more rapid turnover of its target cells is not ideal for a gene therapy vector. In order to make rgRSV a less cytopathic vector we have removed its three glycoproteins which are thought to be responsible for most of the cytopathology seen in cultured cells (Malykhina, O., Yednak, M.A., Collins, P.L., Olivo, P. and Peeples, M.E. manuscript in preparation). The resulting rgRSV replicon is less cytotoxic in many immortalized cell lines and is unable to form infectious virions. Once in a cell, the replicon is able to replicate and produce its encoded proteins but is unable to spread from that cell to others. Insertion of the blasticidin resistance gene, flanked by RSV transcription signals, in place of the glycoprotein genes allows the selection of pure populations of replicon containing cells.

To determine if the rgRSV replicon was capable of CFTR expression and delivery, we inserted the CFTR gene into four different positions in the rgRSV replicon genome. All four rgRSV replicon vectors were rescued in BHK SR19-T7. In contrast to the rgRSV-CFTR vectors which could only express CFTR from the 10th and 12th positions, rgRSVrep-CFTR vectors were capable of expressing CFTR from all four positions (Fig. 11). Our lab has observed that, once established in cells, the rgRSV replicon exhibits reduced gene
expression compared to acute rgRSV infection (Malykhina, O., Yednak, M.A., Collins, P.L., Olivo, P. and Peeples, M.E. manuscript in preparation). A reduction in total gene expression as compared to rgRSV may explain why the rgRSV replicon is capable of expressing CFTR from the two more 3’ positions. The reduction in the amount of CFTR produced from these positions in the replicon is apparently enough to avoid cytotoxicity due to CFTR overproduction. A gradient of CFTR expression, based on its position in the rgRSV replicon genome, was evident as expected.

In order to determine if the rgRSVrep-CFTR vectors were capable of delivering CFTR to fresh cells, they were mobilized from the BHK SR19-T7 cells by supplying the RSV F and G proteins \textit{in trans}. This is accomplished by transiently transfecting the replicon containing cells with plasmids expressing the F and G genes. Expression of the F and G proteins allows the formation of “one step virus” (OSV) with the replicon as its genome. This is somewhat of an inefficient process however; resulting OSV usually does not reach titers above 10^3. The rgRSVrep-CFTR OSV was used to infect HeLa cells in which CFTR was again expressed indicating that the rgRSVrep-CFTR vectors were capable of CFTR delivery (Fig. 11).

The low titers of the rgRSVrep-CFTR vectors prevented the testing of CFTR function in CF HAE cultures, however, non-CF HAE cultures were inoculated with the vectors to determine if they were less cytotoxic than rgRSV in these primary cells as had been observed in immortalized cell lines. On average, the rgRSVrep-CFTR vector containing cells survived in the culture more than twice as long as rgRSV infected cells indicating that these vectors are less cytotoxic in these primary cells (Fig. 13). rgRSVrep-CFTR2 and rgRSVrep-CFTR10 containing cells survived longer than rgRSVrep-CFTR6 and rgRSVrep-CFTR8 containing
cells for unknown reasons. Some rgRSVrep-CFTR10 containing cells remained in the culture for the length of the observation period. The lack of cytotoxicity could be due to the absence of the glycoproteins or the reduction in gene expression exhibited by the replicon. The loss of replicon gene expression in HAE cultures is probably due to the loss of the replicon-containing cell itself. The length of time a replicon-containing cell remains in the culture is partially dependent on when during the ciliated cell’s life span it was transduced.

In addition to being less inherently cytotoxic the rgRSVrep-CFTR vectors should also stimulate less of an immune response. The fusion glycoprotein is the RSV antigen against which most of the host cytotoxic T cell (CTL) response is generated \([1224]\). The absence of this protein should lessen the CTL response to rgRSVrep-CFTR vector containing cells possibly resulting in fewer of these cells being eliminated by these immune cells. The overall reduction in gene expression exhibited by the rgRSV replicon may also lead to a decrease in immune stimulation. A diminished immune response would also improve the likelihood that these rgRSVrep vectors could be re-administrated.

Our results support the further examination of the rgRSV replicon as a gene therapy vector for CF. The rgRSV replicon can express variable levels of CFTR and can deliver it to fresh cells. The rgRSVrep-CFTR vectors are less cytotoxic in immortalized cells and primary airway epithelium. Currently, however, the efficiency of mobilizing rgRSVrep-CFTR into OSV vectors, by the addition of the RSV F and G genes \(\text{in trans}\), is too low to examine the functionality of the rgRSVrep delivered CFTR in CF HAE. The generation of a packaging cell line for the rgRSV replicon may improve OSV production and allow functional testing of the CFTR delivered by these rgRSVrep-CFTR vectors.
CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

The development of gene therapy approaches to treat CF was begun almost immediately following the cloning of the CFTR gene. Multiple viral and non-viral CFTR delivery methods have been evaluated in animal models as well as human trials however, none have yet been able to deliver a sufficient amount of CFTR to the airways of CF patients for an adequate amount of time to be used therapeutically. Obstacles to efficient CFTR delivery and maintenance include the physical barriers of the CF respiratory tract, inefficient targeting of the apical cells of the human respiratory tract, vector cytotoxicity, immune response-mediated elimination of vector containing cells and an antibody response to the vector that prevents re-administration. An efficient gene therapy vector for the treatment of CF must therefore survive in and penetrate the harsh environment of the CF lung; a vector based on a human pathogen known to infect the lungs of CF patients should be able to accomplish this. A vector that can successfully target the apical ciliated cells of the respiratory tract, the cells believed to be most important in determining ASL hydration, must also not induce high titers of neutralizing antibodies that prevent its ability to be re-administered. A vector based on a virus that naturally infects the apical, ciliated cells of the respiratory tract and is able to naturally reinfect patients due to the lack of a long-lived antibody response targets the correct cells and should be able to be re-administered. If the cytotoxic and immunostimulatory properties can be removed from this vector, it should be able to demonstrate sustained CFTR expression. This could be done by mutating or removing the viral genes encoding the proteins most responsible for cytotoxicity and to which the
majority of the immune response is directed. RSV was chosen as a potential vector for the treatment of CF because it possesses these characteristics.

Collectively, the data presented in this dissertation support further investigation of RSV as a vector for CF gene therapy. rgRSV-CFTR vectors efficiently infect the ciliated cells of CF HAE cultures, thus delivering CFTR to the appropriate target cells. rgRSV-delivered CFTR is expressed correctly at the apical surface of these cells and is functional at a level that recapitulates the Cl⁻ channel activity in CF HAE observed in normal HAE cultures. rgRSV-expressed CFTR also improves the ASL dehydration in CF HAE cultures, thus correcting a hallmark defect of CF lung disease. Unfortunately, rgRSV-CFTR infection of these cells accelerates their shedding from the airway epithelium leading to the elimination of vector containing cells within approximately a week, shortening the longevity of any therapeutic effects.

The rgRSV replicon that we developed is a less cytotoxic, second generation, RSV-based vector for CF gene therapy. The replicon is stable and non-cytotoxic in multiple immortalized cell lines and can express CFTR from four different genome positions leading to four vectors with different CFTR expression levels. Once mobilized into OSV, rgRSVrep-CFTR vectors are successful at delivering CFTR to new cells. rgRSVrep-CFTR vectors also seem to be less cytotoxic in HAE cultures as ciliated cells containing these vectors can survive longer than those containing rgRSV-CFTR vectors. However, mobilization of rgRSVrep-CFTR vectors into OSV has been an inefficient process limiting the attainable titers and the ability to test rgRSVrep-expressed CFTR function.
Each vector that has been previously suggested for the treatment of CF lung disease has its own pros (lack of cytotoxicity/immune stimulation, long lived gene expression, etc.) and cons (stimulation of a protective immune response prohibiting vector re-administration, inefficient transduction of apical CF respiratory cells, etc.). These potential vectors should continue to be improved by reducing or eliminating the qualities that inhibit their ability to successfully treat CF lung disease and by improving upon their positive attributes.

Exploiting the natural characteristics of human respiratory viral pathogens could also result in the creation of an efficient CFTR delivery vehicle for the CF respiratory tract. Natural human respiratory viruses, such as RSV, will need to be engineered to remove their cytotoxic and immunostimulatory properties but they are naturally efficient at infecting the cells of the CF respiratory tract that most require CFTR in order to ameliorate the respiratory symptoms of CF, a characteristic lacking from most of the CF gene therapy vectors currently being developed; therefore they deserve further vector development as well. Also, the generation of new, more physiologically relevant CF animal models will certainly aid in the construction and testing of new, more efficient CF gene therapy vectors.

**Establishment of an Inducible RSV G and F-expressing Cell Line**

In order to improve the mobilization efficiency of the replicon we have been attempting to generate an inducible OSV packaging cell line. To accomplish this we are using the Rev-TetOn system (Clontech) to insert the RSV F and G genes into the HeLa cell genome under the control of a tetracycline (tet) inducible promoter. This system is based on two elements from the *E. coli* tet operon, the tet operator DNA sequence and the tet repressor protein (TetR) (Fig. 14A). In the absence of tet, TetR binds to the tet operator DNA sequence repressing the transcription of downstream genes in the operon. When tet is present it
binds to TetR making it unable to bind the tet operator sequence allowing the downstream genes to be transcribed [1384]. The Rev-TetOn system uses a reverse tetracycline transactivator ((r)tTA) which was created by making four amino acid changes in the native TetR gene and fusing it to the herpes simplex virus VP16 protein. The amino acid changes reversed its response to tet. The fusion converted TetR from a transcriptional repressor to a transcriptional activator. Now, when tet is present it binds to the (r)tTA protein, then this complex binds to the tetracycline response element (TRE) promoter consisting of seven repeats of the tet operator sequence and the minimal immediate early promoter of human cytomegalovirus (CMV<sub>min</sub>) activating transcription of the downstream genes. When tet is absent (r)tTA does not bind to the TRE and downstream genes are not transcribed [1385].

We are using a HeLa cell line that constitutively expresses (r)tTA (Clontech).

The RSV F and G genes were cloned into either side of a bi-directional vector, pTRE-Tight-BI (Clontech), using the Bam HI and Hind III, and Sac II and Apa I restriction enzyme sites, respectively, downstream of a TRE promoter flanked on both sides by the CMV<sub>min</sub> (Fig. 14B) [1386]. The inclusion of the CMV<sub>min</sub> on either side of the TRE allows transcription to occur in either direction from the TRE. The TRE Tight-BI fragment containing F and G was then cloned into the cDNA of the pRev-TRE retrovirus, using SbfI restriction enzyme sites after removing the TRE present in this plasmid with Bam HI and Bgl II. The Rev-TRE retrovirus is based on Maloney murine leukemia virus and contains the hygromycin resistance gene. This cDNA was transfected into retrovirus packaging pT67 cells which express the Maloney murine leukemia virus gag, pol and env (10A1) genes. The pT67 cells were then selected and cloned using 200 g/ml hygromycin. Clones were screened for the presence of the integrated retrovirus by harvesting the genomic DNA using a lysis buffer
(10 mM Tris HCl, 5 mM EDTA, 0.2% SDS, 200 mM NaCl, and 100 µg/ml proteinase K) and assaying for the TRE Tight-BI F/G sequence by PCR. Virus was harvested from four pT67 clones positive for the retroviral insert according to the manufacturer's protocol and used to infect the HeLa cells expressing (r)tTA. Currently, we are selecting and cloning these HeLa cells with 200 g/ml hygromycin. We are also attempting to stably transfect the pTRE-Tight-BI vector containing the RSV G and F genes directly into the HeLa cells expressing (r)tTA. In this case the plasmid containing the pTRE-Tight-BI vector and the G and F genes was transfected into the HeLa cells using the TransIT HeLa Monster transfection kit (Mirrus) according to the manufacturer's protocol, along with a linear hygromycin resistance gene. Currently, these HeLa cells are also being selected and cloned with 200 g/ml hygromycin.

Once we establish an RSV F and G expressing cell line, rgRSVrep-CFTR vector OSV can be used to move the vectors into this cell line. Replicon containing cells will be selected with blasticidin and treated with doxycycline, a tetracycline derivative, to mobilize OSV. This system should result in OSV with higher titers because all of the cells in the culture should be producing OSV, compared to the previous method, transient transfection, where only a fraction of replicon-containing cells received the F and G plasmids, and were therefore capable of producing OSV. Once we obtain rgRSVrep-CFTR OSV with higher titers, we will assess the functionality of CFTR being expressed by these vectors in CF HAE.
Figure 14. RevTet-On System and pRev-TRE Tight BI-GF Construct. (A) Comparison of the E. coli tet operon and the RevTet-On system being used to create the inducible RSV packaging cell line. (B) The codon-optimized RSV F and G genes were inserted into the pTRE Tight BI vector with Bam HI and Hind III, and Sac II and Apa I, respectively. The TRE Tight BI-GF sequence was then moved to the pRev plasmid with Sbf I to create the pRev-TRE Tight BI-GF construct.
Development of a Non-replicating RSV Vector for the Delivery of CFTR

Despite the apparent reduced cytotoxicity of the rgRSVrep-CFTR vectors in HAE cultures, it is possible that these vectors will still reduce the lifespan of their ciliated target cells. It is also possible that future in vivo studies will find that the expression of viral proteins by the replicon result in CTL killing of the vector containing cells. To avoid these potential problems, we have constructed a third generation, non-replicating RSV-based vector. This “minigenome” vector expresses only three genes: a marker gene, CFTR and one RSV protein. This vector and will be mobilized into OSV in a manner similar to the replicon. The absence of genes encoding the proteins required for RSV replication will prevent this vector from replicating in its target cell. Transcription, however, will be carried out by the virion associated polymerase proteins. New virion production is not possible because this “minigenome” lacks the genes encoding the proteins needed for virion assembly. A non-replicating RSV-based vector, lacking most of the RSV proteins should circumvent any antigenic or cytopathic properties of RSV infection. Although this vector will produce only small amounts of CFTR in vector-containing cells, only small amounts of CFTR are required in the ciliated cells of the respiratory tract [371, 372]. Also, since we found that our rgRSV-CFTR vectors produced ~100-fold more CFTR mRNA than that produced by their host HAE cells, expressing much less CFTR mRNA may be sufficient.

To create a non-replicating vector for CFTR, one essential gene was removed from the RSV replicon genome and inserted into a minigenome that replicates separately. Segmentation of the genome of a non-segmented RNA virus has been done previously with vesicular stomatitis virus and measles virus, in which cases their ability to replicate and spread were maintained [1387, 1388]. In our RSV constructs, one segment consists of the replicon
lacking the M2-1 gene (ΔM2-1 replicon). The other segment is a minigenome containing the
gene for red fluorescent protein (RFP) from the Discosoma species of coral (Invitrogen), the
CFTR gene and the M2-1 gene (CFTR minigenome) (Fig. 1A). RFP will be used in the CFTR
minigenome to distinguish cells containing it from those containing the “ΔM2-1 replicon”
which expresses GFP. Each gene in both segments is flanked by a GS and GE sequence, and
both segments include the Le and Tr sequences at their termini, a requirement for RSV
replication.

Since RSV transcription is M2-1 dependent, deletion of the M2-1 gene from the replicon will
prevent its transcription, and without protein production it will not be able to replicate.
Likewise the CFTR minigenome cannot replicate on its own due to the absence of the RSV
polymerase complex genes in its genome. However, when combined with the CFTR
minigenome, which expresses the M2-1 protein, this will enable transcription, and therefore
replication, of the ΔM2-1 replicon. The replicon will provide the remaining viral proteins
required for the replication of the minigenome allowing it to replicate as well when both are
present in the same cell. The presence of the blasticidin resistance gene in the replicon will
allow blasticidin selection of cells containing the ΔM2-1 replicon. Because the ΔM2-1
replicon requires the M2-1 protein from the CFTR minigenome for transcription of its
genes, cells containing both the ΔM2-1 replicon and the CFTR minigenome will be selected.

We constructed the ΔM2-1 replicon using the novel “Unrestricted Mutagenesis and Cloning”
(URMaC) technique developed by Dr. Louay Hallak, a member of our laboratory (Fig. 15B).
A small segment including the M2-1 gene unit and the two nearest unique restriction
enzyme sites flanking it (Aar I and Xho I) was amplified by PCR from the plasmid containing
the RSV replicon sequence. This PCR product was self-ligated then two primers, one sitting on the M2 GS and the other on the M2-1/M2-2 overlap sequence were used to amplify this smaller plasmid, thereby deleting the M2-1 gene. This amplified segment was again self-ligated creating a plasmid which is lacking the M2-1 gene and has the M2 GS positioned just before the M2-2 gene start codon. This ligated product was again amplified using the original two primers, then digested with Aar I and Xho I and reinserted into the original replicon plasmid.

The CFTR minigenome was made by replacing the GFP gene unit with one containing the RFP gene using the Kpn I restriction site in a minigenome plasmid already containing the CFTR gene unit and Le and Tr sequences. An M2-1 gene unit, amplified from the same plasmid used to make the ΔM2-1 replicon, was inserted downstream of the CFTR gene unit using the Apa I restriction enzyme site. We also constructed an M2-1 minigenome lacking the CFTR gene unit as a control for these studies.

The ΔM2-1 replicon and CFTR minigenome were transfected into BHK SR19-T7 cells along with RSV support plasmids to launch their replication. BHK SR19-T7 cells successfully transfected with both the ΔM2-1 replicon and the CFTR minigenome were selected and cloned with blasticidin. These cells expressed both GFP and RFP, as anticipated since the replicon and minigenome are co-dependent. Individual cloned cell lines were then tested for CFTR expression via western blot (Fig. 16). Five of the 7 clones tested produced CFTR, with three clones (clones 3, 5 and 7) expressing large amounts.
Cells containing both the CFTR minigenome and the ΔM2-1 replicon, will produce OSV when the RSV F and G proteins are supplied in trans, either by transfection or by induction of the integrated genes in the inducible cell line. Because smaller genomes are known to replicate more rapidly than the larger genomes, we anticipate that the pool of CFTR minigenome nucleocapsids in the cell will be larger than the pool of ΔM2-1 replicon nucleocapsids, resulting in more OSV containing the CFTR minigenome than the ΔM2-1 replicon [1389]. The amount of each type of OSV produced can be quantified by titration on HeLa cells infected with whole RSV prior to inoculation with the OSV. The RSV will supply all of the viral proteins required for replication and transcription of the CFTR minigenome and the ΔM2-1 replicon. By counting red cells, green cells, and cells that are both red and green, we will be able to determine the titer and the relative proportion of OSV containing only the CFTR minigenome, only the ΔM2-1 replicon, and co-packaged minigenome and replicon, respectively.
Figure 15. ΔM2-1 Replicon and CFTR Minigenome Constructs. (A) Depiction of the two co-dependent genome segments making up the bipartite rgRSV genome. The CFTR minigenome is the non-replicating rgRSV-CFTR vector. (B) Schematic of method (URMaC) used to create the ΔM2-1 replicon.
Figure 16. CFTR Expression from the Non-replicating rgRSV-CFTR Minigenome. A western blot to detect CFTR protein in BHK SR19-T7 cell clones containing the ΔM2-1 replicon and the CFTR minigenome. Endogenous CFTR from Calu3 cells was used as a positive control.
Our results indicate that RSV is capable of targeting the ciliated cells of the human airway and of expressing CFTR in these cells. They suggest that some form of RSV vector might be therapeutic in CF patients. However, improvements need to be made to RSV in order for it to serve as a potential gene therapy vectors for CF. Cytotoxicity must be reduced, viral titers must be improved, and the stimulation of and susceptibility to the immune response must be lessened. We have made progress on all of these fronts by developing the RSV replicon and the non-replicating CFTR minigenome vectors and by creating an inducible packaging cell line. The ability to further modify the RSV genome by the insertion of genes that inhibit the host immune response or improve the reinfection capability of RSV, as well as the potential for generating less cytopathic RSV vectors further strengthens the potential of RSV as a gene therapy vector for CF and for other diseases affecting the airways.
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